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J-2753-1

## SUPPLEMENTARY MATERIAL.

# Analysis of the Mechanism of Cation Binding, O State Kinetics and the Proton Pumping Ability of Analog Bacteriorhodopsins Generated by Replacement of Metal Cations with Large Organic Cations

### S.1. Mechanism of Cation Binding

All of the organic cations, when added to the blue membrane, regenerate the purple membrane. Sample spectra taken during titration of the blue membrane with the largest bolaform divalent cation ($C_4Pr_6$) are shown in Fig. 4.

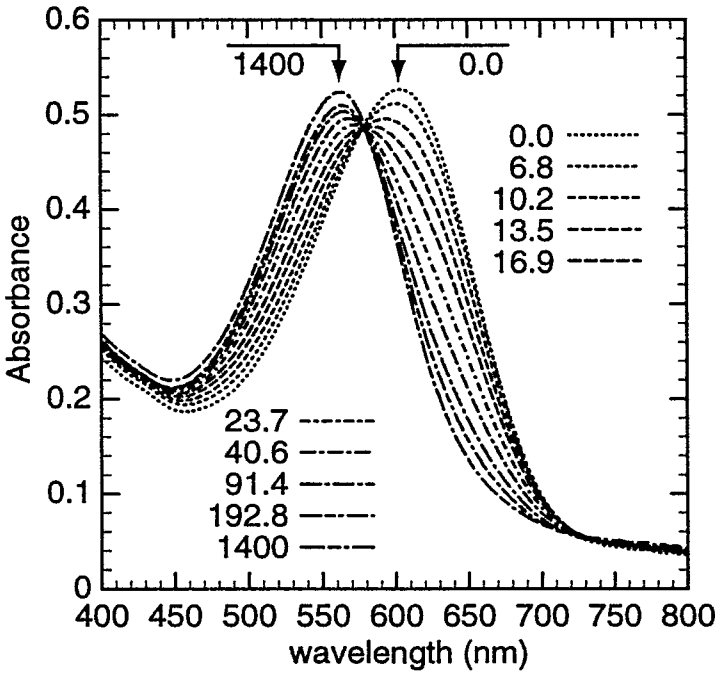


**Figure 4.** Titration of blue membrane solution ($OD_{603}$ = 0.53) as a function of the indicated concentrations of $C_4Pr_6$ in μM at 25°C. A single isosbestic point at ~580 nm is observed [$\lambda_{max}$(blue membrane) = 603 nm; $\lambda_{max}$(purple membrane analog) = 563 nm].

The titration curves shown in Fig. 5 indicate that the bolaform divalent cations bind with much greater affinity as compared to the monovalent organic cations. For example, it takes roughly 60 times more $Me_4N$ as compared to $C_2Me_6$ for complete protein regeneration even though both of these cations have roughly the same charge density. We note that $C_2Me_6$ can be viewed as two $Me_4N$ molecules in terms of chemical structure. Binding studies at the surface of non-specific anionic micelles (long-chain alkyl sulphates) indicate that the ratio of the degree of association between a divalent cation to a monovalent cation is on the order of 1.1- 1.5.[26,27] Thus, mechanisms that involve surface binding (Gouy-Chapman theory[21]) or ionic screening cannot rationalize the observations. We conclude that the bolaform cations bind at specific sites in bacteriorhodopsin to affect the blue-to-purple color transition. Of particular importance is the second high-affinity site which is solely responsible for the blue-to-purple transition in bacteriorhodopsin.[11,15] Studies indicate that this site is located near the retinyl chromophore involving the binding of a cation to two aspartate residues ($ASP_{85}$ and $ASP_{212}$) and two tyrosine residues ($TYR_{57}$ and $TYR_{185}$).[14]

By using molecular orbital simulations, we find that the bolaform cation can be accommodated into this site while approximately preserving the native absorbance maximum of the protein. Fourth derivative spectra of the analog proteins indicate systematic perturbation of the environment of the chromophore within the protein (data not shown) and supports the binding of the organic cations at the chromophore-adjacent cation binding site. In addition, determination of the dissociation constants of the bolaform cations according to a published method[9] gave values that are in the order of ~0.2 and ~15 μM for the first and second cation binding sites respectively indicating high affinity binding of the cations with the protein.

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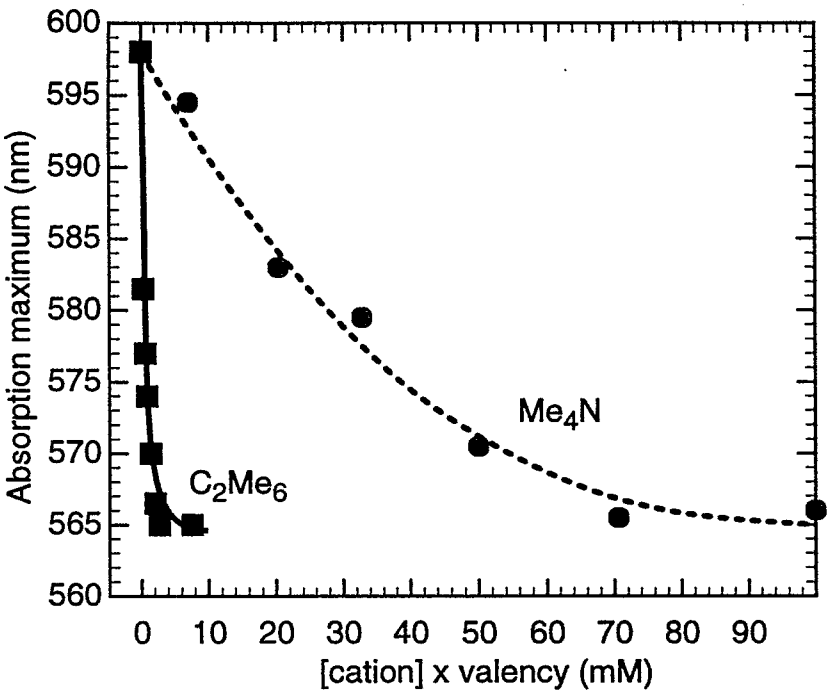


**Figure 5.** The change in the absorption maximum as a function of titration of 3 ml of blue membrane solution ($OD_{598}$~1) with the addition of stock 0.02 M **$C_2Me_6$** or 0.5 M **$Me_4N$** solutions at 25 °C. The data are reported as a function of cation concentration times the charge on the cation. Titration curves for calcium are slightly steeper than the curve shown for **$C_2Me_6$**. The absorption maximum of blue membrane solution varies between 598 and 603 nm depending on the preparation and the solvent. The titration experiments were carried out on the same batch of the blue membrane solution.

## S.2. Flash photolysis measurements of the O intermediate

In this section we provide additional details regarding the experimental methods that were used to generate the data shown in Fig. 3.

A dual wavelength pump-probe spectrometer was used to study the formation and decay of the **O** intermediate. The photocycle of the light-adapted protein was initiated by a frequency double Q-switched Nd-YAG laser (532 nm, Continuum Model PL-9030) operating at 10 Hz with 10-12 ns pulse widths. A continuous-wave probe beam (647 nm, Coherent Innova 90) was directed onto the same spot as the excitation beam on the sample and then through two 650 nm narrow band filters before reaching the photodetector (New Focus Model 2011). The gel sample was sandwiched between two glass plates, and placed in a film-holder in front of the optical filters. The probe signal was digitized by a 2 Gigasample/second - 500 MHz digital oscilloscope(Hewlett Packard 5452-A) triggered by the Nd-YAG laser pulse via a second photodiode. The probe signals were averaged for 256 pump pulses and the temporal signals were highly reproducible.

Gel samples were used to avoid possible aggregation of the protein. Previous studies have shown that the incorporation of **bR** into polyacrylamide gel does not affect the spectroscopic and photochemical properties of the protein.[9,10] Preparation of bacteriorhodopsin-doped polyacrylamide gel has been described previously.[9] The protein in the gel was converted to blue membrane by stirring the purple gels in well-washed cationic exchange resin for ~5 hours. The organic-cation-bR analogs were generated by incubating the blue membrane gel with the salts of interest for ~5 hours in the presence of low concentration of Tris-HCl buffer (pH = 7.6). Completion of the regeneration process was indicated by the stability of the main absorbance band.

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## S.3 Analysis of proton pumping efficiency

The ability of the analog proteins to pump protons was studied by carrying out pH measurements on light-induced alkalinization of **bR**-incorporated azolectin vesicle solution following the procedures of Casadio and Stoeckenius.[28] However, we used the organic salts to regenerate **bR** as well as to generate the vesicles, instead of using high concentrations of KCl as recommended in Ref. 28. The results for calcium versus four difference organic cations are presented in Fig. 6.

Relative proton pumping efficiencies of the analog proteins can be more accurately evaluated by considering the light-induced pH signals together with the amplitude of the **M** state. Correlation between proton pumping efficiency with the **L** → **M** reaction is well established.[29] Based on the data shown in Fig 6 as well as comparable data collected on the other organic cations, we conclude that all of the analog proteins pump protons, as indicated by the abrupt increase in the pH upon irradiation of the solution. Based on these results, we can tentatively conclude that the **$Bu_4N$**-based analog is the least efficient proton pump.

The relative magnitude of the signals may or may not have significance, however. Subtle differences in the vesicle structure may be contributing to the results. For example, pH measurements indicate that **$C_2Me_6$/bR** is more efficient in pumping protons than native **bR** in Ca(II) solution. However, **M** state amplitudes indicate that these two proteins should be similar with respect to efficiency. Calcium is known to have significant effects on the shape and transport properties of vesicles.[30] Thus, further work will be necessary to quantify the relative efficiency of the proton pumping process for the analog proteins. These studies are in progress.

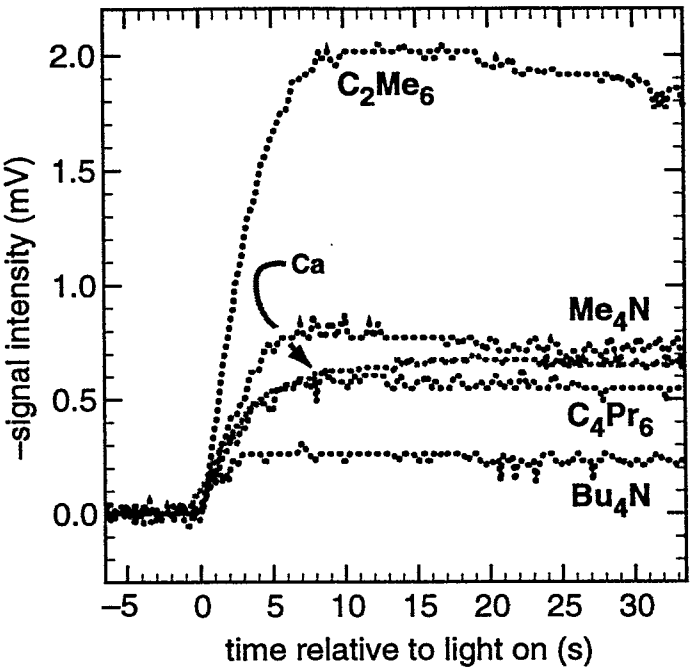


**Figure 6.** Light-induced alkalinization of the bR-incorporated vesicle solutions (initial pH ~6.2) at 25 °C. A decrease in the signal intensity indicates an increase in the pH of the solution. The signal associated with Ca(II) incorporation is in grey for reference.

(1) - (25) *see preceding communication.*

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