Binding of Calcium Ions to Bacteriorhodopsin

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ABSTRACT Adding Ca^{2+} or other cations to deionized bacteriorhodopsin causes a blue to purple color shift, a result of deprotonation of Asp^{85}. It has been proposed by different groups that the protonation state of Asp^{85} responds to the binding of Ca^{2+} either 1) directly at a specific site in the protein or 2) indirectly through the rise of the surface pH. We tested the idea of specific binding of Ca^{2+} and found that the surface pH, as determined from the ionization state of eosin covalently linked to engineered cysteine residues, rises about equally at both extracellular and cytoplasmic surfaces when only one Ca^{2+} is added. This precludes binding to a specific site and suggests that rather than decreasing the pK_a of Asp^{85} by direct interaction, Ca^{2+} increases the surface pH by binding to anionic lipid groups. As Ca^{2+} is added the surface pH rises, but deprotonation of Asp^{85} occurs only when the surface pH approaches its pK_a. The nonlinear relationship between Ca^{2+} binding and deprotonation of Asp^{85} from this effect is different in the wild-type protein and in various mutants and explains the observed complex and varied spectral titration curves.

INTRODUCTION

Bacteriorhodopsin (BR) is a small (26 kDa) integral membrane protein that functions as a light-driven proton pump in halobacteria (reviewed in Ebrey, 1993; Lanyi, 1993, 1997). It is found in membrane patches of extended two-dimensional hexagonal arrays, termed "purple membrane." Its seven transmembrane helices surround the transversely lying retinal that constitutes the chromophore (Grigorieff et al., 1996; Kimura et al., 1997). The cavity enclosed by the helices also contains protein residues with functional roles and bound water (Pebay-Peyroula et al., 1997; Luecke et al., 1998; Essen et al., 1998). Photoisomerization of the all-*trans* retinal to 13-*cis* initiates a reaction sequence (reviewed in Lanyi and Váró, 1995), the "photocycle," in which relaxations of the retinal and the protein are accompanied by proton transfers inside the protein and on its surface that together constitute the translocation of a proton from one side of the membrane to the other.

The first and critical proton transfer step in this sequence is from the protonated retinal Schiff base to the anionic Asp^{85}. The subsequent photocycle reactions and the transport depend, therefore, on whether this aspartate is initially anionic and thus can be a proton acceptor. Because the negative charge of Asp^{85} is an important part of the counterion to the protonated Schiff base, the protonation state of Asp^{85} also affects the absorption maximum of the chromophore. At pH below its pK_a, whose apparent value is ~2.5, the protein changes reversibly from purple (absorption maximum at 558 nm for dark adapted bacteriorhodopsin) to blue (maximum at 605 nm) (Mowery et al., 1979; Fischer and Oesterhelt, 1979; Edgerton et al., 1980; Kimura et al., 1984; Váró and Lanyi, 1989). This is the well-characterized "acid blue membrane" state (Tsuji and Rosenheck, 1979; Kobayashi et al., 1983; Dupuis et al., 1985; Smith and Mathies, 1985; Albeck et al., 1989; Nasuda-Kouyama et al., 1990). The most important difference from the purple membrane is the protonated state of Asp^{85} (Subramaniam et al., 1990; Metz et al., 1992). Because of lack of an acceptor, the Schiff base of blue membranes does not deprotonate upon illumination, and transport does not occur (Moltke and Heyn, 1995).

A variety of treatments, which include deionization with an ion-exchange resin, will remove bound cations from purple membrane (Padros et al., 1984; Chang et al., 1985; Jonas and Ebrey, 1991) and produce a "deionized blue membrane," with properties very similar or identical to the acid blue membrane. The absorption maximum and other properties of the purple membrane are restored upon the addition of cations to deionized blue membrane. For this reason, bacteriorhodopsin was proposed to be a metalloprotein (Dupuis et al., 1985; Yang and El-Sayed, 1995; Yoo et al., 1995; Tan et al., 1996), with bound cations (in vivo most likely Ca^{2+} or Mg^{2+}) playing various critical roles in the proton transport. These would range from keeping Asp^{85} in the anionic state (Chronister et al., 1986; Corcoran et al., 1987) to participating in the reprotonation switch that determines the direction of proton transfers during the photocycle (Tan et al., 1996; Birge et al., 1996). Indeed, the apparent affinity for the binding of Ca^{2+} and other multivalent cations was found to be very high (Chang et al., 1985; Ariki and Lanyi, 1986; Zhang et al., 1992), suggesting specific binding. Titration experiments gave evidence for the existence of numerous binding sites with varying dissociation constants. It appeared that the site with the second highest affinity was responsible for the color shift,

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whereas the one with the highest affinity had no such effect (Ariki and Lanyi, 1986; Zhang et al., 1992). Furthermore, replacement of various residues near Asp^{85} altered the measured dissociation constants (Zhang et al., 1993). However, the idea of specific binding was contradicted by the fact that the blue-to-purple transition was facilitated by virtually all cations that were tried, ranging from small monovalent cations like Na^+ (Chang et al., 1985; Jonas et al., 1990) to very large organic cations that could not possibly penetrate into the interior of the protein and bind near Asp^{85} (Fu et al., 1997). Furthermore, in the latter study the rate constant of the spectral shift was found to be independent of the size of the cation, arguing for surface binding.

In another model, suggested from electrostatic calculations (Szundi and Stoeckenius, 1989) as well as data (Szundi and Stoeckenius, 1987, 1988), the cations bind to the surface of the membrane rather than to its interior. A critical role of the native acidic lipids in the cation-binding dependent spectral shifts was indicated by a lack of cation effects when these lipids were exchanged for neutral lipids (Szundi and Stoeckenius, 1987, 1988). Indeed, removal of a large portion of the lipids decreases the number of cations bound with high affinity to near zero (Griffiths et al., 1996). Deuterium isotope effects on the luminescence of bound lanthanum indicated that several water molecules are ligands to the bound cation (Ariki et al., 1987). In a recent solid-state NMR study (Tuzi et al., 1999), cation binding perturbed the region at Ala^{196} in the E-F interhelical loop, and the cations were found to exchange rapidly. The simplest explanation for all of these results is binding at the surface. By decreasing the large negative surface potential that originates in the pH region in question, mostly from the charge of lipid headgroups, the binding of cations increases the surface pH to values that approach the bulk pH (Szundi and Stoeckenius, 1989). (Given the fact that the electrical field from fixed charges is not homogeneous at the membrane-water interface, "surface pH" is not a well-defined concept. It depends on the assumption of a layer in which both protons and protonating groups experience the same electrical field. Nevertheless, the term "surface pH" is used widely in the study of bacteriorhodopsin, with the definition of $-\log[H^+]$, because the average $[H^+]$ near the negatively charged surface is greater than in bulk. Our measurements report on the ionization state of covalently bound eosin, and for the sake of simplicity we calculate a local or "surface" pH in the same way as one would calculate a conventional pH if the eosin were in bulk.) This causes deprotonation of Asp^{85} without directly changing its pK_a, by simple reequilibration with the higher local pH. Binding at the membrane surface will, naturally, account for the observed lack of cation specificity.

We have reexamined the question of cation binding, and our results strongly suggest that it occurs nonspecifically at the membrane surface and influences the protonation state of Asp^{85} through the surface pH. We can reconcile this model with the observations that had suggested the specific binding model by showing that the complex spectroscopic titration curves do not necessarily require multiple binding sites but are consistent with the expectations of the surface binding model.

MATERIALS AND METHODS

Purple membrane was prepared by a standard method (Oesterhelt and Stoeckenius, 1974) from wild-type *Halobacterium salinarium* and from strains containing the E9A, E194Q, E204Q, E194Q/E204Q, E194D, E204D, E194D/E204D, V130C, S35C, and D36N/D38N/D102N mutations described elsewhere (Dioumaev et al., 1998; Cao et al., 1995; Brown et al., 1999).

Deionized blue membranes were prepared by repeated centrifugation and resuspension of purple membranes in distilled water and passing through an AG50W-X2 cation exchange resin (BioRad, 100–200 mesh) column (e.g., Ariki and Lanyi, 1986). Spectral titrations were in a Shimadzu UV1601 UV-Vis spectrophotometer. Ca^{2+}/BR ratios refer to total (bound plus free) Ca^{2+} concentration.

Bacteriorhodopsin was labeled in the following way. Eosin-5-iodoacetamide was reacted with V130C or S35C bacteriorhodopsin in 0.1 M NaCl, 50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid] (EPPS), pH 8, overnight at room temperature, at an eosin/BR ratio of 50:1. The dye was added from dimethylformamide stock; the concentration of dimethylformamide in the reaction mixture was ~3%. The membranes were then washed extensively to remove excess label.

Enthalpy changes during titration of deionized blue membranes with $CaCl_2$ were measured with a Microcal (Northampton, MA) Isothermal Titration Calorimetric Unit. The measuring temperature was 25°C; the cell volume was 1.35 ml.

RESULTS

Calcium-dependent deprotonation of Asp^{85}

As described before (Chang et al., 1985; Ariki and Lanyi, 1986; Zhang et al., 1992), adding a cation such as Ca^{2+} to deionized blue membrane shifts the absorption maximum from 605 nm (blue) to 563 nm (purple). Fig. 1 *A* shows spectra at various Ca^{2+}/BR ratios for wild-type bacteriorhodopsin. When the spectra in this blue-to-purple transition were measured at increasing pH instead of increasing $[Ca^{2+}]$, the equilibria were found to contain three species (Mowery et al., 1979; Váró and Lanyi, 1989), but one of the components was present in such a small amount that we can describe the spectral shift adequately by the interconversion of only two states. The isosbestic point in Fig. 1 *A*, at 577 nm, indicates that this treatment of the data is valid. The disappearance of the blue state is followed conveniently at 630 nm. Fig. 1 *B* shows that the titration curve for the blue-to-purple transition is distinctly sigmoid, as found before (Ariki and Lanyi, 1986; Zhang et al., 1992). At first sight, this would imply that there are at least two high-affinity binding sites, and occupancy of the first does not shift the spectrum. Comparison of the curve in Fig. 1 *B* with the limiting absorbance change at very high $[Ca^{2+}]$ indicates that there is at least one additional component, which causes a color shift at Ca^{2+}/BR ratios well above 10 (Ariki and Lanyi, 1986; Zhang et al., 1993). If the curve is assumed to be a binding isotherm, the first two calculated binding constants are 0.25 and 35 μM. The second binding

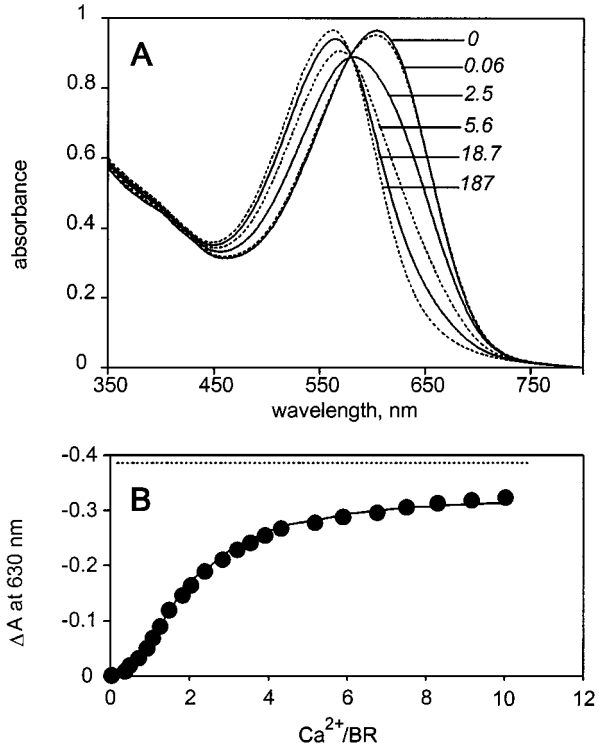

FIGURE 1 Spectroscopic titration of deionized blue membranes, containing wild-type bacteriorhodopsin, with Ca^{2+}. The concentration of added $CaCl_2$ is expressed as the molar ratio of calcium ions to bacteriorhodopsin. (*A*) Spectra at various Ca^{2+}/BR ratios, as indicated. (*B*) Absorption change at 630 nm as a function of Ca^{2+}/BR ratio. Conditions: deionized bacteriorhodopsin, 17 μM, at pH ~5, titrated with 1 mM $CaCl_2$ (stock solution at pH 5). The line represents the binding curve calculated with the equations in the appendix to Ariki and Lanyi (1986), which related the observed absorption changes to the total Ca^{2+} concentration and yielded the binding constants in the text. The dashed line indicates the absorption change at limiting, i.e., 20 mM $CaCl_2$.

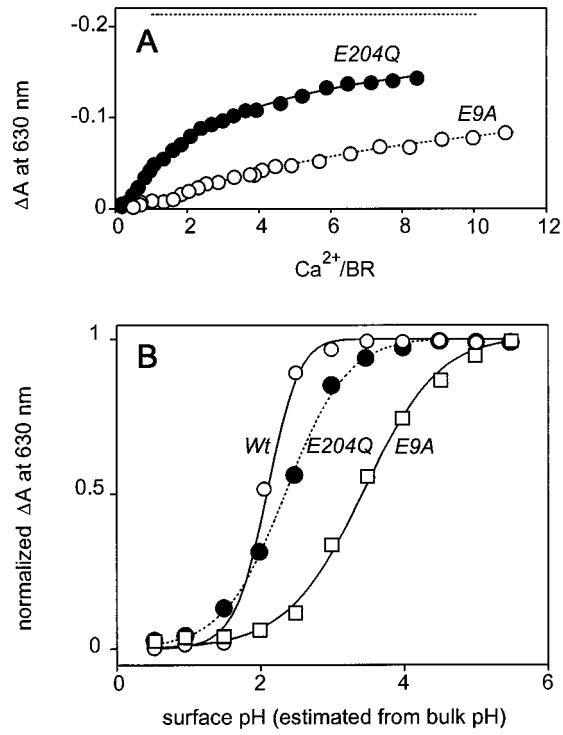

FIGURE 2 Spectroscopic titration of bacteriorhodopsin. (*A*) Titration of deionized blue membranes, containing E204Q (●) and E9A (○) bacteriorhodopsins, with Ca^{2+}, as in Fig. 1 *B*. Conditions are as in Fig. 1. The dashed line indicates the absorption change at limiting, i.e., 20 mM $CaCl_2$. (*B*) Spectroscopic titration of Asp-85 in wild type (○), E204Q (●), and E9A (□) bacteriorhodopsins with H_2SO_4 from an initial pH of 5.5. Conditions: 1.0 M Na_2SO_4, 50 mM citrate, 50 mM phosphate, 17 μM bacteriorhodopsin. In both *A* and *B* the protonation state of Asp^{85} is followed by measuring absorbance at 630 nm.

site is responsible for 75% of the spectral shift, and the rest is due to site(s) of lower affinity.

The interpretation of the spectral titration in terms of binding sites predicts that the sites can be removed, or at least affected, by site-specific mutation of appropriate protein residues. The consequences of some single residue replacements on the spectral shifts have been determined (Zhang et al., 1993). Indeed, the apparent affinities calculated from the curves for mutants of numerous charged or hydrogen-bonding residues in the extracellular region (Asp^{85}, Asp^{212}, Arg^{82}, and Tyr^{185}) were found to be lowered, although the spectral shift was not eliminated. In the present study also, we find that the shape of the titration curves is changed in mutants, specifically in mutants of acidic residues in the extracellular region near Asp^{85}. Glu^{194} and Glu^{204} are buried residues, and their charges have a strong effect on the pK_a of Asp^{85} during the photocycle (Balashov et al., 1996, 1997; Richter et al., 1996; Dioumaev et al., 1998), whereas Glu^{9} is more exposed to the surface, with little such effect. Fig. 2 *A* shows that in E204Q the sigmoid shape of the titration curve is largely eliminated, and in E9A the high-affinity binding site(s) contributes less to the shift than in the wild type, i.e., 50%. Although not shown, the titration curve for E194Q resembles the curve for E204Q. Other mutants tested included E194Q/E204Q, E194D, E204D, E194D/E204D, and D36N/D38N/D102N. All but the last, which resembled the wild type, behaved like E204Q. As before (Zhang et al., 1993), these results would seem, at first, to suggest that although none of the acidic residues in the extracellular region constitute the binding site, the binding of Ca^{2+} must be in their vicinity. The acidic residues at the cytoplasmic surface, Asp^{36}, Asp^{38}, and Asp^{102} appeared to have, on the other hand, no effect on the spectral titrations.

Calcium-dependent increase of surface pH

The alternative model (Szundi and Stoeckenius, 1989) for these titrations is that the deprotonation of Asp^{85} and thus the color shift upon binding of Ca^{2+} originate from an increase in the surface pH. At very low ionic strength these authors calculated the surface pH to be as much as 4 units below the bulk pH. At infinite salt concentration the surface pH will reach the bulk pH, which was 5 under our condi-

tions, and thus Asp^{85} will be essentially fully dissociated. In this model, the titration curves in Figs. 1 *B* and 2 *A* represent partial deprotonation of Asp^{85} as the surface pH approaches the bulk pH with each addition of Ca^{2+}.

In examining this alternative, we first determined the pH dependence of the protonation of Asp^{85}. Fig. 2 *B* shows pH titration of Asp^{85} in wild-type bacteriorhodopsin and in the E204Q and E9A mutants. The salt concentration was 1 M, to ensure that the surface and bulk pH agree within a few tenths of units. The titration of the wild-type protein yields an apparent pK_a of ~2, a value similar to that reported before (e.g., Fischer and Oesterhelt, 1979; Mowery et al., 1979; Váró and Lanyi, 1989). In E204Q and E9A the titration curves are different. At pH < 3 most acidic protein residues are uncharged, and the surface pH will depend on the low pK_a headgroups of lipids, which are not expected to be different in the three membranes tested. The observed differences in this titration curve and those for the two mutants must originate, therefore, from the way in which the local pH at the extracellular surface affects the ionization state of the buried Asp^{85}. In both mutants the apparent number of protons titrated decreases from 1.6 to 0.8–1, and the apparent pK_a's are higher. The latter is particularly evident for E9A.

A way to measure surface pH at low ionic strength and independently of the protonation state of Asp^{85} is with a pH indicator dye covalently attached to the protein surface (Alexiev et al., 1994). The increase in the surface pH as Ca^{2+} is added can thereby be measured. Eosin has the appropriate pK_a (2.5 when bound to Cys^{130} in bacteriorhodopsin; results not shown) for detecting the pH in the region of interest. The calculated pH, defined by the ionization state of the eosin, depends on the geometry of the dye at the surface, i.e., its proximity to charges and distance from the protein/lipid-water interface, but it seems reasonable that it would approximate the effective average surface pH relevant for Asp^{85}. Fig. 3 shows spectra at increasing Ca^{2+}/BR ratios for unlabeled bacteriorhodopsin (Fig. 3 *A*) and for bacteriorhodopsin with eosin covalently linked to the engineered Cys^{130} on the extracellular surface. The differences between these curves and a pH titration curve at 1 M NaCl to calibrate the absorption band of eosin at 537 nm in terms of surface pH yielded the dependency of the surface pH on the Ca^{2+}/BR ratio in Fig. 4 *A*. The pH of 2 for the deionized protein before the addition of Ca^{2+} is approximate, as its value depends on the exact position of the dye at the surface (cf., above). We find that as Ca^{2+} is added, the surface pH changes in such a way as to generate a plausible titration curve for the deprotonation of Asp^{85} (Fig. 4 *B*). Given the fact that it is produced with simplifying assumptions for the response of eosin to surface pH, the pK_a of 2.5 for Asp^{85} from this curve is in remarkable agreement with the value of 2 obtained from the direct pH titration in Fig. 2 *B*. This suggests, by itself, that the effect of Ca^{2+} on Asp^{85} may be through its effect on the surface pH.

The binding site model for the spectral shift could be now put to a direct test. Because the spectral shift is caused by deprotonation of Asp^{85} near the extracellular surface, the observation that it is linked to the second Ca^{2+} that binds (Fig. 1 *B*) requires that either 1) the first and second Ca^{2+} ion both bind at the extracellular surface, but their effects on Asp^{85} are dependent on the exact location of the binding; or 2) the first Ca^{2+} binds at the cytoplasmic surface, where it would not influence Asp^{85}, whereas the second Ca^{2+} occupies a site at the extracellular surface. The surface pH was therefore measured also with eosin covalently linked to the engineered Cys^{35} on the cytoplasmic surface. The absorption changes from eosin in this experiment, as well as from one with eosin linked to Cys^{130} at the extracellular surface, are compared in Fig. 5. It appears from Fig. 5 that even at very low Ca^{2+}/BR ratios (between 0 and 1), the local pH is raised on both surfaces and by about the same extent. This finding indicates that at an average of one Ca^{2+} bound per BR, the Ca^{2+} binding is to both surfaces. It is incompatible therefore with either alternative above and rules out the existence of a specific binding site as the cause of the spectral shift.

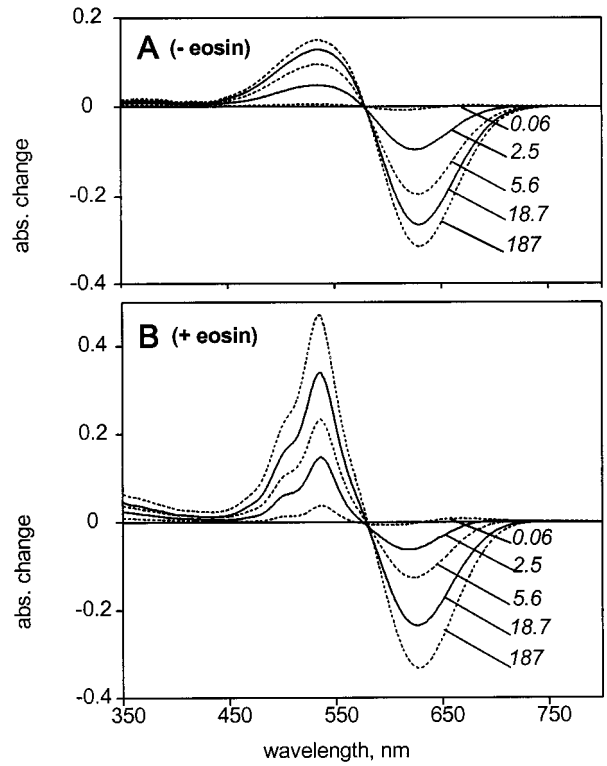

FIGURE 3 Spectroscopic titration of bacteriorhodopsin, with and without eosin covalently linked to Cys^{130} in the V130C mutant. (*A*) Difference spectra relative to deionized blue membrane for the wild-type protein. The spectra originate from the blue shift shown in Fig. 1 *A*. (*B*) Difference spectra relative to deionized blue membrane for the labeled V130C mutant. The spectra contain a pH-dependent contribution from eosin, in addition to the blue shift of bacteriorhodopsin. In both *A* and *B* the spectra are shown at the indicated Ca^{2+}/BR ratios. Earlier results (Cao et al., 1995) had shown that the dye at residue 130 (or at residue 35) does not perturb the protein: the photocycles and the spectra of the V130C and S35C mutants, with or without dye label, were the same as in the wild type. Conditions are as in Fig. 1, but with 13 μM bacteriorhodopsin, and the titration was with 1 mM $CaCl_2$.

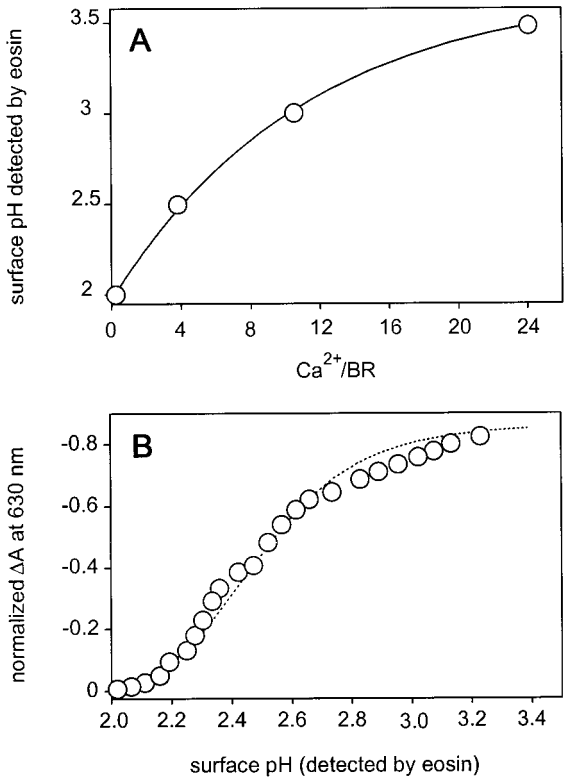

FIGURE 4 Calculated surface pH and the relationship of the protonation state of Asp^{85} to this surface pH. (*A*) Surface pH detected by eosin at various Ca^{2+}/BR ratios in deionized blue membrane, calculated from the spectra shown in Fig. 3. The absorption of bound eosin at various surface pH values was determined by a pH titration in 1 M NaCl (not shown). (*B*) Absorption change at 630 nm at various added Ca^{2+}/BR ratios (from Fig. 1 *B*) as a function of calculated surface pH (from *A*).

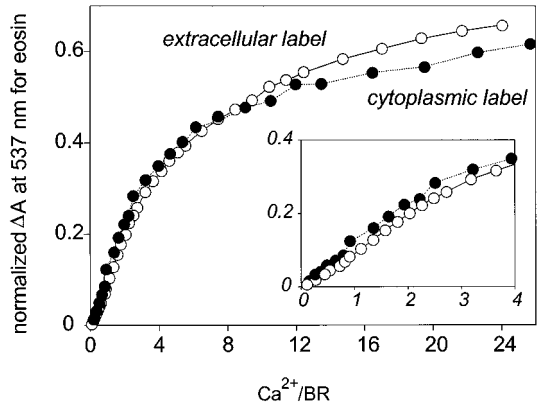

FIGURE 5 Increase in local pH at the extracellular (○) and cytoplasmic (●) surfaces, as detected by eosin covalently bound to either Cys^{130} in the V130C or Cys^{35} in the S35C mutant. The pH-dependent absorption band of eosin at 537 nm (cf. Fig. 3 *B*) is plotted versus the Ca^{2+}/BR ratio. The two sets of absorbance changes were normalized to one another by a correction factor of 0.8 to account for variability among samples. *Inset*: Absorption changes between zero and 4 Ca^{2+}/BR.

Calorimetric titration of Ca^{2+} binding

The heat generated upon binding of Ca^{2+} to deionized blue membranes was measured in a calorimetric titration analogous to the spectral titration in Fig. 1. As Fig. 6 shows, heat evolution is observed at Ca^{2+}/BR ratios well below 1, consistent with the prediction that the sigmoid shape of the spectral titration curves is not caused by lack of binding at low [Ca^{2+}], e.g., from cooperative binding. It is further evident from Fig. 6 *B* that the binding as detected by calorimetry is saturable, like the binding detected from the spectral shift.

However, the comparison of the calorimetry with the spectral titration is more direct when the heat evolved is plotted as a cumulative quantity. Fig. 7 relates cumulative released heat with the calculated surface pH, as in Fig. 4 *B*. The curves for the wild-type protein and several mutants (offset from one another vertically for clarity) appear to describe the upper (higher pH) part of a titration curve with a $pK_a \leq 2$. The data in Fig. 7 show that the binding of Ca^{2+}, as detected by the release of heat, follows the ionization of groups with pK_a's much lower than those of most protein side chains and occurs only when the surface pH is below ~2.5. This directly implicates lipid headgroups in the binding. The fact that different mutants with different apparent affinities for Ca^{2+} (Fig. 2 *A*) exhibit similar titrations when measured calorimetrically is consistent with this model.

Thus the ΔH measured originates at least partly from the deionization of acidic groups of the lipids when Ca^{2+} is bound. There are eight lipid phosphate groups and one sulfate per bacteriorhodopsin (Kates et al., 1982). The estimated heat of ionization is 7.5 kJ/mol for the lowest pK_a (2.1) of phosphate and 21.6 kJ/mol for the lowest pK_a (1.9) of sulfate. (Heats of ionization were calculated from the temperature dependencies of the lowest pK_a's of phosphoric acid and sulfuric acid (Perrin, 1969).) Together, these account for about a third of the heat released as Ca^{2+} is bound and the surface pH is increased from its theoretical lower limit to above 2.5. The rest of the heat of the reaction comes from the binding of Ca^{2+} and deprotonation of Asp^{85} and perhaps other carboxyl groups, such as Asp^{212}, which also has a low pK_a, but the magnitudes of these are uncertain.

DISCUSSION

We have attempted to answer the longstanding unresolved question of the nature of cation binding to deionized bacteriorhodopsin. Calculations of surface electrostatics and a variety of experimental results that appeared to contradict one another have argued for different ways in which cations added to deionized blue membranes might affect the ionization state of Asp^{85}. Deciding between the two conflicting models, i.e., 1) specific binding inside the protein that lowers the pK_a of Asp^{85} and 2) nonspecific binding that raises the pH at the extracellular surface, would be possible if one of these models could be ruled out and all of the varied observations could be interpreted in terms of the other model.

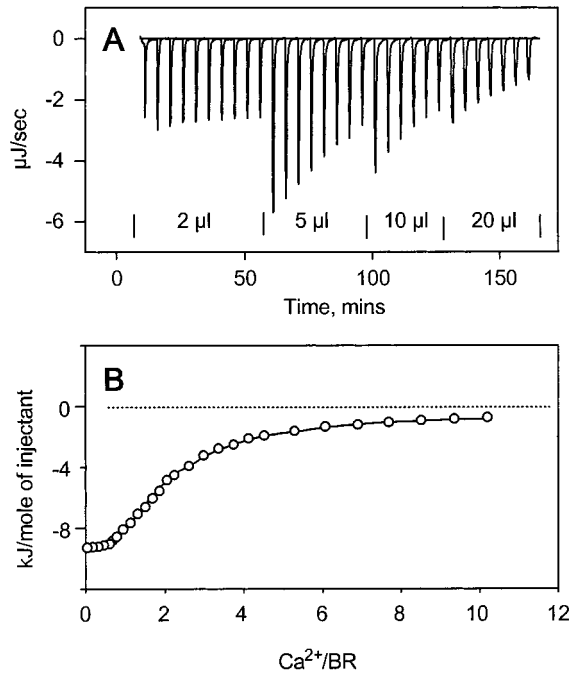

FIGURE 6 Calorimetric titration of deionized blue membrane, containing wild-type bacteriorhodopsin, with Ca^{2+}. Deionized blue membranes (100 μM wild-type bacteriorhodopsin) were titrated with 5 mM $CaCl_2$. (*A*) Traces of released heat measured upon the addition of indicated amounts of $CaCl_2$. (*B*) Calculated heat released in the titration experiment in *A*.

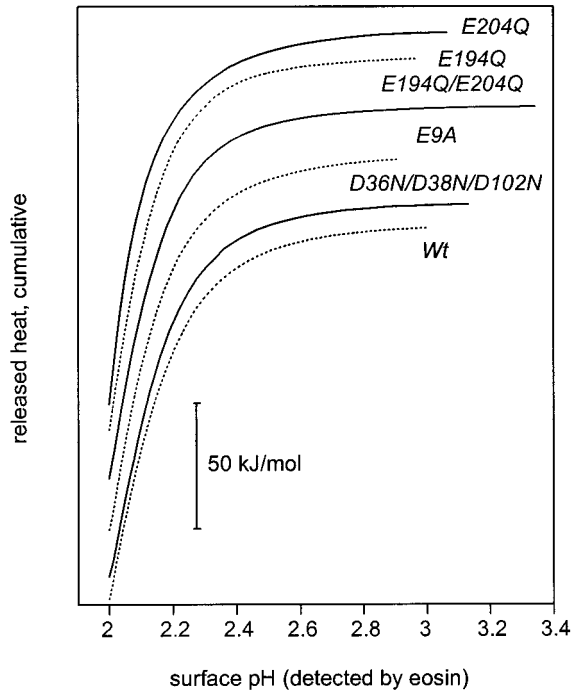

FIGURE 7 Cumulative released heat in the titration of deionized blue membrane with $CaCl_2$, expressed as a function of the increasing surface pH. ΔH is plotted for wild-type bacteriorhodopsin and the E194Q, E204Q, E194/E204Q, and D36N/D38N/D102N mutants. Surface pH is estimated from the relationship of the absorption change of eosin covalently linked to Cys^{130}, as in Figs. 3 and 4, and is assumed to be the same in the titration of all of these samples. The traces are displaced vertically from one another for clarity.

If cation binding is specific, the putative two high-affinity binding sites calculated from the spectral titration (the K_D's for the wild-type protein are 0.25 and 35 μM, respectively; cf. Fig. 1 *B*) will be occupied sequentially as the Ca^{2+}/BR ratio is raised from 0 to 1 and then to 2 (the concentration of bacteriorhodopsin in these titrations was 17 μM). Apart from its effect on Asp^{85}, the positive charge of the cation will lower the negative surface potential on that side of the transmembrane dielectric barrier where the binding site is located. This will raise the surface pH, which is lower than the bulk pH at the negatively charged surface of the membrane. The unexpected observation we now report is that the surface pH is raised equally at both membrane surfaces as [Ca^{2+}] raised, even at a Ca^{2+}/BR ratio of 1 (Fig. 5). This contradicts the model of binding to specific sites. Ca^{2+} binding, as detected by the change in surface pH it causes, is roughly equal at the extracellular and cytoplasmic surfaces. (It has been reported (Heberle et al., 1994; Alexiev et al., 1995) that during the photocycle the released protons rapidly equilibrate between the two membrane surfaces. However, if the charge density were changed at only one of the surfaces, the local pH at the other surface would change very little, because the proton concentrations at the surfaces are in equilibrium with the large reservoir of protons in the bulk.) The cation must bind to sites that exist on both membrane surfaces, i.e., negatively charged lipids. Nonspecific but high-affinity binding is not inconsistent with the curved Scatchard plots reported (Zhang et al., 1992). Thus binding of Ca^{2+} must affect the protonation state of Asp^{85} through its effect on the pH at the extracellular surface.

We find that, although at first the spectral titration of the wild-type protein and the mutants (Figs. 1 *B* and 2 *A*) seemed to argue for the existence of multiple binding sites, the surface pH model accounts well for the complex shapes of the curves. As indicated in Fig. 4 *A*, when Ca^{2+} is added the surface pH is raised monotonically. The surface pH of deionized membrane is well below the pK_a of Asp^{85} (pH $\approx$ 2 from eosin, but its true value is likely to be lower, estimated to be between 1 and 1.5 by Szundi and Stoeckenius (1989)). As the Ca^{2+} is added, the surface pH rises, but the extent of the deprotonation of Asp^{85} is initially small. As the surface pH approaches the pK_a (cf. Fig. 2 *B*), the deprotonation (and therefore the observed spectral shift) becomes greater. Thus a nonlinear function (the dependency of the ionization state of Asp^{85} on pH) is interposed between the effect of Ca^{2+} and the observed spectral changes. This will produce the sigmoid titration curve in Fig. 1 *B*. The different dependencies of the deprotonation of Asp^{85} on pH in the mutants examined (Fig. 2 *B*) will result in different titration curves, as seen in Fig. 2 *A*. A reason for this can be found in the shapes of the pH titration curves (Fig. 2 *B*). The deprotonation in E204Q with increasing pH follows a less steep curve than in the wild type (Fig. 2 *B*), and thus

deprotonation begins already at low Ca^{2+}/BR ratios. This will result in a less sigmoid titration curve (Fig. 2 *A*). Because the apparent pK_a is higher in this mutant, and even higher in E9A (Fig. 2 *A*), the surface pH achieved even at high Ca^{2+}/BR ratios will be insufficient to allow extensive deprotonation until much higher Ca^{2+}/BR ratios are provided than in the wild type (Fig. 2 *A*). These differences in the spectroscopic titrations of the various mutants stand in sharp contrast to the calorimetric titration curves that are similar for all samples (Fig. 7) and suggest that the mutations affect not the cation binding, but the influence of surface pH on the protonation state of Asp^{85}.

These considerations lead to the conclusion that the titration curves with cations should be interpreted as nonspecific binding to the fixed charges of the lipids at the membrane surfaces, which affects the protonation state of Asp^{85} entirely by raising the surface pH.

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