

## The $\text{Ca}^{2+}$ Binding to Deionized Monomerized and to Retinal Removed Bacteriorhodopsin

Difei Yang and Mostafa A. El-Sayed

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400 USA

**ABSTRACT** In our continuing effort to characterize the metal cation binding in bacteriorhodopsin (bR) using  $\text{Ca}^{2+}$ -specific electrodes, potentiometric titration was carried out on deionized solubilized bR (containing monomeric units) and deionized bacterioopsin (bR with its retinal removed). Scatchard plots were analyzed. The monomer was found to have plots similar to those of the trimer, suggesting that the binding sites in bR are localized within the protein monomer unit and not between the molecules within the trimer structure. This also supports the previous assumption that the curvature in the Scatchard plot of regenerated bR is not due to cooperativity of metal cation within the trimer, but rather due to multiple sites. Recent studies further support the finding that the curved Scatchard plot is not due to the cooperativity between the metal ions in the two high affinity sites, wherever they are. The results of the analysis of the Scatchard plot for deionized bacterioopsin have shown a change in the binding characteristics of the high affinity but not the low affinity sites from that observed in bR. This result supports previous conclusions that metal cations in the high affinity sites are not far from the retinal cavity.

### INTRODUCTION

Bacteriorhodopsin (bR), the photosynthetic system other than chlorophyll, is the sole protein pigment found in *Halobacterium salinarium*. The chromophore, an all-*trans* retinal, is bound to the protein moiety through a protonated Schiff base linkage to the  $\epsilon$ -amino group of Lys-216 (Oesterhelt and Stoeckenius, 1971; Bayley et al., 1981). It acts as a light-driven proton pump and converts light into electrical energy by pumping protons across the bacterial membrane. The resulting proton gradient is then used to convert ADP into ATP (for recent reviews see Ebrey, 1993, and Mathies et al., 1991).

Removal of metal ions from purple membrane causes a red shift of the absorption maximum of its retinal from 568 nm to 606 nm and gives deionized blue bR (Fisher and Oesterhelt, 1979; Mowery et al., 1979; Kimura et al., 1984; Moore et al., 1978). Blue bR has an altered photocycle and lacks the ability to pump protons (Mowery et al., 1979; Dunach et al., 1987; Chang et al., 1986). Two different models exist to account for the role that cations play in the color control of bR. The first one is the surface potential model in which metal cations are proposed to be physically adsorbed on the negatively charged surface of bR and thus regulate the color of bR fully by changing the surface pH of the purple membrane (Szundi and Stoeckenius, 1987; Szundi and Stoeckenius, 1988; Szundi and Stoeckenius, 1989). However, other studies have suggested that specific binding between metal cations and bR is also needed to explain their observations (Jonas and Ebrey, 1991; Ariki and Lanyi, 1986). Electron spin resonance investigation of  $\text{Mn}^{2+}$  bind-

ing has indicated that  $\text{Mn}^{2+}$  has different binding sites with different binding constants (Dunach et al., 1987). Recently, Jonas and Ebrey (1991) observed the correlation between color change and one bound  $\text{Ca}^{2+}$ , which suggested that this  $\text{Ca}^{2+}$  might be located within the retinal pocket with a specific binding involving D85, D212, Y57, Y185, R82, and protonated Schiff base (Jonas et al., 1990). Time-resolved fluorescence measurement of  $\text{Eu}^{3+}$ -regenerated bR resolved only two to three emission components (Corcoran et al., 1987) of similar binding constants for samples having four  $\text{Eu}^{3+}$  per bR. This together with the observation that the strong binding site for  $\text{Eu}^{3+}$  is found to have only one  $\text{Eu}^{3+}$  ion suggests that the strong binding site must be near retinal, and thus its fluorescence is quenched and not seen in the emission studies (Sweetman and El-Sayed, 1991). The energy transfer studies between the retinal and different probing molecules such as  $\text{Ru}(\text{bpy})_3^{2+}$  (Wu and El-Sayed, 1994a) and  $\text{DAA}^+$  (3, 6, diaminoacridine) (Wu and El-Sayed, 1994b) again support the finding that at least one or two metal ions are located inside the protein not far from the retinal pocket. An alternative way of looking at  $\text{Ca}^{2+}$  binding is that some of metal ions are on the surface and others are inside the protein.

Using calcium-sensitive electrodes for potentiometric titration (Zhang et al., 1992) combined with site-directed mutagenesis (Zhang et al., 1993) and C-terminus removal treatment of bR (Zhang and El-Sayed, 1993), it was shown that calcium binding sites are divided into three classes. Most (if not all) of the low affinity binding sites of  $\text{Ca}^{2+}$  in bR are surface sites (Zhang and El-Sayed, 1993), whereas the two strong binding sites seem to interact with the amino acid within the active site, i.e., the retinal pocket (Zhang et al., 1993). Furthermore, the presence of several water molecules close to the retinal binding site (Papadopoulos et al., 1990; Cao et al., 1991) makes retinal pockets relatively hydrophilic for cation binding.

Received for publication 4 May 1995 and in final form 7 August 1995.

Address reprint requests to Dr. Mostafa A. El-Sayed, Department of Chemistry/Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332. Tel.: 404-894-0292; Fax: 404-894-0294; E-mail: mostafa.el-sayed@chemistry.gatech.edu.

© 1995 by the Biophysical Society

0006-3495/95/11/2056/00 \$2.00

In order to determine whether the metal cation sites are within the individual protein monomer or whether some are between the monomer units within the trimer structure, we used Ca<sup>2+</sup>-specific electrodes on the titration of deionized solubilized bR. The similarity between the results obtained for the monomer and the trimer bR suggests that the curvature in the Scatchard plots in the latter is not a result of cooperativity between metal ions in different monomer units.

If one or more metal ions are indeed present somewhere close to the retinal, the nature of the metal ion binding sites should change upon removal of retinal from bR. By using Ca<sup>2+</sup>-selective electrodes, we were able to measure the calcium binding to retinal free deionized bR, i.e., to deionized bacteriorhodopsin (bO). The results show the presence of only one metal cation in one high affinity site. No effect is observed on the Ca<sup>2+</sup> binding to the lowest affinity (surface type) site. These observations support the finding that metal cations in the high affinity sites are not far from the retinal cavity.

## MATERIALS AND METHODS

*H. salinarum* was grown from master slants of ET1001 strain generously provided by Professor R. Bogomolni (University of California, Santa Cruz, CA). Bacteriorhodopsin was isolated and purified by a combination of standard published procedures (Oesterhelt and Stoekenius, 1971; Becher and Cassim, 1975).

Bleached bR (bO) was prepared by the hydroxylamine treatment described by Wu et al. (1991). The sample was then washed by distilled-deionized water at least three times to remove the excess amount of hydroxylamine. Monomerized bR was obtained by solubilizing wild type bR suspension in Triton-X-100 (Kovacs et al., 1995; Dencher and Heyn, 1978). The 10 nm blue shift absorption maximum of the protein retinal and the disappearance of the biphasic feature of the bR circular dichroism spectrum in the visible region serve as criteria to confirm the completion of solubilization. Deionization of this bR monomer was carried out by passing the sample through a column prepared by cation exchange resin (AG 50W-X8 resin, Bio-Rad Laboratories, Richmond, CA). Deionized bO was prepared by dialyzing the bO sample against the same cation exchange resin because of the colorless nature of the sample. A small amount of purple bR in a separate dialysis tube was used as an indicator to assure the complete removal of metal ions from bO. The deionized bO and monomerized bR samples were concentrated by centrifugation and then suspended in deionized distilled water. All deionized samples were used without further pH or ionic strength adjustment in order to prevent competition binding from other ions, e.g., Na<sup>+</sup>. The initial pH of such samples was between 4.50 and 4.60. The pH of the solution dropped sharply to ~3.90–4.00 with the addition of 2–6  $\mu$ M Ca<sup>2+</sup>, and then the pH decreased gradually as more and more Ca<sup>2+</sup> was added, with a final pH in the region of 3.60–3.70. Therefore, a reasonable estimation of the average pH during the course of titration should be ~3.80–3.90.

Titration was performed following the same procedure described earlier (Zhang et al., 1992) except that 5 ml instead of 2 ml deionized bO sample with a concentration of 35–50  $\mu$ M was used in order to achieve a better consistency in binding affinity values. The concentration of unbound Ca<sup>2+</sup> in bR suspension was measured 5 min after the addition of Ca<sup>2+</sup> when the equilibrium was found to be established. In a separate experiment, free Ca<sup>2+</sup> concentration was monitored as a function of time (up to 2 h) after the addition of Ca<sup>2+</sup> to bR solution, and the results showed that a Ca<sup>2+</sup>-binding equilibrated state was reached ~2–3 min after Ca<sup>2+</sup> was added to the solution. In a control experiment to determine the sensitivity of the Ca<sup>2+</sup> electrode response, a calibration curve was obtained by adding a

known amount of CaCl<sub>2</sub> to distilled deionized water. The mV reading, which is the potential difference between the ion selective electrode and the reference electrode and pCa where [Ca<sup>2+</sup>] is the concentration of free Ca<sup>2+</sup> in the solution, has a perfect linear relationship. This linearity is essential for an accurate and reproducible determination of calcium binding constants in the protein. The linear region covers the molarity of free Ca<sup>2+</sup> from 3–3.5  $\mu$ M, which is sufficient for our current application. The concentration of bO was calculated from the absorbance of the protein band at 280 nm.

## RESULTS AND DISCUSSION

### Cooperativity or independent binding sites

In previous studies (Zhang et al., 1992, 1993), a concave upward Scatchard plot was observed for the titration of deionized bR with Ca<sup>2+</sup>. It was possible to resolve the curve into three straight lines with different slopes. The fact that these lines extrapolated to exact integers (1, 2, and 6) suggested the presence of three classes with one metal cation in each of the first two classes (with the largest and second largest slope, i.e., highest affinity) and four in the third class (the low affinity one).

The fact that the resolution of the Scatchard plot of Ca<sup>2+</sup> binding to deionized bR into three straight lines made good physical sense alone does not eliminate the possibility of cooperative effects. This would also give rise to curved Scatchard plots. Cooperativity is due to interaction between binding sites. There are two possible origins of the introduction of cooperativity in bR. One arises from binding sites on different bR molecules within the trimer structure. The other could be due to the multiple binding sites being within the same bR molecule. To address the first possibility, Ca<sup>2+</sup> binding to deionized monomer bR was carried out.

The Scatchard plot for the binding to deionized monomer bR is shown in Fig. 1. A gradual upward concave curvature is observed, which is similar to that observed for Ca<sup>2+</sup>-regenerated bR. This rules out the cooperativity due to intermonomer interaction as the source of the curvature in the Scatchard plot of bR.

In a very recent work in our group (Yoo et al., 1995), competitive binding of Mg<sup>2+</sup> and Ca<sup>2+</sup> in bR was studied by both calcium ion selective electrode and UV-visible spectroscopy. The fact that the presence of Mg<sup>2+</sup> in one of the strongly bound sites does not change the value of the binding constant of Ca<sup>2+</sup> in the other binding site (and vice versa) excludes the possibility that the cooperative interaction is between metal cations in the two strong binding sites on the same bR molecule.

### The binding constants of monomer bR

We followed the same procedure as that of Zhang et al. (1992) to determine the binding constants for each binding class. Table 1 shows that in monomerized bR both the number of Ca<sup>2+</sup> ions in each binding class and the values of the corresponding binding affinity constants are close to those found for deionized bR. This leads to the important

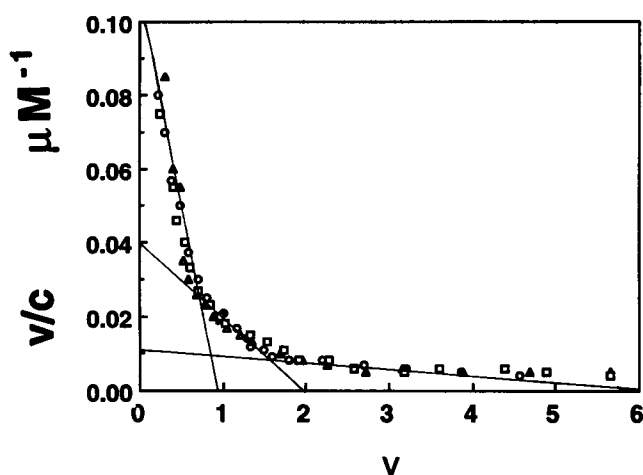


FIGURE 1 Scatchard plot for  $\text{Ca}^{2+}$  titration of deionized monomer bR. The slope of each straight line gives the binding affinity constant of  $\text{Ca}^{2+}$ , and the x-intercept shows the number of  $\text{Ca}^{2+}$  in each class. C is the concentration of free calcium ions in the monomer bR solution;  $V = [\text{bound } \text{Ca}^{2+}]/[\text{bR}]$ , where  $[\text{bound } \text{Ca}^{2+}]$  is the concentration of bound  $\text{Ca}^{2+}$  and  $[\text{bR}]$  is the concentration of deionized monomer bR. The data of three experiments ( $\square$ ,  $\Delta$ ,  $\circ$ ) are plotted to show the reproducibility. The similarity of the plot to that for deionized bR suggests that 1) the curvature in the Scatchard plot is not due to cooperativity between sites on different monomers, and 2) the metal cation binding sites reside within the monomer bR and not between bR monomers.

conclusion that metal cation binding sites in bR are within the monomer unit and that none of them is between the monomers in the trimer.

### Effect of retinal removal

The Scatchard plot of the  $\text{Ca}^{2+}$  binding to deionized aprotin is shown in Fig. 2, and the results are summarized in Table 1. It is clear that there is only one high affinity site with a binding constant somewhat larger than the second binding affinity in  $\text{Ca}^{2+}$ -regenerated bR but smaller than the binding constant of the first site. Both the number of  $\text{Ca}^{2+}$  ions that occupy the low affinity sites and their binding constants do not seem to differ much from those observed in deionized  $\text{Ca}^{2+}$ -regenerated bR.

It is thus concluded that retinal removal leads to changes in the nature of  $\text{Ca}^{2+}$  binding sites of bR. This observation

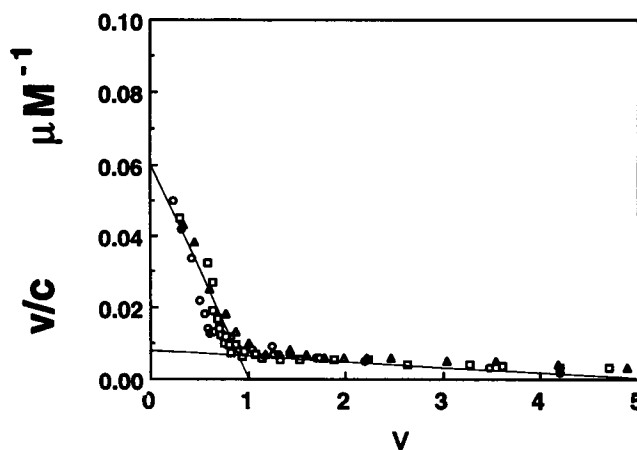


FIGURE 2 Scatchard plot for  $\text{Ca}^{2+}$  titration of deionized bO sample. The slope of each straight line gives the binding affinity of  $\text{Ca}^{2+}$ , and the x-intercept shows the number of  $\text{Ca}^{2+}$  in each category. C is the concentration of free calcium ions in equilibrium with the bO solution;  $V = [\text{bound } \text{Ca}^{2+}]/[\text{bO}]$ , where  $[\text{bound } \text{Ca}^{2+}]$  is the concentration of bound  $\text{Ca}^{2+}$  and  $[\text{bO}]$  is the concentration of deionized bO. The data of three experiments ( $\square$ ,  $\Delta$ ,  $\circ$ ) are plotted to show the reproducibility. The fact that the plot is different from that in bR and monomer bR (Fig. 1) suggests that the high affinity sites are not far from retinal.

supports the previous conclusion reached from the results of  $\text{Ca}^{2+}$  titration (Jonas and Ebrey, 1991) and titration of deionized bR mutant (Zhang et al., 1993). These studies suggested the presence of direct coupling between each  $\text{Ca}^{2+}$  ion in the high affinity sites with charged residues within the retinal pocket. In the studies of Zhang et al. (1993), the observed effect on the  $\text{Ca}^{2+}$  binding resulting from the individual replacement of charged D85, D212, R82, and H-bonding Y185 residues, which are known to be in the active site of retinal (retinal pocket), can be summarized as follows. 1) The two high affinity sites exhibit much reduced binding constants in D85N, D212N, and R82Q mutants, and the effect on the two strong binding sites are comparable, which leads to the conclusion that  $\text{Ca}^{2+}$  in the two high affinity sites interacts with aspartate (Asp) 85 and 212 and with arginine 82 by means of nonspecific long-range electrostatic interaction. 2) On the other hand, Y185F has a different effect on the two strongly bound  $\text{Ca}^{2+}$  ions, which results from short-range interactions. The above conclusion was recently supported by the agreement between the calculation (Stuart et al., 1995) and the observed one- and two-photon spectra of retinal in bR if a  $\text{Ca}^{2+}$  ion is bound to Asp-85 and Asp-212. The calculation uses all valence electron MNDO and MNDO-PSDCI molecular orbital theory in which the binding site for the metal ion near the retinal is formed by Asp-85, Asp-212, and tyrosine 57 and 185, together with three water molecules.

Our present results suggest the presence of only one strongly bound  $\text{Ca}^{2+}$  ion in bO. This provides direct evidence that the high affinity sites are not far from the retinal. It is difficult to conclude from our results whether the lost site belongs to the first or second binding site in native bR. It is possible that the site we observe in bO is structurally

TABLE 1 Apparent binding constants and number of bound  $\text{Ca}^{2+}$  in different classes for regenerated bR, monomerized bR, and retinal removed bR (all of the samples have an initial pH of 4.50–4.60)\*

Sample	$K_1$ ( $\text{mM}^{-1}$ ) ( $n_1$ )	$K_2$ ( $\text{mM}^{-1}$ ) ( $n_2$ )	$K_3$ ( $\text{mM}^{-1}$ ) ( $n_3$ )
Wild type bR	135 (1)	23 (1)	2.0 (4)
Monomerized bR	110 (1)	20 (1)	2.4 (4–5)
Retinal removed bR	57 (1)		2.0 (4)

\*Values shown are the average of 5–10 trials with relative standard deviation <10%.  $n_1$ ,  $n_2$ , and  $n_3$  are the numbers of  $\text{Ca}^{2+}$  in each of these classes.

different from either of the two high affinity sites in bR. Because the effect of a charged residue replacement in bR affects the binding constants of both sites by a comparable amount (Zhang et al., 1993), the fact that one site disappeared by retinal removal suggests that the charges in the structure of the pocket involves more than just displacement of charged amino acid residues. The number and the structure of the water molecules could also change by retinal removal, thus leading to further changes in the structure of the metal cation binding sites on going from bR to bO.

Potentiometric titration on Ca<sup>2+</sup> binding for both deionized native bR, monomerized bR, and bO gave essentially the same values for the binding constant of the lowest affinity class. This supports the previous conclusion that this class is a surface one and is thus far from the retinal.

In conclusion, the fact that bR monomer gave the same binding characteristics as observed from the Scatchard plot for the trimer suggests that the binding sites are made of amino acids (and water molecules) within the monomer and not between the monomers in the trimer structure. Furthermore, the fact that the nature of these sites changes by removing retinal supports the previous proposal that these sites are not far from the retinal cavity. It is not known whether one high affinity (the highest affinity) site is eliminated, leaving the second high affinity site intact, or whether both high affinity sites in bR are sufficiently changed by retinal removal to give a new one.

This work was supported by the Department of Energy (Office of Basic Energy Science) Grant DE-FG03-88ER13828.

## REFERENCES

- Ariki, M., and J. K. Lanyi. 1986. Characterization of metal ion binding sites in bacteriorhodopsin. *J. Biol. Chem.* 261:8167-8174.
- Bayley, H., R. Radhakrishnan, K. S. Huang, and H. G. Khorana. 1981. Light-driven proton translocation by bacteriorhodopsin reconstituted with the phenyl analog of retinal. *J. Biol. Chem.* 256:3797-3801.
- Becher, B. M., and J. Y. Cassim. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep. Biochem.* 5:161-178.
- Cao, Y., G. Varo, M. Chang, B. Ni, R. Needleman, and J. K. Lanyi. 1991. Water is required for proton transfer from Aspartate-96 to the bacteriorhodopsin Schiff base. *Biochemistry*. 30:10972-10979.
- Chang, C. H., J. G. Chen, R. Govindjee, and T. Ebrey. 1986. Mechanism and role of divalent cation binding of bacteriorhodopsin. *Biophys. J.* 49:731-739.
- Corcoran, T. C., K. Z. Ismail, and M. A. El-Sayed. 1987. Evidence for the involvement of more than one metal cation in the Schiff base deprotonation process during the photocycle in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 84:4094-4098.
- Dencher, N. A., and M. P. Heyn. 1978. Formation and properties of bacteriorhodopsin monomers in the non-ionic detergents octyl- $\beta$ -D-glucoside and Triton X-100. *FEBS Lett.* 96:322-326.
- Dunach, M., M. Seigneuret, J. L. Rigand, and E. Padros. 1987. Characterization of the cation binding sites of the purple membrane. Electron spin resonance and flash photolysis studies. *Biochemistry*. 26:1179-1186.
- Ebrey, T. G. 1993. Light energy transduction in bacteriorhodopsin. In *Thermodynamics of Membrane Receptors and Channels*. M. B. Jackson, editor. CRC Press, Boca Raton. 353-387.
- Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211-230.
- Jonas, R., and T. G. Ebrey. 1991. Binding of a single divalent cation directly correlates with the blue-to-purple transition in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 88:149-153.
- Jonas, R., Y. Koumlos, and T. G. Ebrey. 1990. Purple membrane: surface charge density and the multiple effect of pH and cations. *Photochem. Photobiol.* 52:1163-1177.
- Kimura, Y., A. Ikegami, and W. Stoeckenius. 1984. Salt and pH-dependent changes of the purple membrane absorption spectrum. *Photochem. Photobiol.* 40:641-646.
- Kovacs, I., K. Hollos-Nagy, and G. Varo. 1995. Dark adaptation and spectral changes in Triton-X-100 treated bacteriorhodopsin. *J. Photochem. Photobiol. B: Biology.* 27:21-25.
- Mathies, R. A., S. W. Lin, J. B. Ames, and W. T. Pollard. 1991. From femtosecond to biology: mechanism of bacteriorhodopsin's light-driven proton pump. *Annu. Rev. Biophys. Chem.* 20:491-518.
- Moore, T. A., M. E. Edgerton, G. Parr, C. Greenwood, and R. N. Perham. 1978. Studies of an acid-induced species of purple membrane from *Halobacterium halobium*. *Biochem. J.* 171:469-476.
- Mowery, P. C., R. H. Lozier, Q. Chae, Y. W. Tseng, M. Taylor, and W. Stoeckenius. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry*. 18:4100-4107.
- Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature*. 233:149-152.
- Papadopoulos, G., D. A. Dencher, G. Zaccai, and G. Buldt. 1990. Water molecules and exchangeable hydrogen ions at the active centre of bacteriorhodopsin localized by neutron diffraction. Element of the proton pathway? *J. Mol. Biol.* 214:15-19.
- Stuart, J. A., B. W. Vought, C.-F. Zhang, and R. R. Birge. 1995. The active site of bacteriorhodopsin. Two-photo spectroscopic evidence for a positively charged chromophore binding site mediated by calcium. *Biospectroscopy*. 1:9-28.
- Sweetman, L. L., and M. A. El-Sayed. 1991. The binding site of the strongly bound Eu<sup>3+</sup> in Eu<sup>3+</sup>-regenerated bacteriorhodopsin. *FEBS Lett.* 282:436-440.
- Szundi, I., and W. Stoeckenius. 1987. Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 84:3681-3684.
- Szundi, I., and W. Stoeckenius. 1988. Purple-to-blue transition of bacteriorhodopsin in a neutral lipid environment. *Biophys. J.* 54:227-242.
- Szundi, I., and W. Stoeckenius. 1989. Surface pH controls purple-to-blue transition of bacteriorhodopsin. A theoretical model of purple membrane surface. *Biophys. J.* 56:369-383.
- Wu, S., E. S. Awad, and M. A. El-Sayed. 1991. Circular dichroism and photocycle kinetics of partially detergent solubilized and retinal regenerated bacteriorhodopsin. *Biophys. J.* 59:70-75.
- Wu, S., and M. A. El-Sayed. 1994a. Binding characteristics of an organometallic cation, Ru(bpy)<sub>3</sub><sup>2+</sup>, in regenerated bacteriorhodopsin. *J. Phys. Chem.* 98:7246-7251.
- Wu, S., and M. A. El-Sayed. 1994b. Binding of, and energy-transfer studies from retinal to, organic cations in regenerated reduced bacteriorhodopsin. *J. Phys. Chem.* 98:9339-9344.
- Yoo, S., E. S. Awad, and M. A. El-Sayed. 1995. Comparison between the binding of Ca<sup>2+</sup> and Mg<sup>2+</sup> to the two high affinity sites of bacteriorhodopsin. *J. Phys. Chem.* 99:11600-11604.
- Zhang, Y. N., and M. A. El-Sayed. 1993. The C-terminus and the Ca<sup>2+</sup> low-affinity binding sites in Bacteriorhodopsin. *Biochemistry*. 32:14173-14175.
- Zhang, Y. N., M. A. El-Sayed, M. L. Bonet, J. K. Lanyi, M. Chang, B. Ni, and R. Needleman. 1993. Effects of genetic replacements of charged and H-bonding residues on Ca<sup>2+</sup> binding to deionized bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 90:1445-1449.
- Zhang, Y. N., L. L. Sweetman, E. S. Awad, and M. A. El-Sayed. 1992. Nature of the individual Ca<sup>2+</sup> binding sites in Ca<sup>2+</sup>-regenerated bacteriorhodopsin. *Biophys. J.* 61:1201-1206.