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Effects of genetic replacements of charged and H-bonding residues in the retinal pocket on Ca^{2+} binding to deionized bacteriorhodopsin

(bacteriorhodopsin mutants/Asp-212/Tyr-185/Asp-85/Arg-82)

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ABSTRACT **Metal cations are known to be required for proton pumping by bacteriorhodopsin (bR). Previous studies found that bR has two high-affinity and four to six low-affinity Ca^{2+}-binding sites. In our efforts to find the location of these Ca^{2+} sites, the effects of replacing charged (Asp-85, Asp-212, and Arg-82) and H-bonding (Tyr-185) residues in the retinal pocket on the color control and binding affinity of Ca^{2+} ions in Ca^{2+}-regenerated bR were examined. The important results are as follows: (*i*) The removal of Ca^{2+} from recombinant bR in which charged residues were replaced by neutral ones shifted the retinal absorption to the blue, opposite to that observed in wild-type bR or in recombinant bR in which the H-bonding residue, Tyr-185, was replaced by a non-H-bonding amino acid (Phe). (*ii*) Similar to the observation in wild-type bR, the binding of Ca^{2+} to the second site gave the observed color change in the recombinant bR samples in which charged residues were replaced by neutral ones. (*iii*) The residue replacements had no effect on the affinity constants of the four to six weakly bound Ca^{2+}. (*iv*) The two high-affinity sites exhibited reduced affinity with substitutions; while the extent of the reduction depended on the specific substitution, each site was reduced by the same factor for each of the charged residue substitutions but by different factors for the mutant where Tyr-185 was replaced with Phe(Y185F). The above results suggest that the two Ca^{2+} ions in the two high-affinity sites are within interaction distance with one another and with the charged residues in the retinal pocket. The results further suggest that, while the interaction between Tyr-185 and the high-affinity Ca^{2+} ions is relatively short range and specific (with more coupling to the Ca^{2+} ion in the second affinity site), between the charged residues and Ca^{2+} ions it seems to be of the electrostatic (e.g., ion–ion) long range, nonspecific type. Although neither Asp-85, Asp-212, nor Arg-82 is individually directly involved in the binding of Ca^{2+} in these two sites, they might all participate in it. Together with the protonated Schiff base, the charged residues along with Tyr-185 and one or two Ca^{2+} ions (and probably a few water molecules) seem to form an electrostatically coupled system that is part of a cavity that controls the color and function of bR.**

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium halobium* (1, 2). It contains a polypeptide chain of 248 amino acid residues (3), as well as a single retinylidene chromophore bound via a protonated Schiff base (PSB) to the ε-NH_2 group of Lys-216. Upon absorption of a photon, bR undergoes a photocycle (4) during which the all-trans to 13-cis isomerization of the chromophore takes place followed by the deprotonation of the PSB, and protons are pumped across the cell membrane. The resulting electrochemical proton gradient is then used by the bacteria for some metabolic processes (e.g., ATP synthesis) (4, 5).

Well-washed purple membrane patches contain 3–4 mol of Mg^{2+} and ≈1 mol of Ca^{2+} per mol of bR at a bulk pH of ≈5.5 (6). Removal of these cations from purple membrane patches (6, 7) or acidification (2, 8, 9) causes a color transition from purple to blue. The absorption maximum of the retinal chromophore is believed to be primarily regulated by the electrostatic interactions between the charged or polar side chains of amino acids and the PSB, as well as certain carbon atoms along the retinal electronic system (10–14). From the structure (15) and genetic modification studies of bR (16, 17), it has been found that Asp-85, Asp-212, and Arg-82 are charged groups in the retinal pocket. They are suggested to make a major contribution to the regulation of color of bR (17).

Different models have emerged on the role of metal cations in purple membrane patches. Szundi and Stoeckenius (18–20) treated the metal ions as free positive charges distributed uniformly on the membrane surface, which regulate the surface pH via the Gouy–Chapman effect. In this model, the removal of the cations increases the negative surface charge density, which lowers the surface pH and causes the protonation of the aspartate counter ion(s) of the Schiff base. This change induces the purple-to-blue transition. The essential point of this model is that cations affect the color transition only by changing the surface pH. The fact that deionization no longer has an effect on the spectrum of the purple membrane once the acidic lipids are removed or exchanged for neutral lipids gave strong evidence for this model (18) and its implication that metal cations are bound to the negatively charged surface of the purple membrane patch bR via nonspecific electrostatic attraction.

Another model proposed by several other groups (21–32) emphasizes the existence of specific binding between some of the metal cations and negatively charged groups (e.g., carboxylate groups of Asp and Glu side chains) inside the protein, without excluding possible binding of some cations on the membrane surface (12, 18, 31). It was proposed (6, 22–24, 32) that protonation changes near the Schiff base are directly linked to the binding of cations to the bR molecule. Evidence (31–33) has supported the direct involvement of at least one cation in a special binding site, which would aid in maintaining the ionization state of key amino acids in the active site and thereby control the color of the purple membrane.

To characterize the cation binding sites, various methods [e.g., electron spin resonance spectroscopy for Mn^{2+} (26, 28), filtration techniques for Ca^{2+} and Hg^{2+} (27), and steady-state fluorescence methods for Eu^{3+} (30)] have been used to measure the binding constants between deionized bR and different cations. All of them reached the conclusion that there are more than two classes of cation binding sites in

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Abbreviations: bR, bacteriorhodopsin; PSB, protonated Schiff base.

deionized blue membrane, with different binding affinities and therefore different binding environments. In our recent studies (31), we developed a technique for studying the binding characteristics of the naturally bound ion Ca^{2+} to deionized bR. We have used a Ca^{2+}-selective-electrode potentiometric titration technique and found (31) two high-affinity and four to six low-affinity Ca^{2+} binding sites in deionized blue membrane suspended in deionized, distilled water. We determined the association constants for the two most tightly bound Ca^{2+} to be $K_1 = 0.63\ \mu M^{-1}$ and $K_2 = 0.14\ \mu M^{-1}$, respectively at pH 4. In addition, we found that the binding constant of the second site is comparable in magnitude to that determined from spectrophotometric titration of the purple-to-blue color change. This suggests that Ca^{2+} in the second site induces color changes, in agreement with the conclusion of Ariki and Lanyi (32). This conclusion is also consistent with the previous suggestion (29) that the binding to the second site affects the charge distribution within the retinal pocket either directly (by being physically nearby) or indirectly (by changing the protein conformation around the pocket from a distance). Recently, Jonas and Ebrey (33) suggested that the binding site for cation responsible for the color change might involve Asp-85, Asp-212, Arg-82, or Tyr-185. While energy transfer studies between Eu^{3+} (30) and retinal suggest a short retinal–cation distance, a more direct method must be devised to examine whether or not the metal cation responsible for the color change is present within the retinal cavity. In the present work, we have performed spectroscopic and binding studies on some of the individual mutants in which functionally important residues known to be in the retinal pocket are replaced, one or two at a time, by neutral amino acids. The binding constants as well as the effect of removing the metal cations on the chromophore absorption of these mutants are compared with those for wild-type bR.

MATERIALS AND METHODS

Clones containing the three residue replacements studied were constructed from *H. halobium* strain L-33 by transformation with a shuttle vector containing the modified *bop* gene, as described (34). The recombinant bRs were isolated as purple membrane sheets and stored at −70°C in 40% (wt/wt) sucrose.

Steady State Absorption Spectra of the Mutated bRs at Different pH Values. The initial pH of the bR samples in 40% sucrose was ≈6.5. The acidification and alkalization of the sample were done by the addition of microliter quantities of 0.1 M and 1.0 M HCl or NaOH at different stages, respectively. The samples were kept in 40% sucrose to minimize aggregation at low pH values. bR concentrations were ≈10 μM. The pH of the sample was measured by a digital pH meter (Beckman model F71). All steady-state absorption spectra were recorded immediately after pH measurement, with a diode-array spectrophotometer (Hewlett–Packard 8451). All samples studied in these measurements were light-adapted except for mutant Y185F (Tyr-185 replaced with Phe) and double mutant R82Q/Y185F (Arg-82 replaced with Gln and Tyr-185 replaced with Phe).

Binding Experiments. The samples were washed three times by centrifugation to remove sucrose (except for the D85N/R82Q double mutant in which case dialysis was used instead due to the difficulty of pelleting out the small membrane patches). Sample pH was ≈6 before deionization. Deionization was performed by dialyzing the sample in a beaker containing Bio-Rad AG 50W-X4 cation-exchange beads and distilled, deionized water. When small amounts of sample were handled, this method proved to be better than passing the sample through the ion-exchange column. The resulting deionized sample had a pH of ≈4.0 and was used without any further pH adjustment. Only polyethylene and Teflon containers were used to prevent contamination by metal ions from glassware.

The binding experiment was carried out at room temperature as described in our previous paper (31). A sample of 2 ml of deionized bR at a given pH was titrated by addition of microliter quantities of 0.01 or 0.1 M $CaCl_2$. After each addition, the free Ca^{2+} concentration was determined by measuring the electrical potential in millivolts (Beckman pH meter model F71) using a calcium ion-sensitive electrode (model 93-20; Orion, Cambridge, MA) against a double junction reference electrode (Orion 90-02). A duplicate run using the same volume of deionized distilled water in place of the deionized bR sample served as a calibration curve for obtaining the free calcium ion concentration. Each experiment was repeated at least twice to ensure reproducibility. In the case of D212N, Y185F, and R82Q/Y185F, the binding experiment was carried out in the dark because deionized D212N is not stable under light exposure and because the spectra of light-adapted Y185F mutants indicate that they are a mixture of two spectrally distinct species. All other samples were light adapted.

The variation of bulk pH during the titration was monitored in control experiments as described (31).

RESULTS AND DISCUSSION

If the metal cation binding sites are not far from the retinal pocket, we expect the following: (*i*) the shift in the retinal absorption spectrum upon deionization will be sensitive to the charge and location of residues within the retinal binding pocket and, therefore, to their replacements by neutral residues; and (*ii*) if any of the Asp-85, Asp-212, Arg-82, or Tyr-185 residues directly participate in cation binding, isomorphic replacement of these residues will have major consequences on the values of the corresponding association constants or even abolish binding altogether. To understand the relationship between cations and the retinal chromophore, as well as the possible interaction between cations and these charged residues inside the retinal pocket, we investigated the effects of removing metal cations on the retinal absorption of the different bR variants as well as on their binding characteristics.

The Effect of Removing Metal Cations on the Chromophore Absorption Spectra. The absorption spectra of all the samples upon acidification and deionization were studied, and their absorption maxima are summarized in Table 1. From these steady-state absorption data, we made the following observations and possible conclusions:

(*i*) Deionization of D85N, D212N, R82Q/D85N, and R82Q/Y185F blue shifts their absorption maxima by 20, 50, 10, and 10 nm, respectively. This is opposite to what is observed in wild-type bR and in the Y185F mutant, for which deionization red shifts the λ_{max} by 38 and 34 nm, respectively. Thus the direction of the shift (i.e., red or blue) upon deionization is sensitive to whether the residue replaced is charged or neutral. Furthermore, replacement of the neutral residue preserved the direction of the absorption shift as in bR while the replacement of charged residues did not. This points out the importance of the coupling between the charged residues, the Ca^{2+} ion(s), and the PSB in controlling the retinal absorption in bR.

(*ii*) All the mutant samples begin to aggregate at pH 3, and the aggregation becomes very serious at pH 2. No denaturation or bleaching of the chromophore was observed for any of these five mutant samples even at pH values as low as 1.8.

(*iii*) The deionized forms of D85N, R82Q/D85N, Y185F, and R82Q/Y185F absorb at the same wavelength as their acidified forms (pH < 3). Upon deionization or acidification, the λ_{max} of D85N is decreased by 20 nm, from 616 to 596 nm.

Table 1. Absorption maxima of wild-type (wt) bR and the recombinant bRs D85N, R82Q/D85N, D212N, Y185F, and R82Q/Y185F at different pH values and upon deionization

Sample	λ_{max}, nm				
	pH 7.8 ($\Delta\lambda_{Mut}$*)	pH 6.5	pH 4.0	pH 2.0	Deionized ($\Delta\lambda_{Deion}$†)
D85N	616 (+48)	616	606	596	596 (−20)
R82Q/D85N	586 (+18)	586	576	576	576 (−10)
D212N	586 (+18)	576	558	586	536 (−50)
Y185F‡	556 (−12)	556	574	590	590 (+34)
R82Q/Y185F‡	578 (+10)	578	574	568	568 (−10)
wt bR	568 (—)	568	568	606	606 (+38)

*$\Delta\lambda_{Mut}$ = λ_{max} of mutant at pH 7.8 − λ_{max} of wild type at pH 7.8.
†$\Delta\lambda_{Deion}$ = λ_{max} of deionized sample − λ_{max} of sample at pH 7.8.
‡Data shown is for dark-adapted samples.

On the other hand, the λ_{max} of Y185F is increased by 34 nm, from 556 to 590 nm. The two double mutants R82Q/D85N and R82Q/Y185F both have relatively small decreases in absorption maximum (8–10 nm) upon deionization and acidification.

(*iv*) As shown in Table 1, deionization of D212N results in the largest blue shift of all the mutants studied that are made by replacement of the charged residues. This suggests that D212 has a stronger coupling, either with the PSB or with the color-controlling Ca^{2+} ion(s) in bR. The fact that the D85N mutation gives rise to more red shift (48 nm) than D212N (18 nm) suggests that the charge on D85N is more strongly coupled to the positive charge of the PSB than D212. Thus the fact that the effect of deionization causes much larger blue shift in D212N than in D85N must suggest that D212 is much more strongly coupled to the color-controlling Ca^{2+} ion(s) than the other charged residues. This conclusion is also supported by the fact that the D212 replacement gives the largest reduction in the second Ca^{2+} affinity constant.

(*v*) Table 1 shows that, while all other mutants show similar λ_{max} values for the deionized and acidified forms (pH < 3), the λ_{max} for D212N in deionized form is 50 nm blue shifted from its acid form at pH 2. This suggests that the structure of the cavity around the retinal is different for the deionized and low pH species in the D212N mutant but similar for all the other mutants as well as for the wild-type bR.

(*vi*) As shown in Fig. 1, the deionized D212N is bleached after exposure to room light for 30 min. This was not observed for the acidified sample of D212N. This again suggests that the two forms of this mutant do not have identical structures. The addition of Ca^{2+} to the deionized sample in the dark reversed the spectral shift, and the regenerated sample became light insensitive. This suggests that cations play a key role in restoring the photostability of the D212N mutant sample.

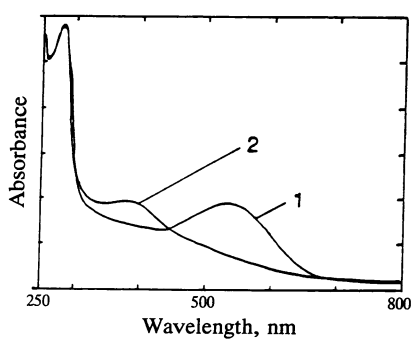

FIG. 1. Photostability of deionized D212N. UV and visible steady-state absorption spectra of the deionized D212N mutant in the dark (curve 1) and after exposure to light for 30 min (curve 2). The latter resembles a bleached bR sample with retinal detached from its binding site.

(*vii*) It is known that Asp-85 is an important counterion of the PSB (16, 17), and as such its protonation state and its distance from the PSB determine the absorption maximum of the retinal chromophore. When the Asp-85 residue is absent in the protein, our results show that the λ_{max} of the retinal is still affected by the pH change, which suggests that in the acid pH region the color of bR is not solely controlled by the protonation of Asp-85, but that the protonation state of the other negatively charged groups also regulates the charge distribution around the active site and thus affects the chromophore.

Ca^{2+} Binding to the Deionized Recombinant bR Samples. Equilibrium association constants for the calcium ion with the deionized samples were obtained potentiometrically as described in *Materials and Methods* and by using Scatchard plots (35).

For the equilibrium deionized bR (D) + Ca^{2+} ⇌ regenerated bR (R), one writes the associated binding constant as $K = [R]/[D][Ca^{2+}]$. Assuming that there exists a number of independent classes of Ca^{2+} binding sites in the deionized molecule, then for a particular class of sites (35, 36) $\nu/c = K(n - \nu)$, where n is the number of sites having the same binding affinity (i.e., in the same class), ν is the number of bound Ca^{2+} per bR molecule, and c is the free equilibrium Ca^{2+} concentration in solution. Therefore in the plot of ν/c versus ν (the Scatchard plot), the slope gives $-K$ and the ν intercept gives n.

Fig. 2 shows Scatchard plots of Ca^{2+} bound to deionized bR and to each of the five deionized bR mutants at an initial pH of 4 in aqueous suspension. The pH change during the experiment was found to be ≈0.2 unit as shown previously (31). The corresponding binding constants for each class of sites in comparison with those for wild-type bR under the same conditions are listed in Table 2.

The important results and possible conclusions are summarized as follows:

(*i*) The five recombinant proteins exhibit similar binding stoichiometry with Ca^{2+} as wild-type bR—namely, two high-affinity sites with distinguishable K values and four low-affinity sites (as shown in Fig. 2). This suggests that none of the amino acids substituted binds directly to Ca^{2+}.

(*ii*) The spectroscopic titration curves of D85N and D212N shown in Fig. 3 suggest that, as observed for native bR (Fig. 3 *Top*), the binding of the second Ca^{2+} causes the color transition to take place in these mutants. This suggests that the order of the affinity constants does not change upon amino acid substitutions. In the cases of Y185F and R82Q/Y185F, addition of Ca^{2+} to deionized samples does not immediately restore the native color during the course of the titration experiment. The color restoration in Ca^{2+}-regenerated Y185F and R82Q/Y185F was observed 2 weeks after the binding experiments were performed. Thus the replacement of Y185 creates a considerable barrier in the binding kinetics of the color-controlling Ca^{2+} ion(s).

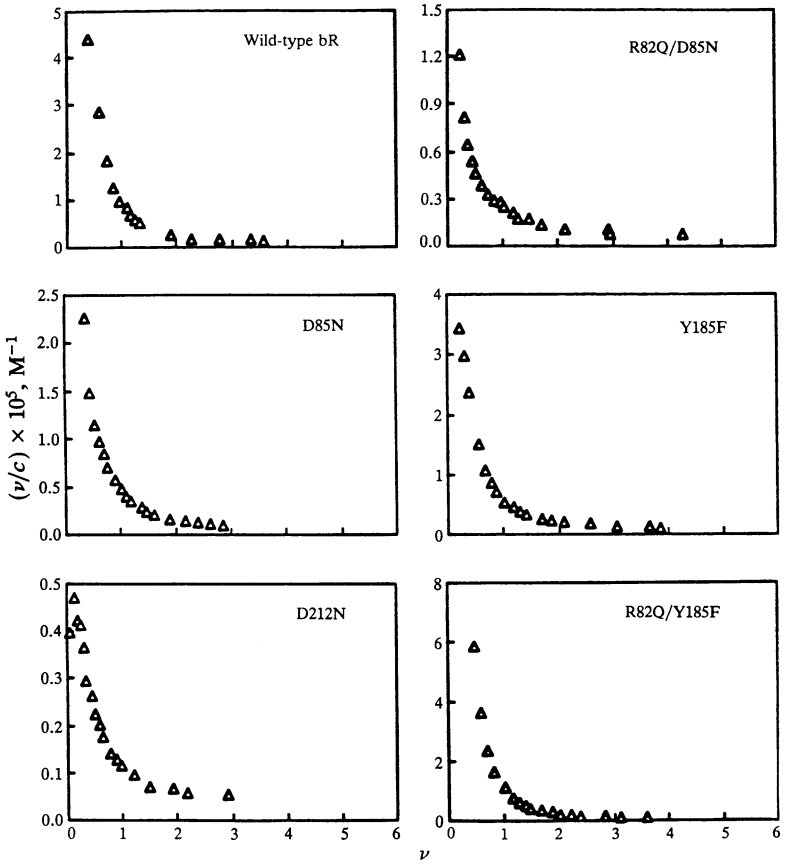

FIG. 2. Scatchard plots of Ca^{2+} binding to 40 μM deionized wild-type bR and recombinant bRs D85N, D212N, R82Q/D85N, Y185F, and R82Q/Y185F (sample pH = 4). *c*, [free Ca^{2+}]; ν, [bound Ca^{2+}]/[bR mutant]. All mutants show two high-affinity sites (but with reduced binding constants from those in regenerated bR) and four to six low-affinity sites with comparable constants to those in regenerated bR (see Table 2).

(*iii*) As shown in Table 2, charged residue replacement of D85N, D212N, or R82Q/D212N reduces both K_1 and K_2 by the same factor whose value depends on the mutant. This suggests that the Ca^{2+} ions in the high-affinity sites are *not* very *far* from the retinal pocket. Furthermore, these results suggest that either these two sites are not far from one another or else they are somehow coupled with each other indirectly via coupling to the charged amino acid residues. The former conclusion agrees with a previous one (37) based on intermetal energy transfer results that placed the distance between the two high-affinity sites at 7–8 Å in regenerated bR.

(*iv*) The charged residue replacement in D212N has the largest effect on the values of K_1 and K_2 (reduction by a factor of $\approx$15) compared to the other mutants (reduction by factors of 3 and 5 for D85N and R82Q/D85N, respectively). This suggests that the charge on D212 is coupled more effectively to the Ca^{2+} ions in these binding sites. This is consistent with conclusion *iv* in the previous section, which is based on the observed effect of removing metal cations on the chromophore absorption maximum. These observations support specific binding for the Ca^{2+} ions in the two high-affinity sites because D212 is too far from the membrane surface to have much surface potential effect.

(*v*) The additional replacement of Arg-82 in R82Q/D85N reduces K_1 and K_2, whereas in R82Q/Y185F it increases them.

(*vi*) In contrast to mutants with charge residue replacements, the extent of the reduction of K_1 and K_2 is different in Y185F (factors of 0.7 and 0.3, respectively). In R82Q/Y185F, K_1 actually increased by 1.6 and K_2 decreased by a factor of 0.6. These results suggest that Y185 interacts with Ca^{2+} ions via shorter range type interaction, which distinguishes between the Ca^{2+} in the first and second affinity sites; it is closer to the one in the second affinity site (the one that controls color).

(*vii*) Mutations have very small effects on the binding affinities of the low-affinity sites. This is consistent with the previous proposal that these sites are located on the membrane surface (31, 37). It is also consistent with recent conclusions made from the observed effect of removing the C terminus on the binding of Ca^{2+} to deionized bR (Y.N.Z. and M.A.E.-S., unpublished results).

Conclusions. Residue replacements of charged and H-bonding (neutral) residues in bR produce opposite behav-

Table 2. Association constants of Ca^{2+} with deionized wild-type (wt) bR and deionized recombinant bRs D85N, R82Q/D85N, D212N, Y185F, and R82Q/Y185F at pH 4

Sample	K_1, μM^{-1}	K_1/K_1 (wt bR)	K_2, μM^{-1}	K_2/K_2 (wt bR)	K_3, mM^{-1}	K_3/K_3 (wt bR)
D85N	0.18 ± 0.02	0.3	0.046 ± 0.006	0.3	3.3 ± 0.5	0.8
R82Q/D85N	0.10 ± 0.01	0.2	0.025 ± 0.003	0.2	2.8 ± 0.5	0.7
D212N	0.04 ± 0.004	0.07	0.01 ± 0.001	0.07	1.7 ± 0.5	0.4
Y185F	0.45 ± 0.05	0.7	0.04 ± 0.1	0.3	4.0 ± 0.4	1.0
R82Q/Y185F	0.95 ± 0.1	1.6	0.085 ± 0.015	0.6	3.7 ± 0.4	0.9
wt bR	0.6 ± 0.05		0.15 ± 0.01		4.0 ± 0.6	

Each binding experiment was repeated at least twice, and the relative deviation was $\approx$10% for K_1 and K_2 and 10–30% for K_3.

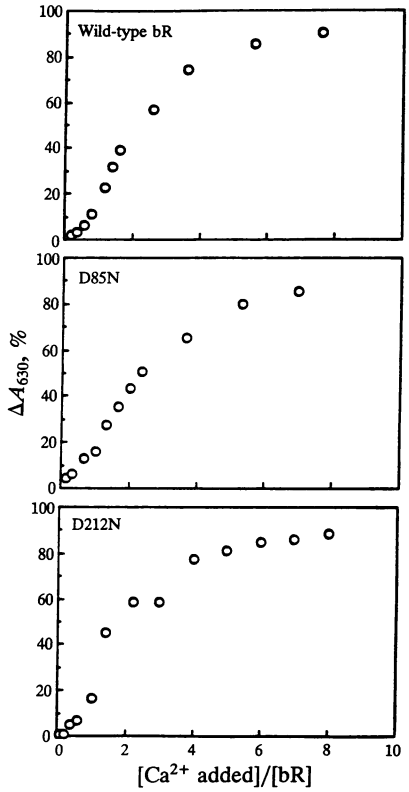

FIG. 3. Titration of deionized wild-type bR, D85N, and D212N with Ca^{2+} ions. Deionized bR sample concentrations were 5 μM. The sigmoidal spectroscopic titration curves suggest that binding to the second site is required for the observed absorption shift (32). This result, together with the determined values of the affinity constants for the different classes of sites, indicates that the order of the affinity sites does not change upon mutation. Unfortunately, we were unable to obtain a similar titration curve for deionized D85N/R82Q sample because the shift in the spectrum was too small or for deionized Y185F and R82Q/Y185F because the color change does not occur on the time scale of the titration.

ior: (*i*) they shift the absorption maximum of retinal in opposite directions; (*ii*) the removal of metal cations from the resulting mutants induces retinal absorption wavelength shifts with opposite signs; and (*iii*) charged residue replacements induce a decrease in the affinity constants of the two high-affinity sites by the same factor, whereas in the Y185F mutant, the reduction of the affinity constant of the second affinity site is larger than that of the first affinity site. We conclude that the interaction between Tyr-185 and cations is more localized (i.e., of shorter range) and stronger with Ca^{2+} in the second affinity site. On the other hand, the interaction of the charged residues studied with the PSB (thus determining the color) and with the Ca^{2+} in the two high-affinity sites (thus determining their binding affinities) is of the nonspecific electrostatic long range ion-ion type. Asp-212 seems to be less shielded from these Ca^{2+} ions and thus is more strongly coupled to them than is the Asp-85 residue.

The fact that the values of the association constants of the two high-affinity sites seem to be affected by the charged residue replacement suggests that the two Ca^{2+} ions in these sites are not far from one another and that both are within interaction range of these amino acids in the retinal pocket. One may then suggest that the two metal cations in the two high-affinity sites and residues Asp-85, Asp-212, Tyr-185, and Arg-82, together with the PSB and a few water molecules, are all part of an electrostatically coupled retinal cavity that controls both the color and function of bR. Due to the relatively low value of the dielectric constant inside the protein, this complex coupling could occur over *not too small* a distance. At this time, it is not known whether the effect of the residue replacement on the Ca^{2+} binding and the retinal color is a result of a direct change in the potential energy function within the cavity or a result of an indirect effect due to slight changes in the cavity conformation.

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