

Nature of the individual Ca^{2+} binding sites in Ca^{2+} -regenerated bacteriorhodopsin

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ABSTRACT The binding constants, K_1 and K_2 , and the number of Ca^{2+} ions in each of the two high affinity sites of Ca^{2+} -regenerated bacteriorhodopsin (bR) are determined potentiometrically at different pH values in the range of pH 3.5–4.5 by using the Scatchard plot method. From the pH dependence of K_1 and K_2 , it was found that two hydrogen ions are released for each Ca^{2+} bound to each of the two high affinity sites. Furthermore, we have measured by a direct spectroscopic method the association constant, K_s , for the binding of Ca^{2+} to deionized bR, which is responsible for producing the blue to purple color change. Comparing the value of K_s and its pH dependence with those of K_1 and K_2 showed that the site corresponding to K_s is to be identified with that of K_2 . This is in agreement with the conclusion reached previously, using a different approach, which showed that it is the second Ca^{2+} that causes the blue to purple color change.

Our studies also show that in addition to the two distinct high affinity sites, there are about four to six sites with lower binding constants. These are attributed to the nonspecific binding in bR.

INTRODUCTION

Bacteriorhodopsin is the only protein in the light energy transducing purple membrane of *Halobacterium halobium*. Upon absorption of light by its retinal chromophore, it undergoes a photocycle during which protons are translocated across the cell membrane. The resulting proton gradient is then used by the bacteria for the synthesis of ATP (1–3).

Light adapted bacteriorhodopsin (bR, purple bR) has an absorption maximum at 568 nm (4). The blue form of bR was described by Oesterhelt and Stoeckenius (4) concurrently with their isolation of the purple membrane, to be found in a purple to blue transition with a pK_a value of 3.2. More recently an intrinsic pK_a of 2.05 has been reported (5). The blue membrane has an altered photocycle and does not pump protons (6). Conversely, as reported by Kimura et al. (7), the addition of calcium or magnesium or other ions converts the deionized blue bR back to purple. According to Chang et al. (8) a well washed purple membrane contains 3–4 mol of magnesium and ~1 mol of calcium per mol of bR. Removal of these cations by means of an ion-exchange column (7–10) or by use of a chelating agent such as EDTA (20), as well as by lowering the pH by addition of acid (5, 7, 8), turns the membrane blue ($\lambda_{\text{max}} = 606 \text{ nm}$).

The binding of various cations to bacteriorhodopsin has been studied by several groups (9–14). A filtration technique was used to monitor the binding of radioactive $^{45}\text{Ca}^{2+}$ to the blue membrane at pH 5 (12). Scatchard plots of the data showed five binding sites. Similar

results were also reported for Mn^{2+} binding to bR at pH 5 and 7 from ESR experiments (11, 13). In addition to Ca^{2+} and Mg^{2+} a variety of divalent and trivalent cations (including lanthanides) were found to restore the purple color to bR (5, 7–12, 16, 17), but not Hg^{2+} and Pt^{4+} (12, 16). Based on the shape of the binding curves of Ca^{2+} and Eu^{3+} to blue bR, Ariki and Lanyi (16) suggested that in addition to a very high affinity site, a site of lower affinity was responsible for the blue to purple color change. A nonsigmoidal shape of the binding curve does not by itself prove the presence of two unequal high affinity sites, because nonsigmoidal curves can also be produced by having two equal affinity sites (18). Studies of the binding of Eu^{3+} to bR by time-resolved fluorescence quenching (10, 18) revealed only three decay components, although the binding studies had shown a stoichiometry of four Eu^{3+} per bR (15). More recently (17), it was suggested that the Eu^{3+} binding site of highest affinity could account for the complete quenching of the 'missing' fourth europium ion.

Another important aspect of cation binding to bR is the number of hydrogen ions that are involved in the process. Chang et al. (19) reported that a maximum of 13–15 H^+ are released as the blue membrane associates with cations. Jonas and Ebrey (5) found that as acid is added to the purple membrane in a 5-mM solution of MgSO_4 , 13 protons are taken up with a pK_a of 4–5 and 2 protons with a pK_a of 2.75. Since the purple to blue transition occurs in the pH range of 2–3, they suggested that the binding of a single divalent cation directly

correlates with the blue to purple color change in bR. Using Scatchard plot studies, the release of 2–3 protons from the first binding site was found in the binding of Eu^{3+} to blue bR (17).

There are two different models describing the binding of metal cations in bR: a specific binding, e.g., to carboxylate groups (16, 20), and a nonspecific binding within the Gouy-Chapman diffuse double layer (21–25). In the nonspecific binding model (23–25), the blue to purple color transition results from the protonation-deprotonation of a counterion due to changes in the surface pH as the metal cation concentration changes (25). More recently, Jonas and Ebrey (5) proposed that the specific binding of one metal cation that causes the color change might involve Asp85, Asp212, Tyr185, and Arg82.

In this work, the binding constant of Ca^{2+} to blue bR was determined in the pH range of 3.5–4.5, both potentiometrically (by using a calcium ion-sensitive electrode to measure directly the free Ca^{2+} in equilibrium with the bR at a given pH) as well as spectroscopically (to follow the blue to purple transition equilibrium). Furthermore, by determining the pH dependence of each binding site, the number of protons displaced upon binding of Ca^{2+} to each individual site was determined. This is especially important in determining the detailed structure of each individual metal cation binding site.

Analysis of the data gave the following important conclusions: (a) there are two specific high affinity binding sites differing by an order of magnitude in affinity to Ca^{2+} at 22°C, as well as four to six sites of much reduced affinity; (b) the second site, the lower of the two high affinity sites, is the one that correlates with the spectral color change from blue to purple in agreement with previous conclusions (16); and (c) the pH dependence of the association constants of each of the two high affinity sites shows conclusively that in each case the binding of one Ca^{2+} to bR displaces two H^+ ions in the pH and temperature range of the experiment.

MATERIALS AND METHODS

Halobacterium halobium was grown from master slants of ET1001 strain provided by Professor R. Bogomolni (University of California, Santa Cruz) and Professor W. Stoerkenius (University of California, San Francisco). Bacteriorhodopsin was isolated by a combination of methods (26, 27). Deionized bR was prepared by passing a sample through a column of Bio-Rad AG 50W-X4 (Richmond, CA) cation exchanger in the hydrogen form (7, 10). The deionized bR sample had a pH of 3.6–3.9 after passage through the column. For experiments at $\text{pH} > 4$, deionized samples were prepared by EDTA treatment (20). Bacteriorhodopsin solutions 10 mM in EDTA and 50 mM in Tris buffer, pH 8.0, were allowed to equilibrate overnight. The sample was washed ten times by centrifugation (19 krpm for 35 min) and resuspended in deionized distilled water to remove EDTA and Tris. All

deionized bR samples were used without further pH or ionic strength adjustment so as to avoid competition with other metal cations, e.g., Na^+ . To avoid any possible leaching by blue bR of metal ions from glass, only polyethylene and teflon containers were used.

A sample of 2 ml of deionized bR at a given pH was titrated by addition of microliter quantities of 10^{-2} or 10^{-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 $\Phi 71$; Fullerton, CA) using a calcium ion sensitive electrode (Orion 93-20; Cambridge, MA) against a double junction reference electrode. A duplicate run using the same volume of water in place of the deionized bR sample served as a calibration curve for obtaining the free calcium ion concentration. The amount of calcium bound to bR was computed from the difference between the known total calcium added and the free calcium measured by the electrode. Spectra were taken at successive stages of the titration of blue bR with Ca^{2+} using a diode array spectrophotometer (model HP 8451A; Hewlett Packard, Palo Alto, CA). The pH was monitored at various times during the titration. A control experiment showing the variation of pH upon addition of Ca^{2+} to a bR solution is shown in Fig. 5. All experiments were done at 22°C using light adapted bR samples.

RESULTS

Scatchard plots. If bacteriorhodopsin is regarded as a macromolecule with a number of classes of cation binding sites, where the binding affinity is essentially the same within each class, and if the sites are noninteracting, then for a particular class of sites (28, 29)

$$\nu/c = K(n - \nu),$$

where n is the number of sites with the same binding affinity, ν is the average number of sites occupied by a cation, K is the association equilibrium constant, and c is the concentration of free cation in the equilibrium system. The Scatchard plot, ν/c versus ν , gives a slope of $-K$ and an x -intercept equal to n . Linear regions of this plot will be seen if the binding affinities of different classes of sites are sufficiently separated in magnitude.

Fig. 1 shows the data of a titration (curve A) obtained by additions of known amounts of CaCl_2 to a solution of blue bR and of the corresponding calibration (curve B) obtained by addition of CaCl_2 to an equal volume of water blank. The y -coordinate is the reading in millivolts of the electrical potential, $\Delta E = E_{\text{ISE}} - E_{\text{REF}}$, the difference in potential between the ion selective electrode and the reference electrode. For a given y -coordinate ($\Delta E/\text{mV}$), pCa (total) is read from curve A (titration) and pCa (free) is read from curve B (calibration). The difference between the two curves represents the calcium bound to bR. These are equilibrium values after a given addition of calcium to the blue bR solution. The Scatchard plot for a titration at pH 4.3 is shown in Fig. 2, where $\nu = [\text{bound } \text{Ca}^{2+}]/[\text{bR}]$ and $c = [\text{free } \text{Ca}^{2+}]$. The value of K (which equals $-\text{slope}$) will have dimensions of M^{-1} . The data show clearly two binding sites, each site

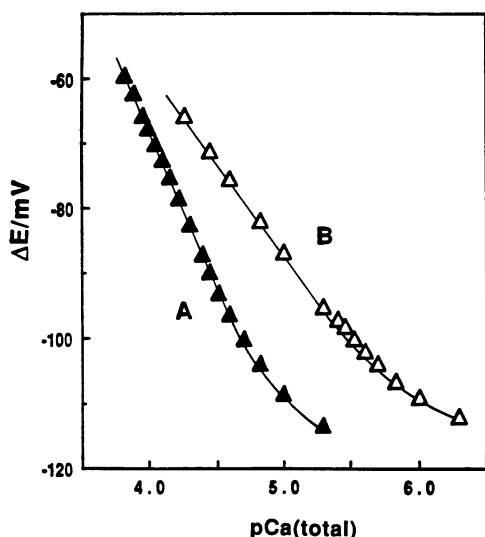


FIGURE 1 Calcium specific ion electrode potentials ΔE obtained (A) after addition of successive amounts of CaCl_2 to $54 \mu\text{M}$ deionized blue bR and (B) to an equal volume of H_2O . The x -axis pCa represents the total amount of Ca^{2+} added.

binding one calcium ion, with affinities differing by an order of magnitude. Upon further addition of Ca^{2+} , more points are obtained on a Scatchard plot (as shown in Fig. 2), which indicate that there are four to six additional calcium binding sites with much lower association equilibrium constants.

Spectral ratio plot. Analogous to the treatment of a multisite prototropic equilibrium (30), the following equation can be written for the equilibrium reaction of a particular class of calcium binding sites associated with

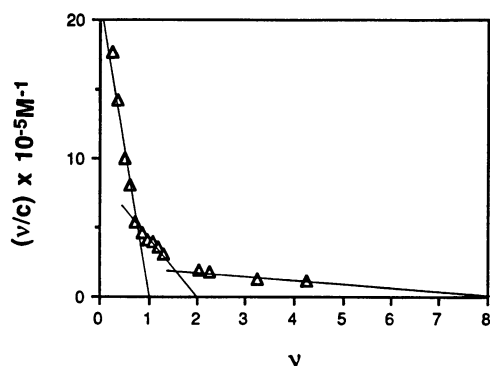
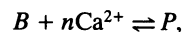


FIGURE 2 Scatchard plot of Ca^{2+} binding to deionized blue bR, where $c = [\text{free } \text{Ca}^{2+}]$ and $v = [\text{bound } \text{Ca}^{2+}]/[\text{bR}]$. In this experiment, $[\text{bR}] = 40 \mu\text{M}$ and initial $\text{pH} = 4.3$. There are two individual high affinity binding sites with association constants $K_1 = 2.4 \times 10^6 \text{ M}^{-1}$ and $K_2 = 4.0 \times 10^5 \text{ M}^{-1}$. The low affinity association constants of each of the four to six nonspecific sites are $\sim 10^4 \text{ M}^{-1}$.

the blue to purple color change in bR:



where B is the blue deionized bR and P is the purple regenerated bR. The association equilibrium constant is

$$K_n = [P]/[B][\text{Ca}^{2+}]^n = R/c^n,$$

where R is the concentration ratio $[P]/[B]$. Taking $-\log_{10}$ and rearranging gives

$$\text{p}R = \text{p}K_n + n\text{pCa},$$

where $\text{pCa} = -\log_{10} [\text{Ca}^{2+}]$ for free calcium ion in the system. A plot of $\text{p}R$ versus pCa gives the number of Ca^{2+} ions responsible for the color change as determined from the slope, because slope = n . The association equilibrium constant for a single site is

$$K_s = [P]/[B][\text{Ca}^{2+}] = R/c.$$

Hence, the x -intercept, when $\text{p}R = 0$, gives

$$\text{p}K_s = -\text{pCa} = \text{p}K_n/n.$$

Fig. 3 shows a set of spectra taken during a titration of blue bR with CaCl_2 at $\text{pH} 4.1$. The value of R at a given point in the titration was determined from the absorbance of 620 nm using the expression

$$R = (A_{620}^B - A_{620}) / (A_{620} - A_{620}^P),$$

where A_{620} is the absorbance after addition of a given amount of Ca^{2+} , A_{620}^B is the absorbance of 100% blue bR, A_{620}^P is the absorbance of 100% purple bR obtained after

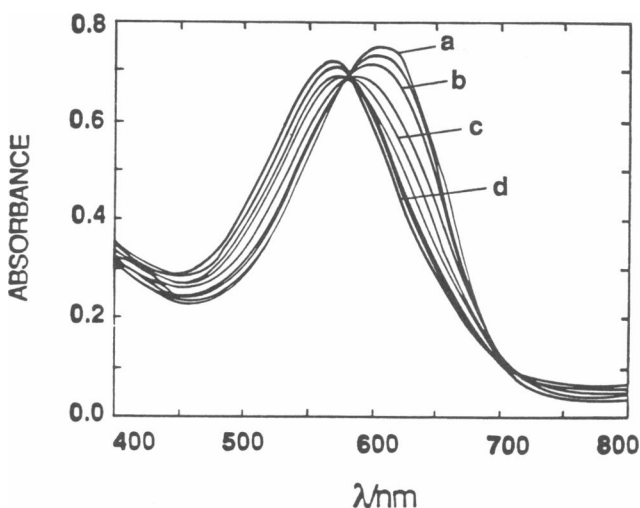
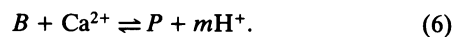


FIGURE 3 Spectra taken after addition of successive amounts of 10^{-2} M CaCl_2 to $54 \mu\text{M}$ initially deionized blue bR at $\text{pH} 4.1$. The Ca/bR molar ratios were (a) 0; (b) 1.0; (c) 2.0; (d) 5.0. The isosbestic point was at 580 nm .

saturation by addition of excess Ca^{2+} to a ratio of 5:1 Ca^{2+}/bR . The spectral ratio plot, pR versus $p\text{Ca}$ (free), shown in Fig. 4, clearly establishes the number of Ca^{2+} ions to be $n = 1$ and the x -intercept gives $pK_s = -5.2$, hence $K_s = 1.6 \times 10^5 \text{ M}^{-1}$ for Ca^{2+} association with blue bR. Comparing the value of K_s (obtained spectroscopically) with K_1 and K_2 (obtained potentiometrically) clearly suggests that the Ca^{2+} , which causes the blue to purple color change occupies the lower affinity site of the two sites of strongly bound Ca^{2+} ions.

It is to be noted that during the titration the spectra begin to deviate from the spectrum of 100% blue bR only after a significant amount of Ca^{2+} (almost 1 mol $\text{Ca}^{2+}/\text{mol}$ bR) has been added, indicating that calcium ions responsible for the color change are binding to bR at sites other than the one with highest affinity.

pH dependence. Consider the equilibrium in which m hydrogen ions are released from bR for every Ca^{2+} bound:



The equilibrium constant for this reaction is

$$K_H = \frac{[P][\text{H}^+]^m}{[B][\text{Ca}^{2+}]} = K[\text{H}^+]^m \quad (7)$$

$$pK = pK_H - mpH, \quad (8)$$

where K is a function of pH, whereas K_H is not. In our experiments, the pH varied only by 0.1 in the range corresponding to the titration of each of the two high affinity sites, as may be seen in Fig. 5. If K is measured under conditions in which the pH is held essentially constant during the calcium titration, then by carrying out titrations at various pH, the value of m can be found from the slope of a plot of pK versus pH. Such pH profiles of K_1 and K_2 are shown in Fig. 6. The stoichiometry $m = 2$ is established unambiguously for both binding sites. Furthermore, it is evident by comparing the values of the association constants (K_s) of the spectral plot with those found from the Scatchard plot that the blue to purple transition is controlled by the binding of calcium ion to the second site (K_2). This is corroborated by the observation that the initial addition of CaCl_2 to blue bR does not induce a color change, as noted above in reference to the spectra shown in Fig. 3. The values of the association constants K_s are much lower than K_1 . This rules out the possibility that the binding of Ca^{2+} ions to the site of higher affinity (site 1) correlates with the color transition. Furthermore, for the four to six sites of very low affinity, it is not possible to determine the association constants accurately. These

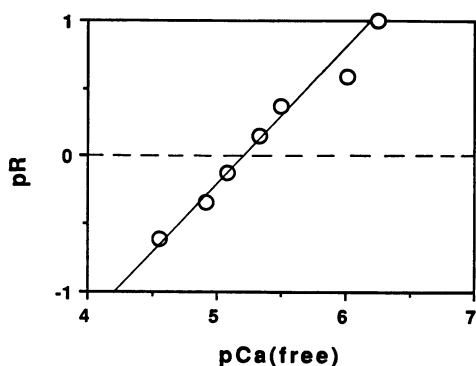


FIGURE 4 Spectral ratio plot, pR versus $p\text{Ca}$ (free). The concentration ratio, R , of blue to purple bR was determined spectrally from the data of Fig. 3. The x -intercept of $-pK_s$ gives a K_s of $1.6 \times 10^5 \text{ M}^{-1}$. Line drawn with slope = 1.00, suggesting that a single Ca^{2+} ion is responsible for the observed blue to purple color change.

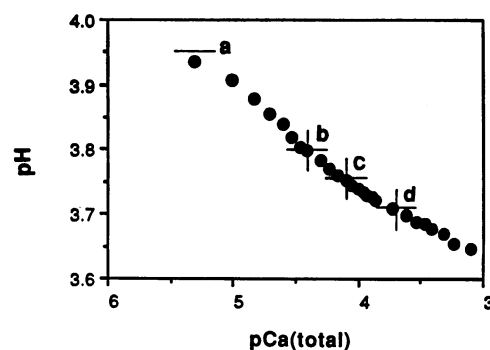


FIGURE 5 Variation of pH when successive amounts of CaCl_2 are added to $40 \mu\text{M}$ initially deionized blue bR. (a) Initial pH = 3.95; (b) pH = 3.80 when $\text{Ca}/\text{bR} = 1$; (c) pH = 3.75 when $\text{Ca}/\text{bR} = 2$; (d) pH = 3.71 when $\text{Ca}/\text{bR} = 5$.

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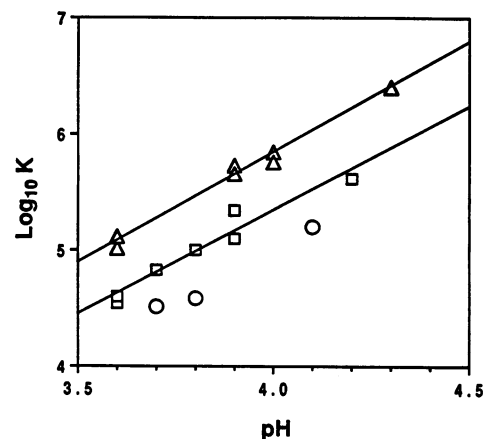


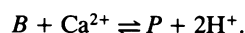
FIGURE 6 The pH dependence of the association constants of the two high affinity sites. Scatchard plot data K_1 (Δ) and K_2 (\square), spectral ratio data K_s (\circ). Lines drawn with slope = 2.00. This suggests that each Ca^{2+} ion releases two protons.

constants are estimated from the Scatchard plots to average $\sim 8 \times 10^3 \text{ M}^{-1}$ and they do not show a clear trend of pH dependence. Also, these are too small to be comparable to K_s . Therefore, the data strongly support the conclusion that the color transition is controlled by site 2.

DISCUSSION

Our results are in agreement with the conclusion of Arika and Lanyi (16) concerning the stoichiometry of the two high affinity binding sites with regard to calcium and hydrogen ions. In addition, we have further shown that the binding in each site at the pH used displaces two protons. The linearity obtained for each site in the Scatchard plot suggests that each site is independent of the other. Thus, at least two Ca^{2+} ions bind independently and specifically, as proposed earlier (16, 20). In addition, we observe that over four Ca^{2+} ions bind with much reduced binding constants.

The specific equilibrium expression for each of the two high affinity sites in the pH range of our experiments (pH 3.5–4.5) can be written as



Riviere et al. (31) have reported a stoichiometry of five H^+ per bR at 20°C associated with the blue to purple color change in the presence of Ca^{2+} . The manner in which their data were obtained allows ancillary prototropic equilibria to be included in the observed stoichiometry. It is important to distinguish the prototropic equilibria of the ligand groups at the binding sites of Ca^{2+} from the other prototropic equilibria with similar $\text{p}K_a$ values in bR that are not involved in the chelation of Ca^{2+} . In order to establish the H^+ participation at the binding site, it is necessary to measure the equilibrium constant by titration of bR with Ca^{2+} at a fixed pH, then to repeat the titration at other fixed pH values.

Two main questions need to be addressed concerning the binding of calcium ions to bR: (a) where does Ca^{2+} bind? and (b) what is the effect of Ca^{2+} binding on structure and reactivity of bR?

The $\text{p}K_a$ of 2–3, at which the purple to blue transition occurs (5), suggests carboxyl groups, most probably Asp, but Glu is not necessarily ruled out. Calcium ions are known to bind strongly to oxalate and to EDTA, but not to acetate. This suggests a steric requirement for the chelate, probably octahedral. At least two aspartyl residues suitably located in the tertiary structure of bR are needed. In addition, water molecules may be involved to give octahedral coordination to calcium.

In blue bR at pH 3.5–4.5, some of the carboxyls must be protonated, otherwise a displacement of two H^+ by Ca^{2+} would not be seen in our experiments.

Calcium ions are also known to bind to phosphate strongly. If this is the case in the purple membrane, then four to six nonspecific bound Ca^{2+} ions could be in the phospholipid bilayer (23, 24) as well as on the flexible COOH-terminus (11). A concentration preponderance in the vicinity of a negatively charged zone according to the Gouy-Chapman theory (7, 23–25) does not constitute a site of specific chelation. However, in addition to the two highly specific high affinity sites in the protein moiety, a Gouy-Chapman effect may well be operative at the surface of the lipid bilayer as a consequence of the negative charge furnished by the phosphate groups involving a variety of mobile ions, including the four to six calcium ions. This would produce a diffuse cationic concentration gradient and control the surface pH (23–25).

Occupation of the second binding site by Ca^{2+} is a necessary condition for the blue to purple transition. But is it a sufficient condition? We may well ask the question: if the first site were blocked would Ca^{2+} binding to the second site still produce purple bR? Would Ca^{2+} still bind at all or with different affinity at the second site? We know that the two high affinity sites are essentially noninteracting because they are separable as linear regions on the Scatchard plot. But is the Ca^{2+} binding to the first site a prerequisite condition for the production of purple bR before perturbation of the chromophore occurs upon calcium binding to the second site?

With the molecular structure described by Henderson et al. (32) in mind, it is plausible to consider Asp85 and Asp212 together as constituting a binding site for Ca^{2+} . It is known that blue bR does not translocate protons (6). Is, therefore, Ca^{2+} bound at this location in the proton channel required for participation in the translocation activity, or does the binding of Ca^{2+} at the first high affinity site and/or second high affinity site (not in the proton channel) lock the tertiary structure of bR into the active conformation by an allosteric mechanism (33–36)? Is the Ca^{2+} located on a conformational hinge, which in the L_{550} to M_{412} kinetic step causes a positive charge to approach the protonated Schiff base, lowering the $\text{p}K_a$, thereby inducing deprotonation? It seems that even with the wealth of structural, spectroscopic, and kinetic information currently available, the exact location and function of metal cations remain to be elucidated. Experiments are planned to examine the effect of replacing different amino acids by genetic mutation, on the association constants of Ca^{2+} binding to bR.

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