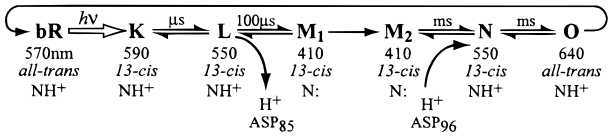
# Large Organic Cations Can Replace $Mg^{2+}$ and $Ca^{2+}$ Ions in Bacteriorhodopsin and Maintain Proton Pumping Ability

Eric H. L. Tan, Deshan S. K. Govender, and Robert R. Birge*

*Department of Chemistry and W. M. Keck Center for Molecular Electronics, Syracuse University Syracuse, New York 13244-4100*

*Received October 17, 1995*

The mechanism of the proton pump of bacteriorhodopsin is a subject of fundamental importance to our understanding of biological structure and function.[1−8] The functioning of light-adapted bacteriorhodopsin ($\lambda_{max} \approx 570$ nm) as a proton pump requires the presence of $Ca^{2+}$ and $Mg^{2+}$ ions which bind at specific locations inside the protein and on the surface.[9−17] Removal of these cations produces the blue membrane ($\lambda_{max} \approx$ 603 nm), a protein which lacks a photocycle and which does not pump protons.[9−16] It has long been known that the spectroscopic and photochemical properties of the protein can be restored by adding either calcium or magnesium to an aqueous solution of the blue membrane.[9−16] We demonstrate in this paper that analog proteins with nearly identical spectroscopic properties and similar photochemical properties can be generated by adding large organic monovalent and divalent cations to the blue membrane. The native photocycle is summarized below.




We generated analog bacteriorhodopsins from the resin-washed, cation-depleted blue membrane[17] with the addition of a series of monoquaternary ammonium cations and bis(quaternary ammonium) or so-called "bolaform" cations (see Figure 1). We were surprised to observe that all of the above salts regenerate the purple color[18,19] and restore the photocycle and proton pumping ability of the protein. The spectra of the organic cation bR analogs are similar to those of the native protein, with absorption maxima within a few nanometers of those of the native protein. To examine systematic variations, the main absorption bands of the protein spectra were fit by using a log-normal distribution function.[8] This procedure fits the band to a skewed Gaussian as a function of the absorption maximum ($\lambda_{max}$), full width at half-maximum ($\Delta\tilde{\nu}$), and skewness ($\sigma$). The results of the least-squares fits are shown in Figure 1 for spectra recorded in polyacrylamide gel (the gel prevents aggregation).

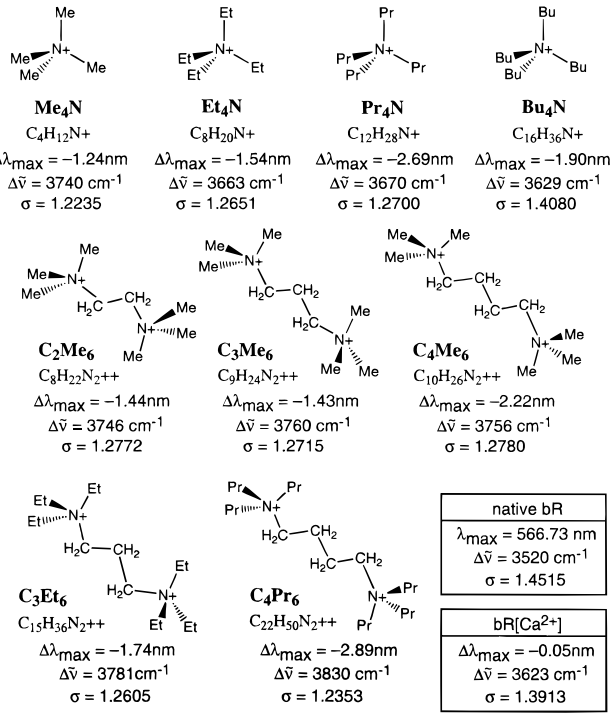



**Figure 1.** Structure of the quaternary ammonium salts and the spectroscopic properties of the light-adapted analog bacteriorhodopsin proteins generated via addition of the organic cations to the protein blue membrane (see text).

Regeneration of bacteriorhodopsin was studied by monitoring $\lambda_{max}$ as a function of salt titration of the blue membrane at nearly constant pH ($\Delta$pH < 0.2) to avoid pH effects.[10,15,17] The divalent bolaforms bind with much higher affinity to the protein as compared to the monovalent $R_4N$ salts. For example, it takes ~60 times more $Me_4N$ to regenerate bR from the blue membrane at pH = 4.02 as compared to $C_2Me_6$, even though both the cations have roughly the same charge density and hydrophobicity ($C_2Me_6$ is roughly two $Me_4N$ molecules in terms of molecular structure; see Figure 1).[20] The Gouy−Chapman or "surface effect" theory (e.g., ref 21) cannot rationalize the large differences observed in the binding ability of the divalent bolaforms versus the simple monovalent salts. We conclude that the boloform cations are occupying divalent cation binding sites within the protein.

Of particular interest is the chromophore-adjacent cation binding site. This site is solely responsible for regenerating the purple color of bR[11] and represents the second high-affinity binding site.[15,16] Recent two-photon studies indicate that the hydrated metal cation occupies a dynamic site involving two aspartate residues ($Asp_{85}$ and $Asp_{212}$) and two tyrosine residues

(1) El-Sayed, M. A. *Acc. Chem. Res.* **1992**, *25*, 279−286.
(2) Ebrey, T. G. In *Thermodynamics of membrane receptors and channels*; Jackson, M. B., Ed.; CRC Press: Boca Raton, FL, 1993; Chapter 10, pp 353−387.
(3) Deng, H.; Huang, L.; Callender, R.; Ebrey, T. *Biophys. J.* **1994**, *66*, 1129−1136.
(4) Honig, B.; Ebrey, T.; Callender, R. H.; Dinur, U.; Ottolenghi, M. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2503−2507.
(5) Lanyi, J. K. *Nature* **1995**, *375*, 461−463.
(6) Mathies, R. A.; Lin, S. W.; Ames, J. B.; Pollard, W. T. *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 491−518.
(7) Oesterhelt, D.; Stoeckenius, W. R. *Nature* **1971**, *233*, 149−152.
(8) Birge, R. R. *Biochim. Biophys. Acta* **1990**, *1016*, 293−327.
(9) Ariki, M.; Lanyi, J. K. *J. Biol. Chem.* **1986**, *261*, 8167−8174.
(10) Chang, C. H.; Chen, J. G.; Govindjee, R.; Ebrey, T. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 396−400.
(11) Jonas, R.; Ebrey, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 149.
(12) Jonas, R.; Koutalos, Y.; Ebrey, T. G. *Photochem. Photobiol.* **1990**, *52*, 1163−1177.
(13) Padros, E.; Dunach, M.; Sabes, M. *Biochim. Biophys. Acta* **1984**, *769*, 1−7.
(14) Stuart, J. A.; Vought, B. W.; Zhang, C. F.; Birge, R. R. *Biospectroscopy* **1995**, *1*, 9−28.
(15) Zhang, Y. N.; Sweetman, L. L.; Awad, E. S.; El-Sayed, M. A. *Biophys. J.* **1992**, *61*, 1201−1206.
(16) Zhang, Y. N.; El-Sayed, M. A.; Bonet, M. L.; Lanyi, J. K.; Chang, M.; Ni, B.; Needleman, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1445−1449.
(17) Kimura, Y.; Ikegami, A.; Stoeckenius, W. *Photochem. Photobiol.* **1984**, *40*, 641−646.
(18) Mukohota and Ihara[19] reported regeneration of the purple color following addition of diamino acridine, a monovalent organic cation, to the blue membrane.
(19) Mukohota, Y.; Ihara, K. In *Retinal Proteins*; Ovchinnikov, Y. A., Ed.; VNU Science Press: Zeist, The Netherlands, 1984; p 195.
(20) Supporting information this paper provides additional spectroscopic data, titration curves, and vesicle-based proton pumping studies as well as details regarding the pump-probe measurements of the O-state decay kinetics.
(21) Szundi, I.; Stoeckenius, W. *Biophys. J.* **1989**, *56*, 369−383.

0002-7863/96/1518-2752$12.00/0 

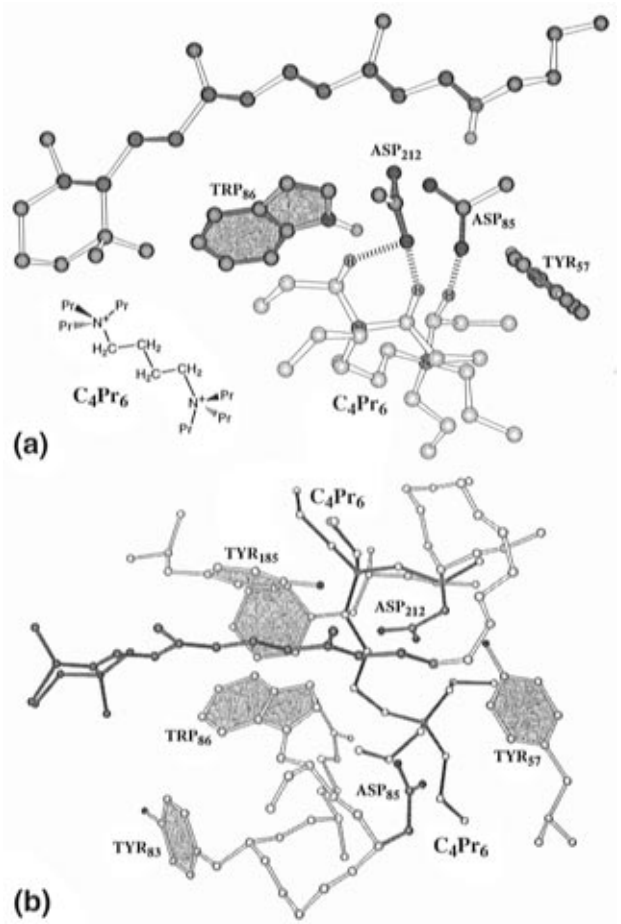



**Figure 2.** Nominal location of $C_4Pr_6$ in the chromophore-adjacent cation binding site of light-adapted bacteriorhodopsin based on MNDO/PM3 molecular orbital theory (a) and semiclassical MM2 force field (b) geometry minimization. The two structures differ significantly, but both methods predict that the organic divalent cation can be accommodated within the binding site in a fashion which closely mimics the electrostatic characteristics of a bound calcium ion (see text). Only selected hydrogen atoms are shown.

($Tyr_{57}$ and $Tyr_{185}$).[14] The key question is how a large organic cation can occupy the same binding site. We used both semiclassical (MM2)[22] and semiempirical quantum mechanical (PM3)[23] procedures to model the process. We discovered, to our surprise, that all of the organic cations, with the exception of the $Bu_4N$, could be accommodated with only minor shifts in the positions of the amino acid residues from those proposed by Henderson et al.[24] Our simulations for the largest bolaform divalent cation, $C_4Pr_6$, are shown in Figure 2. We note, however, that the organic cation does not occupy the site proposed for calcium.[14] MNDO/CI calculations[14] predict a 5 nm blue shift associated with replacement of $Ca^{2+}$ by $C_4Pr_6$. The observed shift is ~3 nm (see Figure 1). The blue shift is associated with two factors: a more diffuse positive charge on the organic cation and a shift of the organic cation further from the retinyl chromophore.

Although all of the analog proteins pump protons, the efficiency decreases as the size of the cation increases.[20] Preliminary experiments indicate that the L → M transition is partially shunted by a competitive L → bR reaction, with the proportion of the latter increasing as the size of the organic cation increases. The decay of the M intermediate is biphasic, composed of a fast and a slow component. While the formation and slow-decay are similar for all of the analog proteins, the fast-decay is slower for the larger cations. These results suggest that the

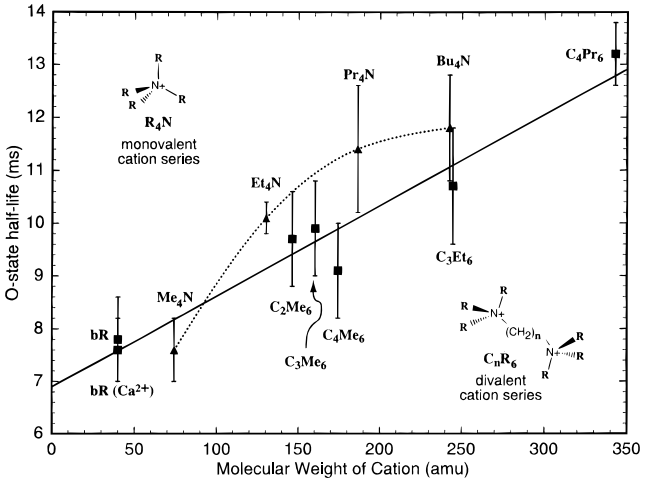



**Figure 3.** Half-life of the O intermediate plotted as a function of the molecular weight of the organic cation.[20] The kinetic data for the divalent bolaform amines are linear with respect to molecular weight within experimental error (straight line shows a least-squares fit). The monovalent tetraalkylamines must be bound in pairs to simulate the divalent metal cation, and the kinetics display a quadratic dependence on molecular weight.

fast-decay involves cation movement. [Microwave absorptivity experiments suggest that, in native bR, the calcium enters the proton channel and provides an electrostatic gate, preventing back-transfer of the proton during the latter stages of the photocycle (results to be published).] The reformation of bR from the O state, as measured by O-state absorption at 647 nm, is linearly proportional to the molecular weight of the organic cation (see Figure 3).[20] We conclude that the cation within the chromophore-adjacent binding site drifts away from the binding site when M is formed in response to protonation of $Asp_{85}$. During the O-to-bR reaction, $Asp_{85}$ must transfer its proton to an acceptor group, and this proton transfer requires motion of the cation into a position of electrostatic stabilization of the $Asp_{85}$ anion.

We conclude by noting that organic cations may provide an important method for improving the reliability of protein-based artificial retinas. In one design, the oriented bacteriorhodopsin protein is placed directly on top of a charge-sensitive semiconductor array.[25] When this design is implemented using native protein, however, the metal cations migrate into the semiconductor and poison it, rendering it insensitive to charge differentials on the surface. The organic bolaform cations maintain photochemical activity of the protein and do not appear to damage the semiconductor array. Thus, incorporation experiments involving the organic cations shown in Figure 1 not only provide new insights into the structure and function of bacteriorhodopsin but may enhance the utility of bacteriorhodopsin as a photochromic material in hybrid semiconductor protein devices.

**Acknowledgment.** The authors thank C. R. Birge for preparing purified bacteriorhodopsin and J. A. Stuart, J. R. Tallent, B. W. Vought, and M. Yamazaki for helpful discussions. This work was supported by the NIH (GM-34548).

**Supporting Information Available:** Additional spectroscopic data, titration curves, and proton pumping studies (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA953491+

(22) Buckert, U.; Allinger, N. L. *Molecular Mechanics*; ACS Monograph 177; American Chemical Society: Washington, DC, 1982.

(23) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 221−245.

(24) Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *J. Mol. Biol.* **1990**, *213*, 899−929.

(25) Chen, Z.; Birge, R. R. *Trends Biotechnol.* **1993**, *11*, 292−300.