The C-Terminus and the Ca^{2+} Low-Affinity Binding Sites in Bacteriorhodopsin†

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ABSTRACT: Bacteriorhodopsin (bR) is found previously to have two high-affinity and four to six low-affinity Ca^{2+} binding sites. Our previous studies with site-directed mutation suggested that the two high-affinity sites are located within the protein and close to the retinal pocket. In order to investigate the location of the four to six low-affinity binding sites, we studied Ca^{2+} binding to deionized bR as well as its variant in which its C-terminus is removed by papain treatment. Potentiometric titration with Ca^{2+}-selective electrodes were carried out and Scatchard plots were obtained from the titration data. We found that most of the low-affinity sites are eliminated upon removal of the C-terminus in bR. This suggests that the low-affinity sites in bR are on the surface. The involvement of the C-terminus in these sites is discussed.

Bacteriorhodopsin (bR),[1] the other photosynthetic system besides chlorophyll, is the sole protein pigment in the purple membrane (PM) of *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971). Upon absorption of visible light, bR undergoes a photochemical cycle (Stoeckenius & Bogomolni, 1982) during which protons are translocated from inside to the outside of the cell membrane. The resulting proton gradient is then used by the bacteria for its metabolic processes, involving ATP synthesis (Stoeckenius & Bogomolni, 1982).

Well-washed PM contains ~3–4 mol of Mg^{2+} and ~1 mol of Ca^{2+}/mol of bR (Chang et al., 1985). Removal of these cations (either by deionization or by acidification) causes a color transition from purple to blue (Chang et al., 1985; Kimura et al., 1984; Kobayashi et al., 1983) and alters the bR photocycle (Dupuis et al., 1985; Kobayashi et al., 1983; Chronister & El-Sayed, 1987). Two different types of binding of the metal cations in bR have been previously proposed. From the observed effect of lipid surface charges on the pK_a of the purple-to-blue transition of bR, it was suggested (Szundi & Oesterhelt, 1989) that cations are physically attached to the negatively charged surface. Other studies provided evidence that specific binding of the cations to protein does exist in bR and that cations might actually be chelated to the carboxylate groups of amino acid side chains (Dupius et al., 1985; Chronister & El-Sayed, 1987; Mitra & Stroud, 1990; Dunach et al., 1987; Ariki & Lanyi, 1986; Jonas & Ebrey, 1990).

In order to understand the role of metal cations in bR function, it is essential to determine their binding sites. Previous ESR binding studies of Mn^{2+} to deionized bR have concluded (Dunach et al., 1987) that there are five high- to medium-affinity Mn^{2+} binding sites and five low-affinity ones in native blue membrane. More recently, using calcium-sensitive electrodes for potentiometric titration and combining this technique with amino acid replacements, we have concluded (Zhang et al., 1992) the presence of two high-affinity sites and four to six low-affinity sites for Ca^{2+} binding to deionized bR and provided experimental evidence (Zhang et al., 1993) which suggests that the two Ca^{2+} high-affinity sites are located within the protein near the retinal pocket (active site). We proposed that the four low-affinity binding sites are probably located on the membrane surface.

The water-exposed C-terminus of bR contains three acidic groups and hence might host the sites for those loosely bound cations. ESR studies on Mn^{2+} binding showed (Dunach et al., 1987) that removing the C-terminus by papain treatment of bR caused the loss of five low-affinity sites but left the five high- and medium-affinity sites unaffected. Since the number of sites found for Ca^{2+} is different (Zhang et al., 1992) from that observed for Mn^{2+} (Dunach et al., 1987), and since Ca^{2+} is one of the physiologically active metal cations in bR, we have decided to study the effect of papain treatment on the binding sites of Ca^{2+}. The results show that all but one of the low-affinity sites found previously by us (Zhang et al., 1992) are eliminated without affecting the value of the affinity constants for the two high-affinity sites. These results support the previous assignment (Zhang et al., 1992) that most (if not all) of the low-affinity binding sites of Ca^{2+} in bR are surface sites.

MATERIALS AND METHODS

Halobacterium halobium was grown from master slants of ET 1001 provided by Professor R. Bogomolni (University of California, Santa Cruz). bR was purified by using a combination of previously described methods (Oesterhelt & Stoeckenius, 1974; Becker & Cassim, 1975). Removal of the C-terminal segment of bR was performed by papain proteolysis of PM according to Liao and Khorana (1984) and checked by SDS–polyacrylamide (15%) gel electrophoresis. It was shown that treatment of the purple membrane with papain results in the cleavage of a maximum of about 17 amino acids out of a total of 22 that form the C-terminus (Liao & Khorana, 1984). Deionized samples were prepared from PM suspensions by dialysis against cation-exchange resins (Dowex 50W).

Binding experiments were carried out as described elsewhere (Zhang et al., 1992). A sample of 2 mL of deionized PM 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 measured by a calcium-sensitive electrode (Orion 93-20; Cambridge, MA) combined with a double-junction reference electrode. 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 electrodes. Scatchard plots were then plotted for each titration.

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[1] Abbreviations: PM, Purple membrane; bR, bacteriorhodopsin; ESR, electron spin resonance; SDS, sodium dodecyl sulfate; PSB, protonated Schiff base.

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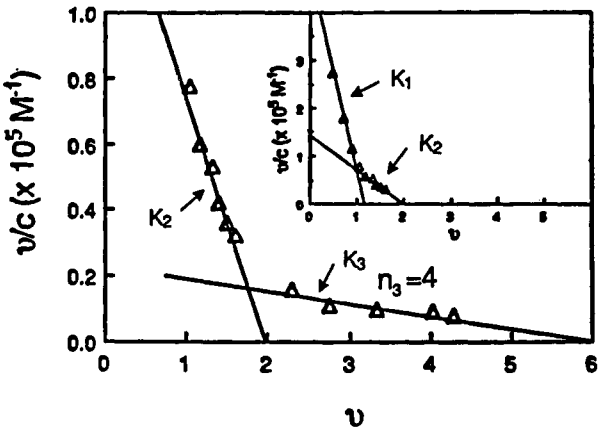

FIGURE 1: Scatchard plots of the titration of deionized PM with Ca^{2+} in the high concentration region (involving the equilibrium with the low-affinity sites) and in the high-affinity region (inset). c = [free Ca^{2+}] and ν = [bound Ca^{2+}]/[bR]. The starting pH of the deionized sample is 3.7 and concentration of the sample is 50 μM.

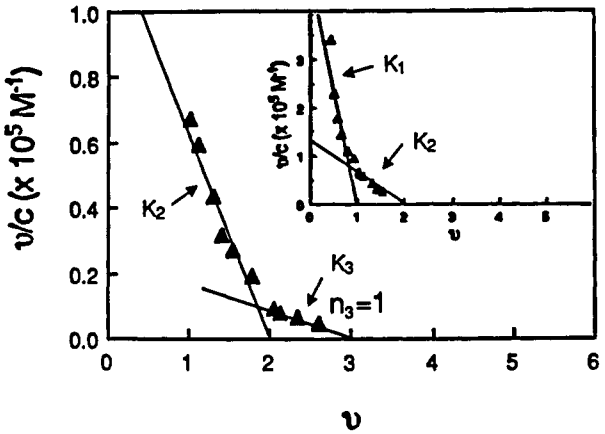

FIGURE 2: Scatchard plots of the titration of deionized, papain-treated PM with Ca^{2+} in the high concentration region (involving the equilibrium with the low-affinity sites) and in the high-affinity region (inset). c = [free Ca^{2+}] and ν = [bound Ca^{2+}]/[bR]. The starting pH of the deionized sample is 3.7 and concentration of the sample is 50 μM. From the comparison with Figure 1, it is clear that deionized papain-treated PM, which lost its C-terminus, lost also most of the low-affinity sites, suggesting that they are surface-type sites.

Typical sample concentration for the binding experiment was ~50 μM. Sample pH was monitored at various times during the titration. Both deionized pM and deionized papain-treated PM had the same initial pH of 3.7 and final pH of about 3.5; this enables us to compare their apparent binding constants directly. Each experiment was repeated at least three times and the relative average deviation was less than 10%.

RESULTS AND DISCUSSION

The Scatchard plots (Scatchard, 1949) for Ca^{2+} binding to deionized PM are shown in Figure 1, and that of Ca^{2+} binding to deionized papain-treated PM is shown in Figure 2. By plotting ν/c vs ν, where ν = [bound Ca^{2+}]/[bR] and c = [free Ca^{2+}], a Scatchard plot gives a slope of $-K$ (apparent binding affinity) and an x-intercept of n (the number of sites with the same binding affinity). As shown previously (Zhang et al., 1992), Figure 1 can be resolved to give two high-affinity sites with one Ca^{2+} ion each. In addition, 4–6 Ca^{2+} ions occupy a low-affinity site(s) with a constant which is 100 times smaller than that for the highest affinity site.

Table 1: Apparent Binding Constants[a] and Number of Sites of Ca^{2+} Binding for Native bR and Papain-Treated bR, both at pH = 3.7

sample	K_1 (μM^{-1}) [n_1]	K_2 (μM^{-1}) [n_2]	K_3 (mM^{-1}) [n_3]
native PM	0.35 [1]	0.063 [1]	3.5 [4]
papain-treated PM	0.45 [1]	0.044 [1]	6.4 [1]

[a] Values shown are averages of at least three trials with relative deviation less than 10%.

For papain-treated PM (Figure 2), the two high-affinity sites are clearly resolved with slopes (and thus affinity constants given in Table 1) that are not different from those observed for Ca^{2+}-regenerated PM. However, only one site at best can be resolved, with a slope that is much reduced from those observed for the first two but is similar to those obtained for the low-affinity sites in regenerated PM. From these observations one concludes that the removal of the C-terminus seems to eliminate most of the low-affinity sites for Ca^{2+} leaving the two high-affinity sites unaffected, in general agreement with the conclusion made for the low-affinity sites of Mn^{2+} using ESR techniques (Dunach, 1987).

Among the 17 C-terminal tail amino acids that are released with papain, there are three acidic groups (Asp, Glu, Glu). It is rather interesting to find that 3–5 low-affinity cation binding sites were lost due to the loss of these residues. There has been speculation by Renthal et al. (1983) that the C-terminus tail is rigidly held at the membrane surface. They attached the danzylhydrazine fluorophore to PM with a water-soluble carbodiimide and found that most of the probe was attached to the C-terminus. At a pH of 8.0, they found that the steady-state polarization of the dansyl fluorescence on the tail was 0.24 at 25 °C, indicating a rigid environment of the C-tail. The fact that the kinetics of anisotropy decay showed no reorientation of the dansyl probe for 25 ns after excitation gave yet another piece of evidence for a restricted C-terminal tail. However, other studies suggested that the C-terminus of bR is free to assume many positions (Wallace & Henderson, 1982; Marque et al., 1986). Using electron and X-ray diffraction, Wallace and Henderson (1982) showed that the carboxyl terminus of bR is generally disordered in the native two-dimensional crystalline purple membranes. This conclusion was supported by the studies of steady-state and time-resolved fluorescence spectroscopy of a dye molecule attached to the C-terminus (Marque et al., 1986). The carboxyl terminus has a large negative charge, which may provide a means of repelling other membranes. In fact, aggregation of the purple sheets after removal of the C-tail was observed by electron microscopy (Wallace & Henderson, 1982).

Taking into consideration the possible location of the C-terminus in bR discussed above, two possible descriptions can be proposed for the surface binding sites that were eliminated by the removal of the C-terminus. First, the loss of the 3–5 cation binding sites may result from the fact that the membrane cytoplasmic surfaces of the membrane patches used in the present studies tend to adhere to one another (i.e., aggregate) upon removal of the negatively charged C-terminus, thus eliminating the original surface sites for cations which were bound physically to the *membrane surface*. This does not require any restrictions on the C-tail orientation with respect to the surface. The other possibility is that metal cations are physically adsorbed at sites in between the C-terminus and the surface. Upon the removal of the C-terminus these adsorption sites disappear. In both cases, our results have indicated that these low-affinity Ca^{2+} binding sites are indeed located on the membrane surface.

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