Contribution of Extracellular Glu Residues to the Structure and Function of Bacteriorhodopsin

PRESENCE OF SPECIFIC CATION-BINDING SITES

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The elucidation of three-dimensional structures of bacteriorhodopsin (BR)\(^1\) at high resolution and the increased use of mutants have improved greatly the knowledge of the proton transport mechanism, allowing the identification of the principal side chains undergoing protonations/deprotonations during this process (1–4). Among key groups, Asp\(^{85}\) and Asp\(^{96}\) are the primary proton acceptor and proton donor of the Schiff base, respectively. An important achievement has been the description of several water molecules in the extracellular region, forming a hydrogen-bonded network with key residues (2, 5, 6). This network serves most likely to transmit protonation changes and to conduct the proton. The role of Asp\(^{85}\) in the extracellular region is not limited to be the first acceptor of the Schiff base proton. According to a coupling model, protonation of Asp\(^{85}\) induces the deprotonation of the proton release group through a relationship existing between their pK\(_a\) values (7, 8). Although this model is useful in understanding protonation/deprotonation mutual influences between ionizable groups, it cannot give the answer about the exact mechanism of proton transfer steps. In fact, several important questions remain still elusive. One of these refers to the identity of the assemblies involved in proton release, the proton release group. Recent studies suggest that it is a complex including at least Glu\(^{194}\), Glu\(^{204}\), and some water molecules (9–11). It should be noted, however, that these two Glu side chains do not behave equivalently, as demonstrated by the inhibition of the second increase of the Asp\(^{85}\) pK\(_a\) during the photocycle in the E194Q mutant but not in the E204Q mutant (12). This is a clear indication that Glu\(^{194}\) forms part of XH, the group coupled to the Asp\(^{85}\) pK\(_a\).

From the time when the presence of divalent cations in the purple membrane was described, numerous efforts have been devoted to identify their role and location (13–26). Some authors claim a nonspecific location of the cations in the double layer (18, 21, 22) or in the lipid phase (26). However, the majority of works argue for the existence of specific binding sites in the purple membrane, including a site near Asp\(^{85}\) (24, 27, 28), or a more external location (29, 30). Among the most outstanding results pointing toward specific cation locations in the protein moiety are: (i) the evidence for carboxyl participation in cation binding from studies using specific reagents (13, 17); (ii) the presence of fewer cation-binding sites and with lower affinity in the bleached and pink membranes as compared with purple membranes (14, 16, 19), even that the electric surface potential of bleached membrane remains unchanged (31); (iii) extended x-ray absorption fine structure data describing a different environment for the bound Mn\(^{2+}\) with respect to free Mn\(^{2+}\) in water and ruling out the interaction of Mn\(^{2+}\) with P or S atoms (32); (iv) Fourier transform infrared studies showing that binding of Mn\(^{2+}\) to deionized membrane induces changes in the reverse turns, located in the loops (33);...
and (v) $^{13}$C NMR studies detecting changes in the Ala$^{106}$ environment upon divalent cation binding (25).

Despite the substantial experimental background suggesting the existence of specific cation-binding sites, none of the recent bacteriorhodopsin models reflects their presence (2, 5, 34). One possible exception is the electron microscopy structural data of Mitsuoka et al. (35), which detected the presence of charges and polarized water molecules in some locations of their structure.

Recently we have described some spectral and functional properties of the quadruple mutant E9Q/E74Q/E194Q/E204Q (4Glu), including high hydroxylamine and Cl properties of the quadruple mutant E9Q/E74Q/E194Q/E204Q. These results suggest a more open structure for the extracellular region of the 4Glu mutant and reduced affinity of H$_2$SO$_4$ solutions. To avoid contamination, pH adjustment of deionized samples was done using duplicates. All experiments were performed in the dark using dark-adapted samples. Because of abnormal dark adaptation kinetics of the E194Q, 3Glu, and 4Glu mutants (36), the samples were kept in the dark for about 25 days.

Absorbance changes at 630 nm as a function of pH were used to monitor the purple-to-blue transition. Experimental points were normalized with respect to the largest value at 630 nm and fitted to the Boltzmann equation.

Calcium binding experiments were done in darkness with deionized samples adjusted to pH 4–4.5. Absorbance changes induced by the addition of small quantities of calcium were monitored spectrophotometrically. BR concentration was in the 1.5–2 × 10$^{-5}$ M range.

DSC experiments were performed using a Micro-Cal MC2 instrument. The samples were dialyzed previously in water adjusted at pH 6.5–7, giving a final concentration of 1.5–2 mg/ml. Experiments were done under 1.7 atm nitrogen pressure to avoid sample evaporation at high temperatures. Scanning speed was set at 1.5 K/min. Three thermograms were registered for each sample. The first informs about the heat released or taken by the protein upon temperature increase. After cooling down to room temperature, second and a third thermograms were run to check the reversibility of the transitions. Two corrections were applied to the first thermogram: (i) subtraction of the second thermogram, which acts as a blank, and (ii) subtraction of the chemical base line using the Takahashi and Sturtevant method (44). $T_m$ was defined as the point where the $C_v$ value is maximal.

Thermal denaturation experiments were carried out on dark-adapted samples in H$_2$O and pH 7.0 by following changes in the UV-visible absorption spectra upon temperature increase. Spectra were taken every 5 °C in the range 250–800 nm, starting at 20 °C and allowing 8 min for stabilization at each temperature. BR concentration was 1 × 10$^{-5}$ M.

**RESULTS**

**Spectral Behavior of Extracellular Glu Mutants upon Acidification: the Purple-to-Blue Transition**

As has been widely described, acidification of purple membrane samples causes the formation of the blue form, because of Asp$^{45}$ protonation (45, 46). Fig. 1 (A and B) shows the absorbance and difference spectra of the dark-adapted E74Q mutant in 150 mM KCl upon acidification of the medium from pH 5.7 to 2.1. As in wild type, the difference spectra of E74Q reveal the presence of two absorbance maxima: one around 610 nm and a second at 570 nm. The isosbestic point at about 620 nm reflects a transition induced by the addition of small quantities of calcium were monitored spectrophotometrically. BR concentration was 1 × 10$^{-5}$ M.
BR<sub>acid</sub> form. The second isosbestic point at 578 nm corresponds to the red shift of the spectrum because of protonation of Asp<sup>85</sup>, giving the principal transition (the purple-to-blue transition). Similar spectral changes upon pH decrease are obtained for E74Q in H<sub>2</sub>O and for dark-adapted E9Q and E194Q single mutants in 150 mM KCl or in H<sub>2</sub>O (not shown).

Acidification of the dark-adapted E204Q mutant reveals more complex spectral changes. Whereas in 150 mM KCl a similar behavior to wild type is obtained, in H<sub>2</sub>O or in 75 mM Na<sub>2</sub>SO<sub>4</sub> where Cl<sup>-</sup> ions are not present, the absorption maximum undergoes a small blue shift and band broadening in both sides (Fig. 1, C and D). This gives rise to two positive bands at 640 and 460 nm in the difference spectra (Fig. 1D). Further decrease of the pH leads to the main transition, where all species are converted to the blue form. This produces a positive band at 628 nm and a negative band at 538 nm.

Titrations of deionized wild type and deionized forms of the single mutants E9Q, E74Q, and E194Q by increasing the pH from 4 (the initial pH after deionization) to 8 gives only one isosbestic point at 577 nm in the difference spectra (Fig. 2A for the spectra of deionized E9Q). However, titration of deionized E204Q shows not only that the red form persists but also that it is better distinguished than in the presence of Na<sub>2</sub>SO<sub>4</sub> (Fig. 2B). Thus, E204Q is unique among wild type and the single Glu mutants, in that titration of the deionized sample displays changes in the protonation state of more than one group. Moreover, the λ<sub>max</sub> never reaches 603 nm in any of the condition examined (not shown).

Upon acid titration, the multiple extracellular Glu mutants undergo spectral changes different from those of the single mutants. The difference spectra of 3Glu or 4Glu mutants do not show the red band at about 460 nm in any of the conditions analyzed (salt, H<sub>2</sub>O, deionized; see Fig. 2C for difference spectra of 3Glu in 75 mM Na<sub>2</sub>SO<sub>4</sub>). However, acidification of the E194Q/E204Q mutant in Na<sub>2</sub>SO<sub>4</sub> or in water gives rise to the red band (Fig. 2D), arising from a small blue shift and broadening of the absorption band, similar to the single E204Q mutant.

**Determination of the Apparent Asp<sup>85</sup> pK<sub>a</sub>**

As is known, the plot of the absorbance increase at 630 nm as a function of pH yields the apparent pK<sub>a</sub> of Asp<sup>85</sup> (45, 46). Table I shows the calculated values for pK<sub>a</sub> of Asp<sup>85</sup> for all mutants and wild type in three different conditions: 150 mM KCl, water, and deionized membranes. Values for the Asp<sup>85</sup> pK<sub>a</sub> similar to those found in the presence of 150 mM KCl were obtained upon titration in 75 mM Na<sub>2</sub>SO<sub>4</sub> (not shown). In low salt concentration (150 mM KCl) all single mutants gave similar values (pK<sub>a</sub> of 2.7, although E194Q shows slightly lower pK<sub>a</sub>), whereas the pK<sub>a</sub> values of the multiple mutants are elevated (around one pH unit above wild type). In water, the absence of ions in the medium induces more negative electrical surface potential and increased proton concentration, giving rise to an apparent pK<sub>a</sub> value as compared with the salt-containing samples. In water, all samples give higher pK<sub>a</sub> values as compared with the same samples in salt, except the E74Q mutant (pK<sub>a</sub> of 2.8, similar to that in salt). Multiple mutants E194Q/E204Q and 4Glu have an identical and elevated pK<sub>a</sub> of 4.7, whereas 3Glu has a pK<sub>a</sub> of 5.2 (Table I).

Analysis of deionized membrane samples permit the evaluation of the effect of mutations themselves over the Asp<sup>85</sup> pK<sub>a</sub>, regardless of the presence or absence of endogenous cations. Additionally, comparison of the sample properties in water with those of deionized form allows estimation of the effects of the endogenous cations. As Table I shows, all deionized mutants give slightly lower pK<sub>a</sub> values as compared with deionized wild type, except for E9Q, which presents a increased pK<sub>a</sub> of 6.0. On the other hand, the absence of endogenous cations in the deionized form as compared with the sample in water cause an increase of the pK<sub>a</sub> of more than 2 pH units for wild type (13, 49) and similar values for the single mutants except for E204Q. It is noteworthy that the multiple mutants exhibit differences of less than 1 pH unit between pK<sub>a</sub> values of the deionized form and in water. This suggests that the nondeionized forms of these samples have low content of endogenous cations, thus affecting only slightly the apparent Asp<sup>85</sup> pK<sub>a</sub> in comparison with deionized sample.

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**FIG. 2.** Purple-to-blue transition of dark-adapted BR mutants. A, curves 1–7, difference spectra between the sample at pH<sub>i</sub> minus the sample at pH 8.8 of deionized E9Q, where pH<sub>i</sub> is 7.6, 6.9, 6.5, 5.6, 5.3, 5.0, and 4.6. B, curves 1–9, difference spectra between the sample at pH<sub>i</sub> minus the sample at pH 7.6 of deionized E204Q, where pH<sub>i</sub> is 7.1, 6.6, 6.3, 5.9, 5.4, 5.2, 4.9, 4.6, and 4.0. C, curves 1–8, difference spectra between the sample at pH<sub>i</sub> minus the sample at pH 5.9 of 3Glu mutant in 75 mM Na<sub>2</sub>SO<sub>4</sub> where pH<sub>i</sub> is 5.6, 5.4, 4.8, 4.4, 4.2, 3.6, 2.9, and 2.3. D, curves 1–8, difference spectra between the sample at pH<sub>i</sub> minus the sample at pH 7.4 of E194Q/E204Q mutant in 75 mM Na<sub>2</sub>SO<sub>4</sub> where pH<sub>i</sub> is 6.9, 5.2, 4.6, 4.1, 3.8, 3.5, 3.0, and 2.5.
**Addition of Calcium to Deionized Bacteriorhodopsin**

When calcium or some other cations are added to blue deionized samples, the purple form of bacteriorhodopsin is recovered, because of the deprotonation of Asp$_{85}^{	ext{ss}}$ (13, 17, 49). Fig. 3A shows typical difference spectra obtained upon calcium addition to the wild type blue form. The presence of only one isosbestic point at 578 nm strongly indicates that only the deprotonation of Asp$_{85}^{	ext{ss}}$ is involved in the process, leading to a sigmoidal curve in the plot of absorbance change as a function of pCa (Fig. 3B).

The pCa values for 50% of the blue-to-purple transition are shown in Table II. As can be seen, the most drastic changes in Ca$^{2+}$ binding, as compared with wild type, are obtained for the multiple mutants where the quadruple mutant 4Glu needs more than 10 Ca$^{2+}$/BR molecule, 3Glu needs about 8 Ca$^{2+}$/BR, and E194Q/E204Q needs 3.5 Ca$^{2+}$/BR. Among the single mutants, only E204Q and E9Q need higher Ca$^{2+}$ amounts compared with wild type.

**Thermal Denaturation Experiments**

**Differential Scanning Calorimetry**—It has been demonstrated previously that BR samples with decreased cation content have lower thermal stability (14, 50). To study the involvement of the mutated Glu side chains on thermal stability, DSC experiments were carried out. Fig. 4 shows thermograms of extracellular mutants in H$_2$O, obtained after correction for instrumental and chemical base lines (see “Experimental Procedures”). A known feature of the thermal scan of native purple membranes is the presence of two transitions (51). The main transition at about 98 °C has been interpreted as resulting from disorganization of the hexagonal para-crystalline arrangement (51, 52).

As is seen in Fig. 4, the main transition of all mutants appears in the range 90–100 °C, decreasing in the order E74Q > E194Q > E9Q = E204Q > E194Q/E204Q > 4Glu. Besides that, the transitions are less cooperative except for E74Q. In comparison with the main transition, the temperature of the pretransition of the mutants is more variable. It appears at lower temperatures and with low cooperativity for E194Q, E204Q, and E9Q (at about 68 °C for this latter sample), and it is absent for all multiple mutants.

**Visible Spectroscopy**—Heating the purple membrane suspension induces first the appearance of the blue form, because of cation release (14). This is followed by the appearance of the red form ($\lambda_{\text{max}}$ at about 460 nm) and finally by the release of retinal above 80–85 °C. Fig. 5 shows the difference spectra obtained for E9Q and 4Glu on temperature increase, in H$_2$O at pH 7.0. Similar to wild type, an increase of temperature from 25 to 55 °C of E9Q causes a progressive red shift of the absorption spectrum, which gives rise to the appearance of the band at 630 nm in the difference spectrum. Moreover, the thermally induced blue form is a reversible process because lowering the temperature from 55 °C (temperature for which maximum accumulation of blue form is observed) back to 25 °C recovers the initial purple form. Importantly, whereas the rest of the single mutants (E74Q, E194Q, and E204Q) also show the temperature-induced band at 630 nm, the multiple mutants lack formation of the blue form, as represented in Fig. 5 for 4Glu.

**DISCUSSION**

In the first part of this work, we performed acid titration experiments to acquire new information about the influence of...

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**Table I** Specific Cation-binding Sites in Bacteriorhodopsin

| Sample         | 150 m M KCl | H$_2$O | Deionized | $\Delta$Dei-H$_2$O$^d$ |
|----------------|-------------|--------|-----------|------------------------|
| Wild type      | 2.7         | 3.2    | 5.5       | 2.3                    |
| E9Q            | 2.7         | 3.0 (60%) | 6.0     | 3.3                    |
|                |             | 3.7 (40%) |         | 2.6                    |
| E74Q           | 2.7         | 2.8    | 5.2       | 2.4                    |
| E194Q          | 2.3         | 3.9    | 5.2       | 1.5                    |
| E204Q          | 2.8         | 4.1    | 5.2       | 1.1                    |
| E194Q/E204Q    | 3.7         | 4.7    | 5.3       | 0.6                    |
| 3Glu           | 3.8         | 5.2    | 6.0       | 0.8                    |
| 4Glu           | 3.9         | 4.7    | 5.1       | 0.4                    |

$^d$ Difference between Asp$_{85}^{	ext{ss}}$ pK$_a$ in deionized samples and in H$_2$O. These values indicate the relative differences in cation binding among the BR samples.

**Table II** Calcium titration of deionized blue membranes.

| Sample         | Ca$^{2+}$/BR |
|----------------|-------------|
| Wild type      | 2.1         |
| E9Q            | 3.5         |
| E74Q           | 2.0         |
| E194Q          | 1.8         |
| E204Q          | 2.9         |
| E194Q/E204Q    | 3.5         |
| 3Glu           | 8.0         |
| 4Glu           | >10         |

$^c$ This titration was carried out at pH 6.
the extracellular Glu side chains on Asp\textsuperscript{85} protonation. Fisher and Oesterhelt (53) and Mowery et al. (47) already noted that the plot of the absorbance changes in the purple-to-blue transition of wild type BR is too steep to be considered as a transition controlled by the titration of one single group, Asp\textsuperscript{85}. Later on, Váró and Lanyi (48) corroborated this observation and proposed a model postulating that more than one proton is bound cooperatively during acid titration. They identified a new species, the BR\textsubscript{acid} form, that results from BR by proton binding and appears before the blue form, BR\textsubscript{blue}. Although the additional group titrated in this process was not identified at that time, neither it is yet known whether it is worthy of mention that the authors suggested Glu\textsuperscript{9} and Glu\textsuperscript{204} residues as a possibility. However, in the majority of the following work dealing with the purple-to-blue transition, these early observations have been ignored, and only the protonation of Asp\textsuperscript{85} has been considered.

As indicated under “Results,” single extracellular Glu mutants show similar pH titrations as wild type, with the minor transition caused by protonation of a second group remaining masked but already suggested by the presence of two isosbestic points. The only exception to this behavior corresponds to the E204Q mutant in the absence of Cl\textsuperscript{−} ions, which shows clearly a band at 460 nm (red form). However, in the presence of Cl\textsuperscript{−} ions, the red band disappears. One reasonable explanation is that Cl\textsuperscript{−} binds near Glu\textsuperscript{204}, restoring the negative charge and the water network. Therefore, unlike the rest of single mutants, E204Q senses the presence of Cl\textsuperscript{−} ions in the medium and exhibits different spectral changes upon acid titration. Although at present we are not able to identify the second protein residue protonating cooperatively with Asp\textsuperscript{85}, comparison of titration results for single and multiple mutants suggest that it can be one of the acidic side chains located in the extracellular region. Moreover, the fact that the red form is observed for E194Q/E204Q but not for 3Glu or 4Glu points to Glu\textsuperscript{9} as being responsible for the second transition.

Our data provide new evidence for cation binding to the extracellular side of the purple membrane. In low salt concentrations, the apparent pK\textsubscript{a} values of Asp\textsuperscript{85} of the single mutants are similar to that of wild type, being independent of the type of salt in the medium (sodium sulfate or chloride). However, titrations in water or in deionized membranes exhibit perturbed pK\textsubscript{a} values of Asp\textsuperscript{85} as compared with wild type. The increased pK\textsubscript{a} obtained for a particular sample in water, as compared with the same sample in salt can be explained essentially by the increased proton concentration over the negatively charged membrane surface because of the absence of counter ions. Especially in the multiple mutants, the pK\textsubscript{a} increase is considerably higher than in wild type. This effect can be attributed to a loss of endogenous cations and a subsequent increase of proton concentration on the surface. Similar pK\textsubscript{a} values found for E74Q in water and in salt can be explained by loss of a negative charge in the membrane surface upon mutation of this external residue.

Comparison of the pK\textsubscript{a} of the purple-to-blue transition in water with that of the deionized sample gives an indication of the effects of bound cations on this transition and thus reveal their affinities, analogous to the comparison of pK\textsubscript{a} values between salt and water. Reasonably, large differences in pK\textsubscript{a} between a sample in water and in the deionized state reflect mainly strong affinity of cations, whereas small differences reflect low affinity of cations. Therefore, the difference of about 2.3 pH units found for wild type indicates the presence of cations bound with high affinity. This difference is strongly reduced to less than 1 pH unit for all multiple mutants, indicating clearly that cations have a low affinity in these samples (Table I). On the other hand, Ca\textsuperscript{2+} binding experiments to deionized samples give further support to the suggested role of extracellular Glu side chains in cation binding. As described...
under “Results,” the single mutant E9Q and the multiple mutants require higher cation concentrations for reaching the 50% of the blue-to-purple transition as compared with wild type. Thus, both acid titration and Ca$^{2+}$ binding experiments point to Glu$^9$, Glu$^{194}$, and Glu$^{204}$ as being involved in cation binding.

Thermal denaturation experiments can provide further information about the role of extracellular side chains in cation binding. Therefore, we analyzed thermal behavior of extracellular mutants by DSC experiments and UV-visible spectrophotometry. In some mutants, the DSC main transition appears at slightly lower temperature as compared with wild type, indicating somewhat decreased stability of the secondary and/or tertiary structures. However, the most interesting effect was obtained for the pretransition, resulting from disorganization of the hexagonal para-crystalline arrangement (51, 52). As presented under “Results,” there is a concurrence between the lack of the pretransition and the absence of cations. Particularly, the deionized wild type membrane lacks the pretransition but, upon cation addition of at least 2 Mn$^{2+}$/BR, it is partly recovered (50). On the other hand, the recently reported decrease in the content of the BR-specific α11 helical structure upon temperature increase (54) is likely to be due also to cation release. First, there is a coincidence of the temperature of the pretransition, indicating either that the hexagonal arrangement occurs continuously as temperature increases, thus lacking any cooperativity and becoming unobservable by DSC. Most likely, these effects do not come directly from the loss of the Glu negative charges themselves but from the loss of cation-binding sites. There is a complete correlation between DSC and calcium titration results; as more calcium is needed to re-establish the Glu negative charges, it is also needed to recover the purple form in the different mutants, the DSC pretransition appears at lower temperatures. These results, which are a sign of the absence of cations in these multiple mutants, correlate closely with the observed lack of pretransition.

In agreement with the location of cations in the extracellular region, previous DSC results established the absence of the pretransition when the extracellular BC loop is cleaved but its presence when the cytoplasmic loop EF is cleaved (55). On the other hand, normal pretransition is obtained for the cytoplasmatic mutants D36N/D38N and D102N/D102N,2 indicating that neutralization of Asp side chains in the cytoplasmatic region of bacteriorhodopsin.

Proposed model for cation-binding sites in the extracellular region of bacteriorhodopsin. The model is based on the experimental data and on the crystallographic structure of Luecke et al. (5). It is suitable for pH > pK, of cation release. For simplicity, only the side chain ligands to Ca$^{2+}$ are drawn; the other Ca$^{2+}$ ligands are supposed to be water molecules. Location of cations is indicated schematically to illustrate that one divalent cation is linked to both Glu$^{194}$ and Glu$^{204}$, whereas the second divalent cation has only one side chain, Glu$^9$, as ligand. According to the results, the cation linked to Glu$^{194}$/Glu$^{204}$ is assumed to be of higher affinity than that linked to Glu$^9$ (see the text). The protonated water molecule interacting with Glu$^{194}$ represents the proton release group, which can consist of several water molecules. We use Ca$^{2+}$ to symbolize the divalent cations, but the physiologically bound Mg$^{2+}$ may occupy these sites instead.

The two cations placed in the extracellular side may have not only structural significance by helping to maintain the correct structure in the extracellular region (36) and the para-crystalline arrangement, but most probably they are also involved in the functioning of the protein. Especially, the cation linked to Glu$^{194}$/Glu$^{204}$ may be implicated in the maintenance of the optimal spatial relationship between these two residues and the water molecules that form the hydrogen-bonded network. This cation may constitute an essential element of the proton release machinery, by providing the electrical potential necessary to release the proton. In other words, the cation may help to regulate the pK of the proton release group for it to be decreased sufficiently in the proton release step. What is more, the cation may have a gate-like function, allowing the proton release group to be reprotolated from the interior of the protein in the last steps of the photocycle but keeping it isolated from the exterior. In this context, it is known that the blue form does not have a normal photocycle, because of the presence of the already protonated Asp$^{85}$ (13, 47). On the other hand, the purple-deionized form has a normal-like photocycle (56) with a yield of M intermediate around 50% (57), but its proton trans-

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2 C. Sanz and E. Padrós, unpublished experiments.
port capacity has not been measured yet. The possibility of a normal photocycle lacking proton transport has been raised before based on electron-optical studies of purple deionized BR (58).

An intriguing question is why the endogenous cations are not identified in the high resolution crystallographic structures published so far. One reason may be that the methods applied for crystal preparation cause loss of bound cations, which are then substituted by monovalent cations. This is likely to occur, because purple membrane is first delipidated by detergent solubilization and finally placed in a highly concentrated Na/K-Pi (59). It is well established that to recover purple membrane from blue, about 50 times more monovalent than divalent cations are needed (17). Moreover, it is known that monovalently regenerated BR turns blue by dilution (49). These facts indicate that monovalent cations have low affinity for BR and thus have high mobility around the binding sites, making them unlikely to be observed by diffraction techniques.

Finally, it should be indicated that other workers have also proposed the occurrence of cation-binding sites in the extracellular region. A site near Glu194 was proposed based on 13C NMR studies (25). Cation-binding site(s) near the membrane surface was proposed by Fu et al. (29), and a site involving Glu204 was anticipated because of theoretical considerations (30). While this article was in the revision process, a new work appeared claiming for a cation site located at less than 9.8 Å from Glu74 (60). Thus, although no cations have been seen in the crystallographic structures, accumulated evidence suggests the presence of cation-binding sites in BR. Our results place two of them in the extracellular region, linked to Glu9, Glu194, Glu204, and several water molecules.

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