Supporting Information

Distortion and a Strong Hydrogen Bond in the Retinal Chromophore Enable Sodium Ion Transport by the Sodium Ion Pump KR2

Nao Nishimura,† Misao Mizuno,† Hideki Kandori,‡ and Yasuhisa Mizutani†

† Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

‡ Department of Life Science and Applied Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya, Aichi 466-8555, Japan

Contents

1. Data corrections

Sensitivity calibration of the CCD detector

The sensitivity of the detector was calibrated using the fluorescence spectra of standard samples, as described previously[1-2] with modifications. The sensitivity of the M intermediate spectra was calibrated using an aqueous solution of the fluorescence standard quinine sulfate (Wako Pure Chemical) by excitation at the Raman probe wavelength of 405 nm and measurement using a fluorescence spectrometer (Hitachi, F-2700). The fluorescence intensity in the spectral window of the Raman measurements of the standard sample was obtained by polynomial fitting of the fluorescence spectrum measured by the fluorescence spectrometer. Then, the fluorescence spectrum of quinine sulfate was measured using exactly the same setup as used for the Raman measurements, thus allowing calibration of the pixel-to-pixel sensitivity variations of the CCD camera. The sensitivity of the O intermediate spectra was calibrated using an ethanol solution of the fluorescence standard sample 1,2,3,5,6,7-hexamethyl-8-cyanopyrromethene-difluoroborate (Exiton, pyrromethene 650). Before obtaining the fluorescence intensity of pyrromethene 650 in the spectral window of the Raman measurements, the sensitivity of the fluorescence spectrometer was calibrated by normalizing to the absolute fluorescence intensity of [2-[2-[4-(dimethylamino)phenyl]ethenyl]-6-methyl-4H-pyran-4-ylidene]-propanedinitrile (Exciton, DCM) in methanol excited at 460 nm.[3]

Elimination of spectral contributions due to the chromophore in incorrectly folded protein

Unexpected additional bands were observed in the Raman spectra obtained by excitation at 405 nm. For example, a strong additional band at 1577 cm^{-1} appeared in the Raman spectrum without pump beam irradiation (traces a in panels A and B of Figure S1). To discuss the chromophore structure of the KR2 intermediate, we had to eliminate the contribution of unexpected additional bands from the obtained spectra.

Intensities of these additional bands increased as the protein sample degraded using high pH conditions (trace b in panel A of Figure S1). Based on this observation, we assigned the additional bands to the deprotonated chromophore in incorrectly folded protein because the deprotonated chromophore generally has an absorption band around 400 nm and hence its Raman bands are resonantly enhanced by the 405 nm excitation. We obtained spectral contribution of the deprotonated chromophore in incorrectly folded protein (trace c in panel A of Figure S1) by calculating difference between the spectra at neutral and high pH.

The intensity increase of the 1577 cm^{-1} band was also observed upon long irradiation with 514.5 nm beam. Traces a and b in panel B of Figure S1 shows the spectra prior to and after 300 minute irradiation with 514.5 nm beam, respectively. In trace b, the intensity of the 1577 cm^{-1} band significantly increased. We obtained a difference spectrum between traces a and b. The difference spectrum as shown in trace c in panel B was similar to trace c in panel A, indicating this can be due to the deprotonated chromophore in incorrectly folded protein. The S/N ratio of the former was higher than that of the latter because the effect of the 514.5 nm irradiation on the intensity increase of the 1577 cm^{-1} band was larger than that of the pH increase.

We next subtracted the spectral contribution of the deprotonated chromophore in incorrectly folded protein (trace c) from the raw spectrum (trace a), and then obtained the spectrum of the retinal chromophore in correctly folded protein as shown in trace d in panel B. The spectral pattern of the trace d in panel B was nearly identical to that of the spectrum obtained by excitation at 514.5 nm as shown in panel C of Figure S1, providing evidence that subtraction of the spectral contribution due to incorrectly folded protein was adequately performed. For the transient spectrum, we eliminated spectral component due to the deprotonated retinal chromophore in incorrectly folded protein in this procedure.

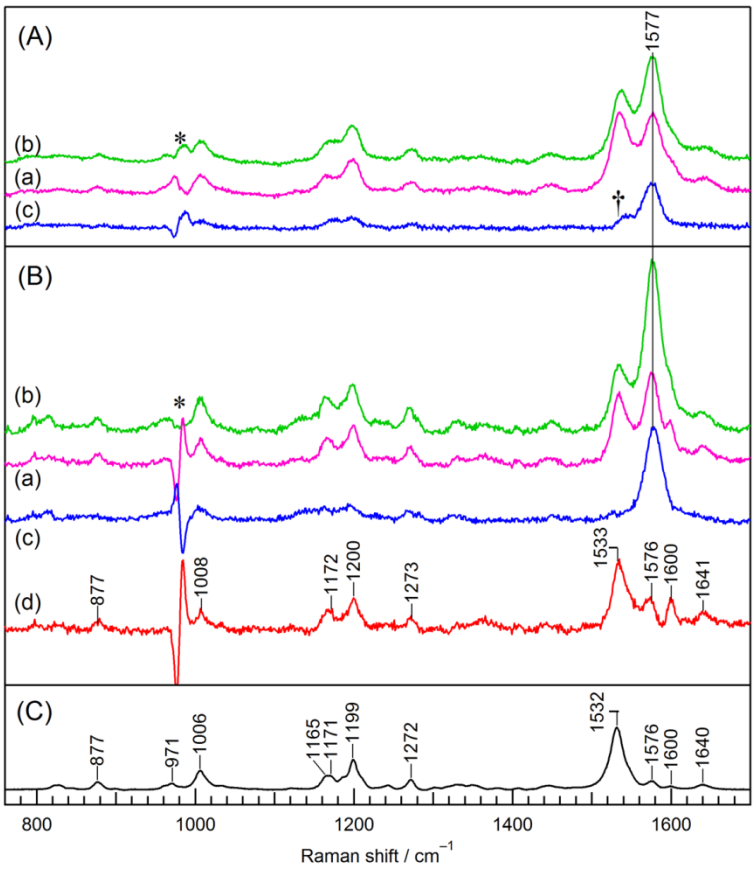

Figure S1. (A) Raman spectra of KR2 excited at 405 nm at pH 8.0 (a) and pH 10.0 (b). (c) Difference spectrum obtained by a function of ((b) − (a) × 0.77) representing the spectrum of the retinal chromophore in the incorrectly folded protein. (B) Raman spectra of KR2 excited at 405 nm prior to (a) and after (b) the 300 minute irradiation with 514.5 nm beam. (c) Difference spectrum obtained by a function of ((b) − (a) × 0.87). (d) Difference spectrum obtained by a function of ((a) − (c) × 0.7) representing the spectrum of the retinal chromophore in the unphotolyzed state in the correctly folded protein. (C) Raman spectra of KR2 excited at 514.5 nm. The spectrum of buffer including the sulfate ion has been subtracted from each spectrum. The derivative-like features marked as an asterisk and a dagger are due to small shifts of the sulfate band at 980 cm^{-1} and the C=C stretch band at 1533 cm^{-1}, respectively, arising from a few micrometer fluctuation of the beam position at the sample cell. Relative intensity of the 1577 cm^{-1} band to the 1533 cm^{-1} band was different in every purification of the sample solution due to fluctuation in purity of the protein.

Close examination of spectral contamination in the traces of Figure 4

In traces a of Figure 4, HOOP bands were observed at 877 and 880 cm^{-1} in H_2O and D_2O buffer, respectively. These bands were not observed in traces b-e, showing no spectral contamination due to the unphotolyzed state in the traces. In the same way, we rule out the possibility that the spectral contamination of the K intermediate is involved in traces a, c, d, and e on the basis of HOOP bands at 778 and 796 cm^{-1} in traces b, which are attributable to the K intermediate.

Bands at 799 and 800 cm^{-1} attributed to the L intermediate was observed in traces c and not seen in trace a, b, d, and e, meaning that the spectral contamination of the L intermediate is negligible in the traces. Bands at 805 and 803 cm^{-1} attributed to the O intermediate was observed in traces e and not seen in traces a–d, meaning that the spectral contamination of the O intermediate is negligible in the traces. The spectral contamination due to the M intermediate is highly unlikely because of its low transient accumulation and its absorption maximum wavelength far from the wavelengths of the probe lights for traces a, b, c, and e. Consequently, the HOOP bands observed in traces a–e are attributed to the unphotolyzed state, the K, L, M, and O intermediates, respectively.

2. Transient absorption spectra of KR2

Experimental

The purified protein sample was suspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 300 mM Na_2SO_4, and 0.1% DDM. Transient absorption spectra were measured while circulating the sample solution in a rectangular-shaped (1 × 4 mm^2) quartz cell. A xenon arc lamp (Hamamatsu Photonics, L8004-01) was used as a white probe light source. We initiated the photoreaction of KR2 with a 532 nm nanosecond pulse (the second harmonic of the output of a Q-switched Nd:YAG laser; Continuum, Minilite) operating at 10 Hz. The pulse width of the pump laser was 7 ns. The energy of the pump pulse was set to 200 μJ at the sample point. The pump pulse was irradiated onto the sample cell with grazing incidence excitation against the probe light. Intensity changes in the transmitted probe light upon photoreaction were monitored using a photonic multichannel analyzer equipped with an image intensifier (Hamamatsu Photonics, C10029-01) and a gate time of 10 μs. Timing between the pump pulse and the gate of the detector was controlled with a pulse generator (Stanford Research Systems, DG-535). Absorbance changes were calculated based on the intensity ratio between the transmitted probe light with and without pump pulse irradiation.

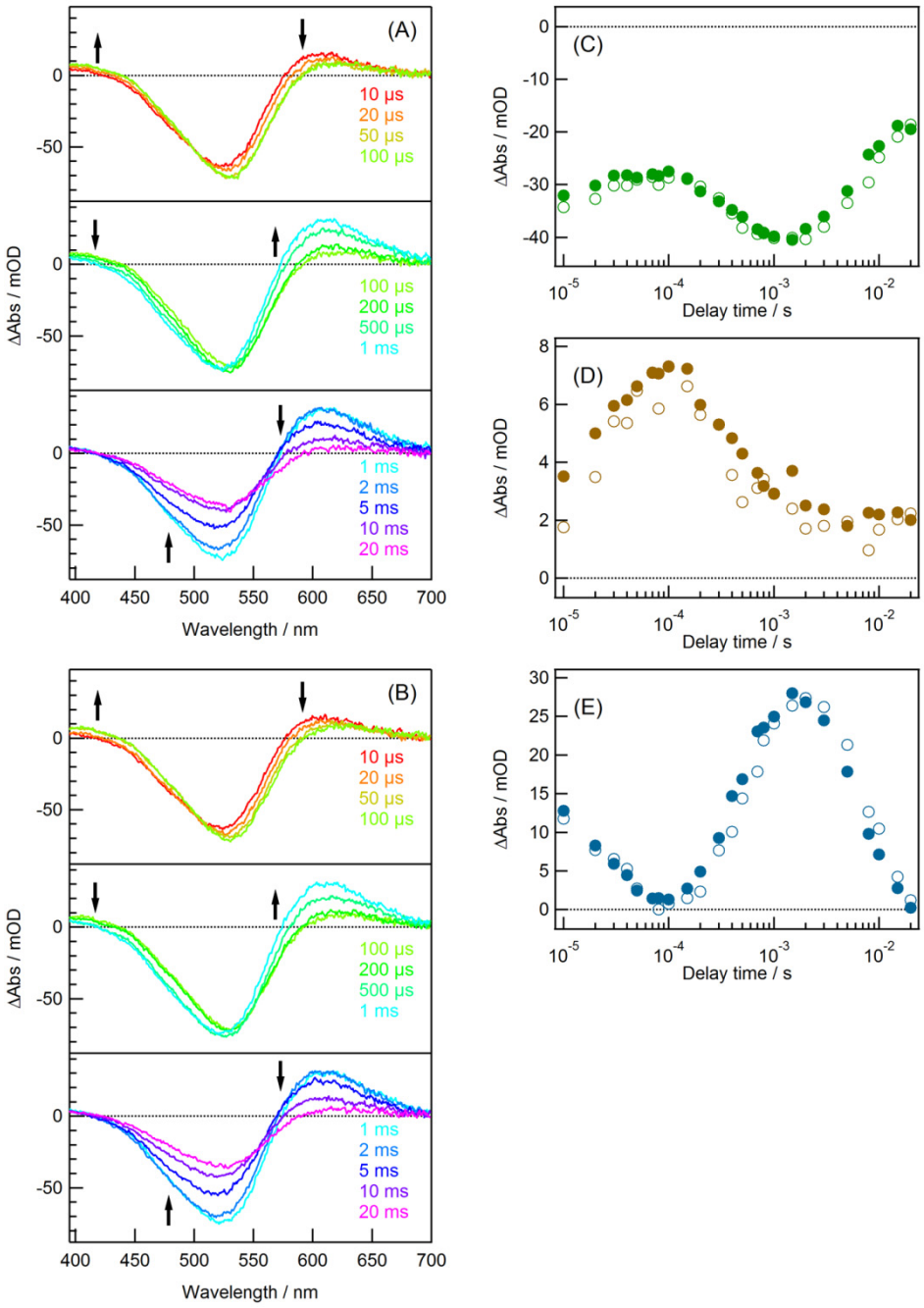

Figure S2. Transient absorption spectra of KR2 in H_2O (A) and D_2O (B). Temporal behaviors of absorbance changes at the excitation wavelengths for measurement of the resonance Raman spectra. (C) L intermediate, 475 nm, (D) M intermediate, 405 nm, and (E) O intermediate, 594 nm. Filled and open circles indicate the absorbance changes in H_2O and D_2O, respectively.

3. Resonance Raman spectra of the KR2 chromophore in D_2O

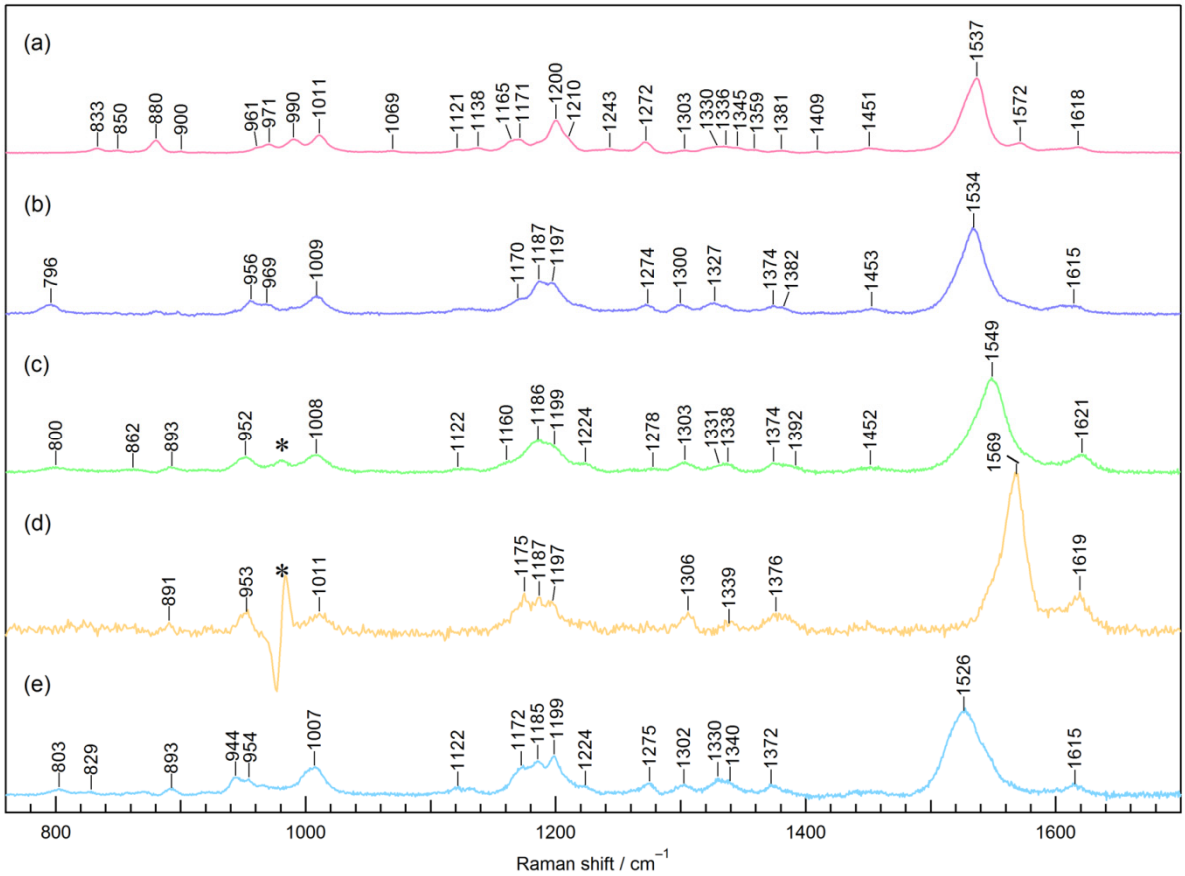

Figure S3. Resonance Raman spectra of the KR2 retinal chromophore in D_2O. (a) RR spectrum of the unphotolyzed state probed at 532 nm. (b) Transient RR spectrum of the K intermediate probed at 532 nm using the single-beam time-resolved method (delay time < 20 ns). (c) Transient RR spectrum of the L intermediate probed at 475 nm after photoexcitation by 532 nm pump beam irradiation (delay time 80 μs). (d) Transient RR spectrum of the M intermediate probed at 405 nm after photoexcitation by 514.5 nm pump beam irradiation (delay time 100 μs). (e) Transient RR spectrum of the O intermediate probed at 594 nm after photoexcitation by 514.5 nm pump beam irradiation (delay time 1 ms). Spectral contributions of the buffer and emission background were subtracted from each spectrum. The spectra were normalized to the intensity of the band at 1200 cm^{-1} for the unphotolyzed state and to that of the band at 1185–1187 cm^{-1} for each intermediate. The asterisks represent the band due to the sulfate ion added as an intensity standard.

4. Probe wavelength dependence of spectral features of the C=C and C=N stretch bands

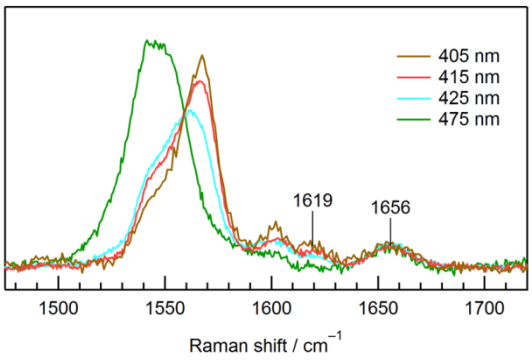

Figure S4. Probe wavelength dependence on spectral features of the C=C and C=N stretch bands. The probe wavelengths are shown in the figure. The spectra were normalized to the intensity of the band at 1656 cm^{-1}, assigned to the C=N stretch band in the L intermediate.

5. Band fitting analysis of the C=C stretch band

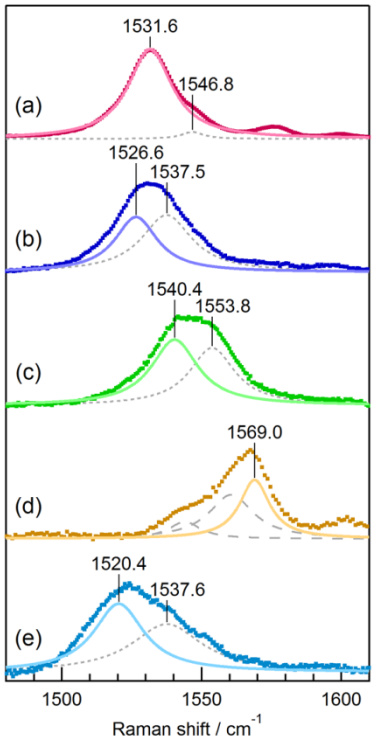

Figure S5. Expanded view of the resonance Raman spectra of the C=C stretch band region, 1480–1610 cm^{-1}. (a) Unphotolyzed state, and (b) K, (c) L, (d) M, and (e) O intermediates. Dotted traces are the raw spectra. The best-fit results using Lorentzian functions are described by lines. Solid lines represent the in-phase C=C stretch band in each state. Dotted lines in traces a, b, c, and e indicate the out-of-phase C=C stretch band. Broken lines in traced is due to the C=C stretch bands of the coexisting L intermediate at 100 μs. Fitting error of peak position was ±0.3 cm^{-1} at maximum.

6. Deuteration effects on the widths of the C=N stretch bands

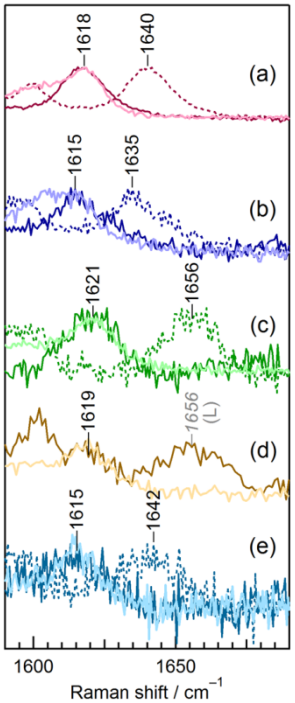

Figure S6. Expanded view of the resonance Raman spectra of the C=N stretch band region, 1590–1690 cm^{-1}. (a) Unphotolyzed state, and (b) K, (c) L, (d) M, and (e) O intermediates. Darker colored traces represent the spectra measured in H_2O. Dotted traces are the raw spectra. Solid traces are the shifted spectra to overlap the C=N stretch band in H_2O onto the band in D_2O, shown as a lighter colored trace for each state.

7. HOOP bands in resonance Raman spectra of BR

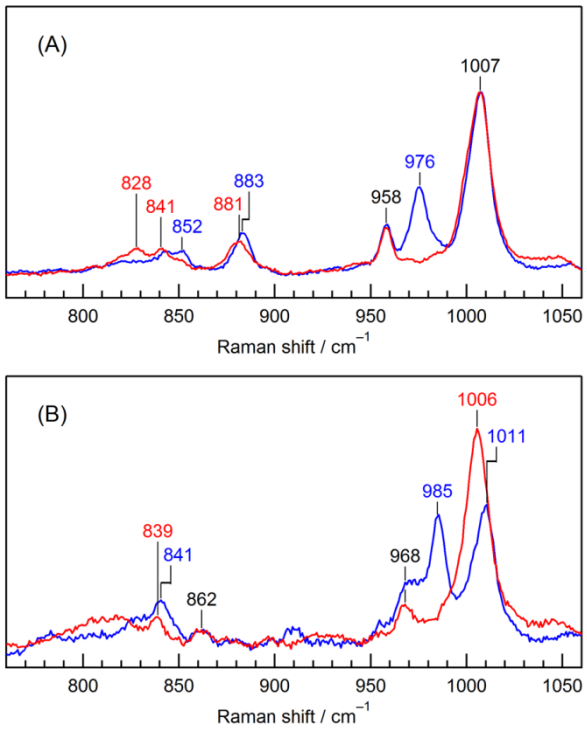

Figure S7. Resonance Raman spectra of the BR chromophore in the unphotolyzed state (A) and the L intermediate (B). Red and blue traces represent the spectra measured in H_2O and D_2O, respectively. Probe wavelength was 532 nm. These spectra of the L intermediate were obtained using single-beam time-resolved measurements, as described previously.[4]

Reference

(1) Iwata, K.; Hamaguchi, H.; Tasumi, M. Sensitivity Calibration of Multichannel Raman Spectrometers Using the Least-Squares-Fitted Fluorescence Spectrum of Quinine. *Appl. Spectrosc.* **1988,** *42*, 12-14.

(2) Higashino, A.; Mizuno, M.; Mizutani, Y. Chromophore Structure of Photochromic Fluorescent Protein Dronpa: Acid-Base Equilibrium of Two *Cis* Configurations. *J. Phys. Chem. B* **2016,** *120*, 3353-3359.

(3) Gardecki, J. A.; Maroncelli, M. Set of Secondary Emission Standards for Calibration of the Spectral Responsivity in Emission Spectroscopy. *Appl. Spectrosc.* **1998,** *52*, 1179-1189.

(4) Mizuno, M.; Nakajima, A.; Kandori, H.; Mizutani, Y. Structural Evolution of a Retinal Chromophore in the Photocycle of Halorhodopsin from *Natronobacterium pharaonis*. *J. Phys. Chem. A* **2018,** *122*, 2411-2423.