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Characterization of Arginine-82 Mutants With Non-Native Chromophores

Vivek Alaigh

University of Connecticut - Storrs, vivek.alaigh@gmail.com

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Characterization of Arginine-82 Mutants With Non-Native Chromophores

Presented by
Vivek Alaigh

Honors Thesis Advisor: Dr. Robert R. Birge

Honors Academic Advisor: Dr. Wolf-Dieter Reiter

Department of Molecular and Cell Biology

Honors Program

The University of Connecticut

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Acknowledgments

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I would also like to thank Megan Sandberg, a graduate student in Dr. Robert Birge's lab who directly supervised my research and provided the assistance, guidance, and encouragement I needed to both understand the topic of my research as well as to run the experiments necessary to obtain my final results. This thesis would not have been possible without her help

I would also like to thank members of Dr. Birge's lab who also helped me along the way in my research, including Nicole Wagner, Matthew Ranaghan, and Jordan Greco.

Finally, I would like to thank my honors advisor, Dr. Wolf-Dieter Reiter, for his help and advice, as well as Dr. Sharyn Rusch, for her advising.

Abstract

Bacteriorhodopsin, found in most halobacteria, is an integral protein that contains seven transmembrane alpha helices and an organic chromophore, all-*trans* retinal. Light energy is captured by the protein and results in a series of spectrally discrete intermediates that conclude with a proton being pumped across the membrane from the cytoplasmic side to the extracellular milieu. The most blue-shifted photo-intermediate, the M state, has been of interest for protein-based holographic memory storage devices. Bacteriorhodopsin mutants were prepared with either a 4-hydroxy retinal or 3,4-dihydro retinal analog: R82A, R82C, R82H, R82K, R82N and R82Q. The objective of this research was to investigate the effect of the combination of genetic and organic modification on the bacteriorhodopsin protein with respect to the formation and lifetime of the M state. Time-resolved UV-vis spectroscopy of the 4-hydroxy R82 analogs revealed an enhanced M state, potentially making these R82 analogs an effective candidate for device applications involving holographic memory.

Introduction

Bacteriorhodopsin is an integral multi-pass protein that is often seen in halobacteria (such as *Halobacterium salinarum*), a type of archaea that are found in salt-water environments. The structure of the protein is a single amino acid chain consisting of 248 amino acids. The chain's folded secondary structure encompasses seven long transmembrane alpha helices. The protein is oriented in the cell with the amino terminus at the extracellular end and the carboxyl terminus at the cytosolic endⁱ. Figure 1 shows the structure of the protein.

The main function of the bacteriorhodopsin protein is to pump protons from the cytoplasmic side to the extracellular milieu, using light energy to fuel the process. Once the protein absorbs a photon of light energy, an isomerization occurs at a molecule known as retinal. Retinal is a chromophore that gives the protein its distinct purple color. Furthermore, the lipid membrane that contains the protein is known as the purple membrane because bacteriorhodopsin is its only protein constituent. It contains a beta-ionone ring, a polyene chain containing five carbon-carbon double bonds as well as an aldehyde group at the end of the polyene chain. The retinal that is observed in the bacteriorhodopsin protein contains all trans-configured double bonds, which is important to know when discussing the function of the moleculeⁱⁱ.

The retinal molecule is covalently attached at the Lysine-216 site (via a protonated Schiff base) in Helix G. The all-trans retinal molecule photo-isomerizes to 13-cis retinal, which allows the retinal to transfer a proton to the side chain of Aspartate-85, positioned towards the extracellular side of the cellⁱⁱⁱ. Figure 2 shows the isomerization cycle of the retinal. The proton is then carried out of the cell through the use of proton release groups,

while the negatively charged retinal takes up a proton from another amino side chain, Aspartate-96, which is positioned closer to the cytosolic side of the cell. The proton travels using water molecules at each amino acid. Asp-96 is then reprotonated from the cytoplasm^{iv}.

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There are four main intermediates during the photo-cycle at which a photon is transferred: L, M, N, and O. The L to M state transition occurs as the proton is released from the Schiff base to Asp-85, which is positioned to release the proton to the extracellular medium. The actual formation of the M state occurs once the proton is in the extracellular space. The transition from the M state to the N state occurs as the Schiff base is reprotonated by Asp-96 at the cytoplasmic side. It has been suggested that the unidirectionality of the reprotonation of the Schiff base occurs because of the increased proton affinity of Asp-85 in the M state via the coupling of the proton transfer with the deprotonation. The N to O state transition follows, in which the retinal molecule re-isomerizes back to the all-trans form, and Asp-96 is reprotonated by a free proton in the cytoplasm^v. There are also other intermediate forms at different wavelengths, such as K and L^{vi}. Figure 3 is a diagram that shows the cycle of intermediates. Figure 4 shows the physical pathway of the proton in the protein.

The M and O states of the bacteriorhodopsin photocycle are crucial for device applications involving holographic memory and three-dimensional memory, respectively.

The M-state is the most blue-shifted state, while the O state is red-shifted. The O-state, through the absorption of a photon of red light, enters a branched photocycle that includes the P1, P2 and Q intermediates. Without red light, the O state transitions back into the resting state, as seen in Figure 3. Taken together, bacteriorhodopsin has two photocycles (a main one and a branched one), creating a bi-stable system. This can be used as a possible medium for information storage for three-dimensional paged memory. Similarly, bacteriorhodopsin has

been thought of as a possible candidate for holographic device applications based on the refractivity difference and diffraction efficiency between the resting state and the M State. In order to achieve optimal conditions for both of these device applications, the lifetime of the M and O States must be increased [together with](#) the yield. Furthermore, the efficiency of the transition between the O and P States [s](#) must be increased^{vii}. This [might](#) be accomplished via modification of the protein via site-directed mutagenesis and by modification to the retinal chromophore.

Arginine-82 is considered to be involved in the proton release group of bacteriorhodopsin; however, its exact role in the photocycle is unclear. Previous data suggested that Arg-82 is the primary counterion in the photocycle. Arg-82 interacts with Asp-85 and/or Asp-212, perhaps through a salt bridge. The idea that Arg-82 interacts with Asp-212 [is](#) favored, with Arg-82 responding to changes in the protonation state of Asp-85, Glu-204, or the chromophore. In other words, the movement of Arg-82, whether upwards or downwards, is a function of the protonation state of the amino side chains it is associated with, as well as the protonation state of the chromophore. These protonation states [define](#) what intermediate the protein is currently in. For example, in the N intermediate, the Asp-85 and the chromophore are protonated and Glu-204 is deprotonated. This leads to Arg-82 forming a strong interaction with Asp-212, allowing a pathway for the proton to travel^{viii}.

Although this is only one study done to identify the role of Arg-82 in the bacteriorhodopsin photocycle, it is clear that Arg-82 works towards proton release. Several studies have looked at the effects of altering the Arg-82 through substituting arginine with another amino acid^{ix}. For example, one study done in a laboratory substituted glutamine and alanine for arginine and noticed changes in the photocycle, namely that there was no proton

release in the M state^x. The most common mutants of Arg-82 that [have been used](#) in laboratory [studies](#) are R82A (substituting alanine), R82C (substituting cysteine), R82Q (substituting glutamine), R82N (substituting asparagine), and R82K (substituting lysine). R82K, like wild-type R82A, is positively charged. The other four mutants are either non-polar or polar/uncharged.

Besides substituting the actual amino acid, another alteration that could potentially affect the photocycle would be [the modification of](#) the retinal, allowing for spectral tuning. Chromophore modification involves the cleavage of the native retinal in the protein through photo-induced hydroxylaminolysis. Briefly, the protein is exposed to hydroxylamine under intense light conditions resulting in the cleavage of the protonated Schiff base linkage and the formation of a retinal oxime. The retinal oxime is removed from the opsin through a series of washes with bovine serum albumin. Once the opsin is purified and the retinal oxime is removed, the altered (synthesized) retinal is incorporated^{xi}. A few modifications to the retinal include: an additional double bond in the beta-ionone ring (the A2 reaction), attaching a keto-group to the carbon-4 position (the 4-keto reaction), and attaching a hydroxyl group to the 4-carbon position (the 4-hydroxy reaction). Figure 5 shows the reaction scheme for the synthesis of the A2-retinal, 4-keto retinal and 4-hydroxy retinal analogs.

Modification of the [retinal](#) chromophore can result in a shift in the absorption maximum for the protein, leading to a change in the color [of](#) the protein. One laboratory used A1 and A2 retinal analogs to replace the wild-type retinal in the protein and noticed shifts in the wavelength for maximum wavelength absorption^{xii}. Table 1 shows a table of the different λ_{Max} 's for each R82 mutant.



Figure 1. 1C3W structure of bacteriorhodopsin. The yellow molecule in the middle of the protein is the retinal molecule, which isomerizes during light transduction.

(<http://guweb2.gonzaga.edu/faculty/cronk/biochem/images/bacteriorhodopsin.jpg>)

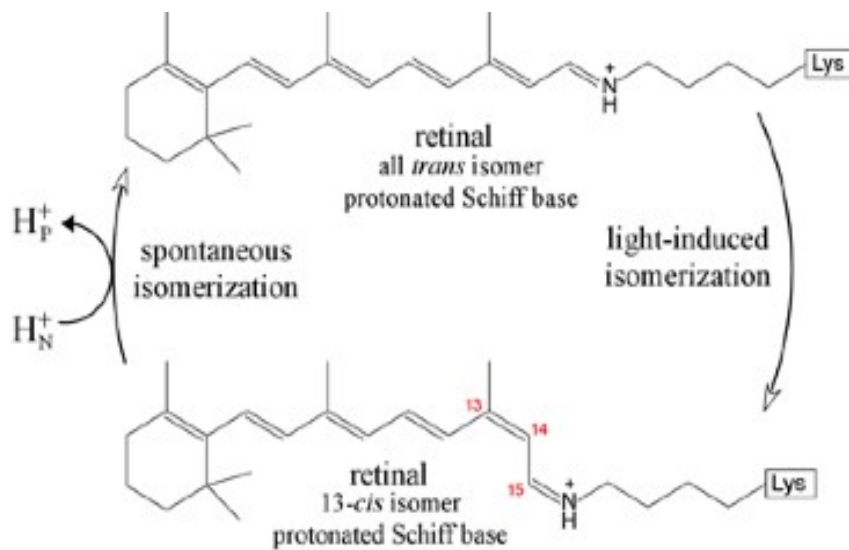


Figure 2. The isomerization of all-trans retinal to the 13-cis isomer. The reaction is induced by light, which causes the retinal to be in a position to transfer a proton to the extracellular space.

(http://guweb2.gonzaga.edu/faculty/cronk/biochem/images/retinal_isomerization.gif)

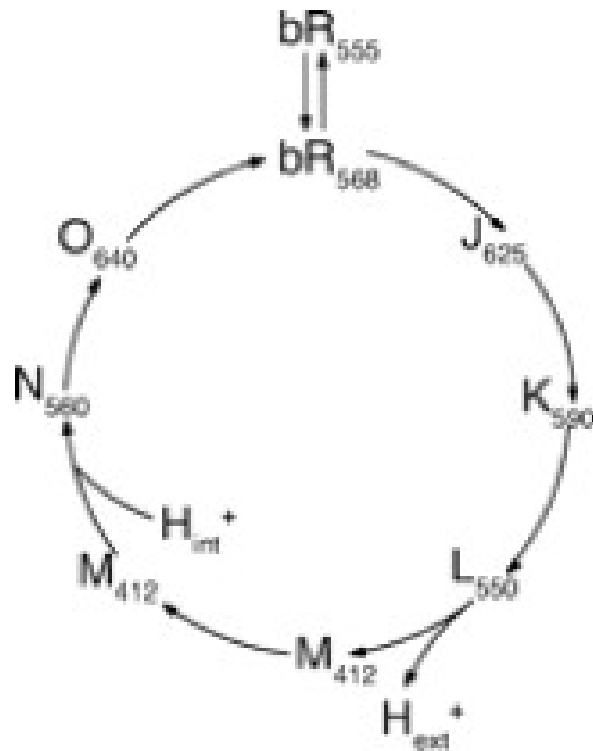


Figure 3. A diagram of the bacteriorhodopsin photocycle, with each intermediate form and

the wavelength that the intermediate takes place at. At the M state of the photocycle, the
 proton is released from the bacteriorhodopsin into the extracellular. The proton uptake occurs
at the N state of the photocycle. (<http://www.pnas.org/content/105/3/883/F1.small.gif>)

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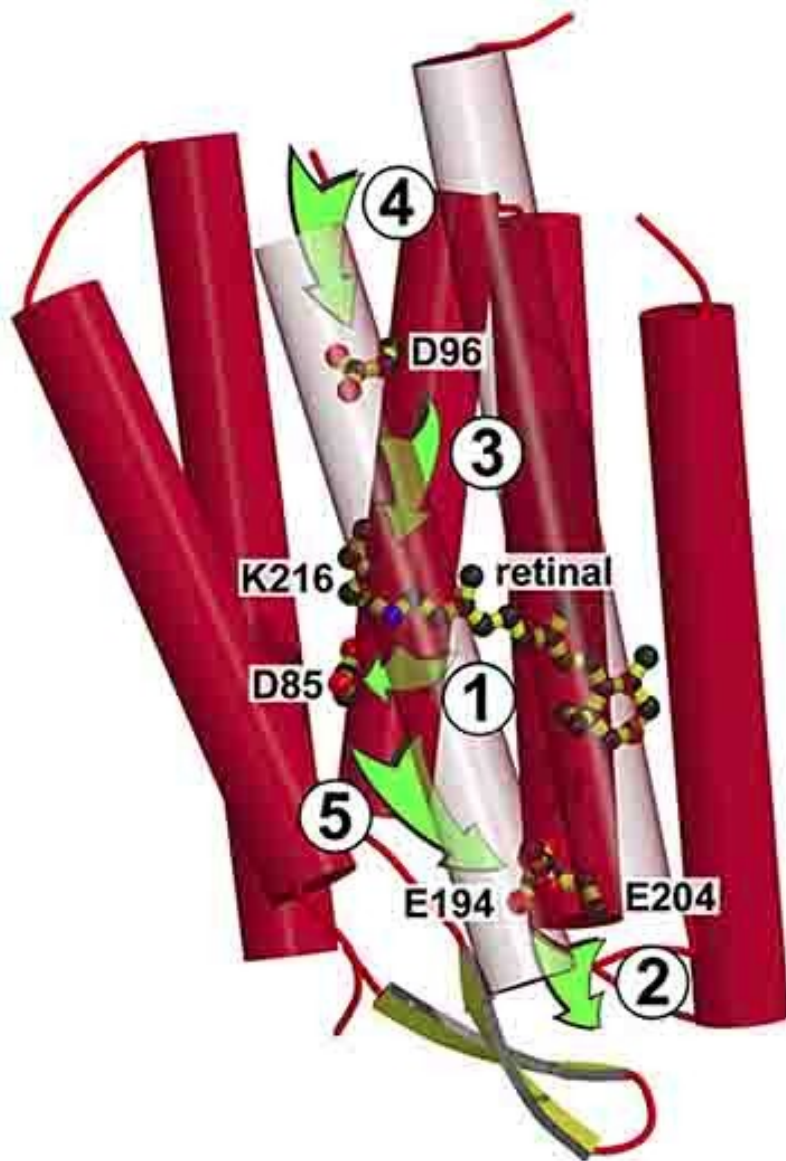


Figure 4. The proton pathway in bacteriorhodopsin, with each number representing the steps to the proton release. The retinal is included in the diagram, which isomerizes to the 13-cis

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form when the protein is exposed to visible light.

(<http://www.bioscience.org/2004/v9/af/1264/fig1.jpg>)

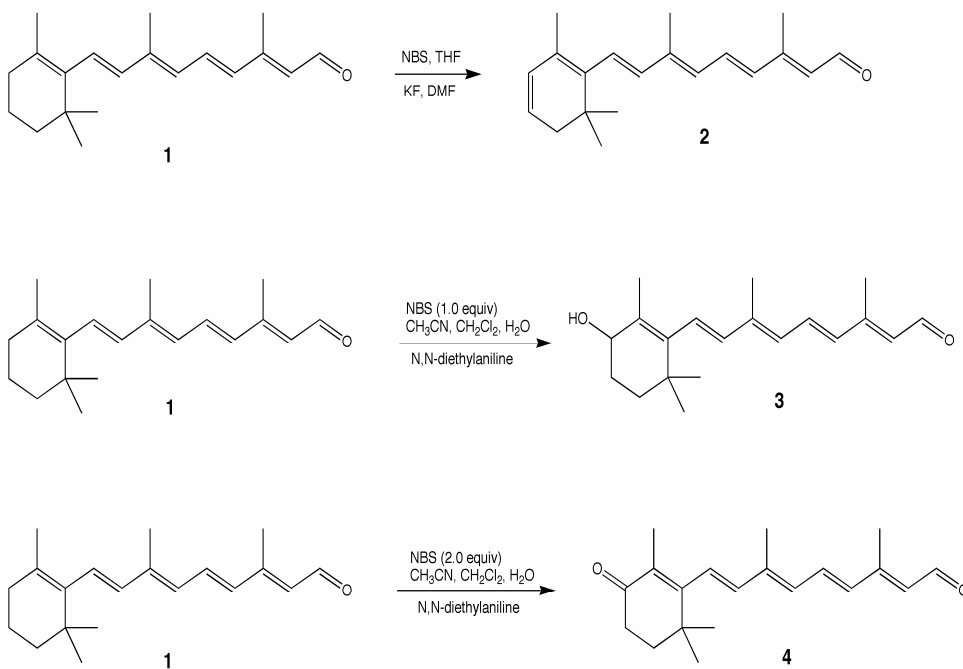


Figure 5. The schematic for **modifying 1) the retinal molecule to 2) the A2 retinal via the A2 reaction, 3) the 4-hydroxy retinal via the 4-hydroxy reaction and 4) the 4-keto retinal via the 4-keto reaction.** Each modified retinal changes the **photophysical** properties of the bacteriorhodopsin.

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Materials & Methods

Protein preparation. Protein cultures of *Halobacterium salinarum* strain MPK409 (1 mL) were grown from frozen stocks in 5 mL of Culture Medium (CM; 4.28 M NaCl, 81 mM MgSO₄, 10.2 mM sodium citrate, 26.8 mM KCl, 5 g/L Tryptone, 3 g/L yeast extract, pH 7.2) supplemented with 500 µL of uracil stock solution (1.5 mg/mL). The cultures were incubated for 4-7 days at 40°C with shaking at 200 rpm. Once turbid, 1 mL of the *H. salinarum* strain MPK409 culture was inoculated in 50 mL of CM, and grown for 4-7 days under continuous illumination at 40°C with orbital shaking at 200 rpm. Large-scale preparation of the mutant protein was then conducted by inoculation of Rich Medium (RM; 4.28 M NaCl, 81 mM MgSO₄, 10.2 mM sodium citrate, 26.8 mM KCl, 10 g/L peptone, pH 7.2) supplemented with uracil (100 mg/ 2L RM) with 10 mL of CM culture per RM flask. Cultures were grown in a 2 L Erlenmeyer flask, containing 1.8 L RM, under continuous illumination at 40°C with orbital shaking at 200 rpm for 7-10 days.

Protein purification. The protein culture was poured into JLA 9.1 tube (1L) and centrifuged at 5,000 rpm (JLA rotor) for 30 minutes at 4°C. After the clear supernatant was discarded, the purple pellet was resuspended in 40 mL dH₂O and 10 mL of a DNase stock solution (0.03 g DNase, 0.125 g MgSO₄, 50 mL ddH₂O) was added. The suspension was transferred to a sterile 250 mL Erlenmeyer flask that was left to shake at 150 rpm overnight at room temperature. The suspension was then poured into JA-17 tubes and was centrifuged at 5,000

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Comment [H6]: Halobacterium cultures? Please specify precisely what bacterial strain was used.

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rpm in a Beckman Ultracentrifuge (JA-17 rotor) for 10 minutes at 4° C. The white pellet was discarded and the purple supernatant was poured into Ti-45 tubes, which were then centrifuged at 32,000 rpm in a Beckman Ultracentrifuge (45-Ti rotor) for 35 minutes at 4 °C; afterwards, the supernatant was discarded and the purple pellet was resuspended in dH₂O, making sure to avoid resuspending the white debris located at the center of the pellet. This process was repeated until the supernatant layer was colorless and the white debris in the center of the pellet was no bigger than 5 mm in diameter (approximately 4-5 spins). The pellet should only contain fragments of the purple membrane in which the protein is embedded in. The purified protein was resuspended in a minimal amount (5 – 10 mL) of dH₂O, transferred to a sterile falcon tube, and stored at 4 °C.

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Protein quantification. Protein quantification was done using Beer's Law, which follows the formula, $A = \epsilon b c$, where A is the absorbance (based on the readings from the UV-Vis), ϵ is the molar absorptivity (63,000 L/mol*cm), b is the path length (1 cm) and c is the concentration (what is being determined). Once the concentration was determined by using this equation, it had to be converted from mol/L to mg/mL using unit conversions. The molecular weight of BR is 26,000 g/mol. The concentration was multiplied by the molecular weight and the units were changed to mg/mL.

Comment [H9]: What is "dimensional analysis"?

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Absorption spectroscopy: All spectra were collected with a Varian Cary-50 UV-Vis spectrophotometer (Palo Alta, CA). Measurements were conducted in either distilled water or 10 mM TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid sodium salt) (pH 8.5) at ambient temperature.

Comment [H10]: Please specify the pH of this buffer.

Bleaching procedure: Fragments of the purple membrane (containing the protein) were pelleted in a tabletop centrifuge using a TLA 55 rotor (20 minutes, 50,000 rpm, 4°C). The pelleted protein was resuspended in 10 mM TAPS, pH 8.5, containing 1 M NH₂OH-HCl to yield a final protein concentration of 1.5 mg/mL. The sample was illuminated at room temperature with an *in house* protein illuminator constructed using Amber LEDs. During the bleaching process, the retinal is converted to a retinal oxime and can be observed spectroscopically by the appearance of a 360 nm peak. Once the conversion was complete, observed spectroscopically by the disappearance of the 570 nm peak, the apo-protein was pelleted and purified by a series of 10mM TAPS washes to remove the hydroxylamine solution. The retinal oxime was removed by a series of 2% BSA (Bovine Serum Albumin) in 50mM TAPS (pH 8.5) washes with a one hour incubation period at 4°C between spins. Lastly, the BSA solution is removed by a series of 10mM TAPS washes.

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Comment [H11]: I don't see how you can pellet a protein of 26 kDa. Was it embedded in liposomes?

Reconstitution of retinal analog into bacteriorhodopsin: The retinal analog, dissolved in a minimal amount of ethanol, was added to the apo-protein in 2-4 molar excess. Regeneration of the bacteriorhodopsin protein was observed spectroscopically by the appearance of the 570 nm peak. Regeneration times vary with the protein mutant and are typically less than 24 hours. The excess retinal analog was removed by a series of 2% BSA washes (typically 4-5) with a one-hour incubation time at 4°C between spins. The BSA was removed by a series of 10mM TAPS washes (typically 4-5). For Wild-Type BR samples, the TAPS solution was removed by two washes with water. Mutant BR samples were stored in the 10mM TAPS buffer at 4°C.

General: The ^1H NMR spectra were recorded on a Bruker 400 NMR spectrometer using tetramethylsilane (TMS, δ 0 ppm) as the internal standard. All syntheses were carried out under a nitrogen atmosphere. N-bromosuccinimide ([NBS](#)) was recrystallized using distilled H_2O . Pet. ether refers to low boiling petroleum ether 40-60 $^\circ\text{C}$. Unless otherwise stated, purification was performed by flash column chromatography (Dynamic Adsorbents flash silica gel) using pet. ether/ ethyl acetate mixtures. The progress of the reactions was monitored by thin layer chromatography (TLC) with a 3:1 ratio of pet. ether/ethyl acetate using 0.25 mm precoated silica gel plates, which were visualized by ultraviolet light and stained with phosphomolybdic acid (PMA). Tetrahydrofuran (THF), diethyl ether (Et_2O) and N,N-dimethylformamide (DMF) were used without further purification unless otherwise stated.

Comment [H12]: Is this a mix of alkanes?

A2-retinal (2): To a stirred solution of all-*trans* retinal (100 mg, 0.35 mmol) in anhydrous THF (9 mL) at 0°C under dim red light, a solution of N-bromosuccinimide (100 mg, 0.56 mmol) [was added](#) in anhydrous THF (4 mL) dropwise. The reaction mixture was [allowed](#) to stir at 0°C for 30 minutes, then a mixture of potassium fluoride (100 mg, 1.72 mmol) in [20 mL](#) DMF [was added](#). The reaction mixture was [allowed](#) to stir for 24 hours at room temperature in darkness. After 24 hours, the reaction mixture was quenched by the dropwise addition of saturated sodium bicarbonate solution (10 mL) and let to stir at room temperature for 30 minutes. The resulting mixture was extracted with diethyl ether (3 x 20 mL). The organic layers were combined and washed with distilled water until neutrality was reached (3 x 20 mL). The organic fraction was then dried (NaSO_4), filtered, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography to give A2-retinal (**2**).

4-hydroxy retinal (3): To a stirred solution of all-*trans* retinal (100 mg, 0.35 mmol) in a mixture of acetonitrile (15 mL), dichloromethane (1.0 mL), and distilled water (100 μ L) was added N-bromosuccinimide (0.35 mmol) at -15°C. The reaction mixture was let to stir for 20 minutes under dim red light. After 20 minutes, N,N-diethylaniline (100 μ L) was added. The reaction mixture was stirred at 0°C for 20 minutes, then poured into 5% aqueous Na₂S₂O₃ solution and extracted with diethyl ether (3 x 15 mL). The combined organic layers were washed with 1 M aqueous HCl solution (10 mL), H₂O (10 mL) and ~~saturated NaCl solution~~ (10 mL), respectively. The organic fraction was then dried (MgSO₄), filtered, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography to give 4-hydroxy retinal (**3**).

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4-keto retinal (4): To a stirred solution of all-*trans* retinal (100 mg, 0.35 mmol) in THF (9 mL) at 0°C under dim red light was added a solution of N-bromosuccinimide (100 mg, 0.56 mmol) in THF (4 mL) dropwise. Benzoyl peroxide (0.5 mg) was then added to the reaction flask. The reaction mixture was let to stir at 0°C for 2 hours, then a mixture of potassium fluoride (100 mg, 1.72 mmol) in DMF (20 mL) was added. The reaction mixture was let to stir overnight at room temperature in darkness. After 24 hours, the reaction mixture was quenched by the dropwise addition of saturated sodium bicarbonate solution (10 mL) and let to stir at room temperature for 30 minutes. The resulting mixture was extracted with diethyl ether (3 x 20 mL). The organic layers were combined and washed with distilled water until neutrality was reached (3 x 20 mL). The organic fraction was then dried (NaSO₄), filtered, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography to give 4-keto retinal (**4**).

Time-Resolved UV-Vis Spectroscopy: Time-resolved kinetics experiments were performed with the protein analogs [at](#) room-temperature [suspended in either](#) distilled water (pH 6.8) [or](#) 10mM TAPS (pH 8.5). The protein analogs were in 1-cm path length plastic fluorescence cuvettes, covered with caps. The samples were exposed to 532-nm, 3.3-ns pulse-width emission from an optical parametric oscillator (OPO) pumped by the third harmonic of a Neodymium:YAG laser system (Coherent Infinity-XPO). The energy of each pulse was measured by a calibrated energy meter (Ophir PE50-BB) interfaced with an Ophir Laserstar acquisition unit. A rapid-scanning monochromator (RSM) system (OLIS Instruments, Inc. RSM-1000 stopped flow) collected spectra of these samples orthogonal to the incident beam. The range of the spectra was from 360 to 740 nm. The RSM system monitored the difference spectrum, with 1000 scans averaged per second.

Results and Discussion

Table 1: Absorption maximum of R82 analogs.

Protein	H ₂ O		TAPS, pH 8.5	
	DA λ_{\max} (nm)	LA λ_{\max} (nm)	DA λ_{\max} (nm)	LA λ_{\max} (nm)
WT-BR	558.9	570.0	561.0	568.9
A2 WT-BR	592.0	598.0	594.0	601.0
4-hydroxy WT-BR	538.1	545.9	538.1	542.9
R82A	574.0	574.0	565.1	561.9
A2 R82A	N/A	N/A	581.0	593.0
4-hydroxy R82A	N/A	N/A	528.0	536.1
R82C	596.9	594.9	566.0	565.1
A2 R82C	N/A	N/A	N/A	N/A
4-hydroxy R82C	N/A	N/A	N/A	N/A
R82H	568.1	568.1	571.9	566.0
A2 R82H	589.1	589.1	596.9	599.1
4-hydroxy R82H	543.0	543.0	541.1	545.0
R82K	552.0	552.0	552.9	561.9
A2 R82K	584.0	584.0	585.1	585.0
4-hydroxy R82K	529.0	529.0	541.1	527.9
R82N	561.0	561.0	N/A	N/A
A2 R82N	589.1	589.1	587.0	599.1
4-hydroxy R82N	543.0	543.0	541.1	545.0
R82Q	580.0	580.0	566.0	566.0
A2 R82Q	N/A	N/A	N/A	N/A
4-hydroxy R82Q	N/A	N/A	N/A	N/A

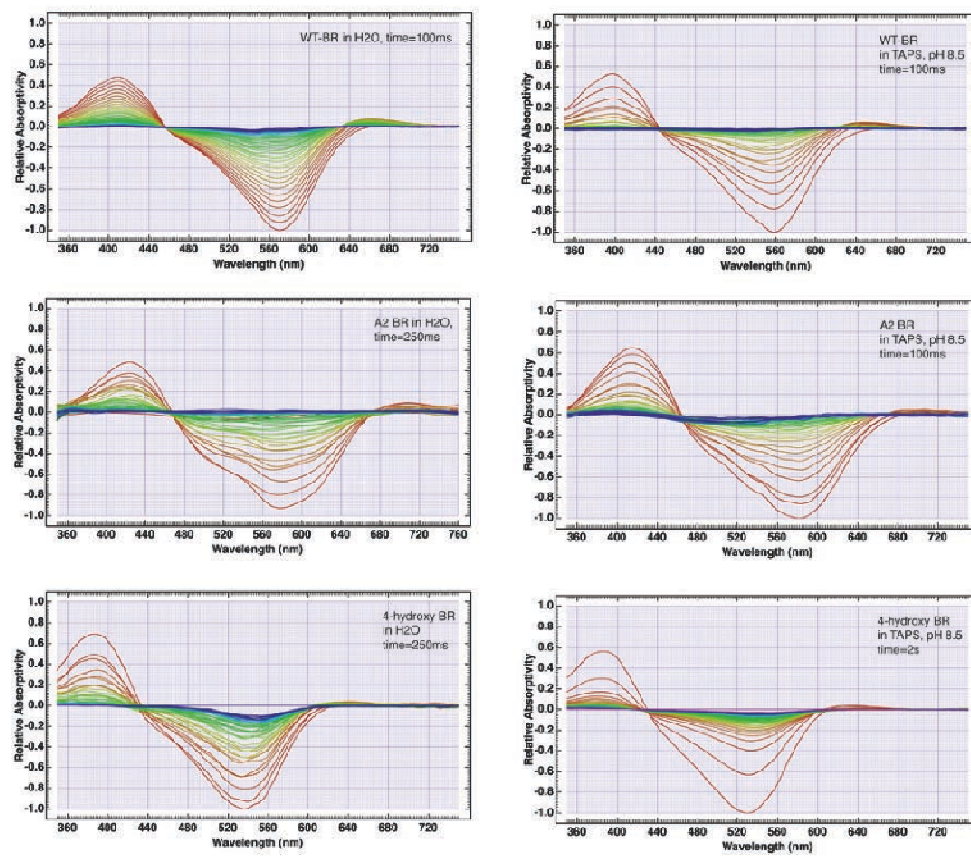


Figure 6. Time resolved difference spectra of wild-type BR (top), A2 BR (center) and 4-hydroxy BR (bottom). The measurements were recorded on samples in H₂O (left column) and pH = 8.5 (right column) at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

Comment [H14]: Please see to it that the figures and their respective legends are on the same page.

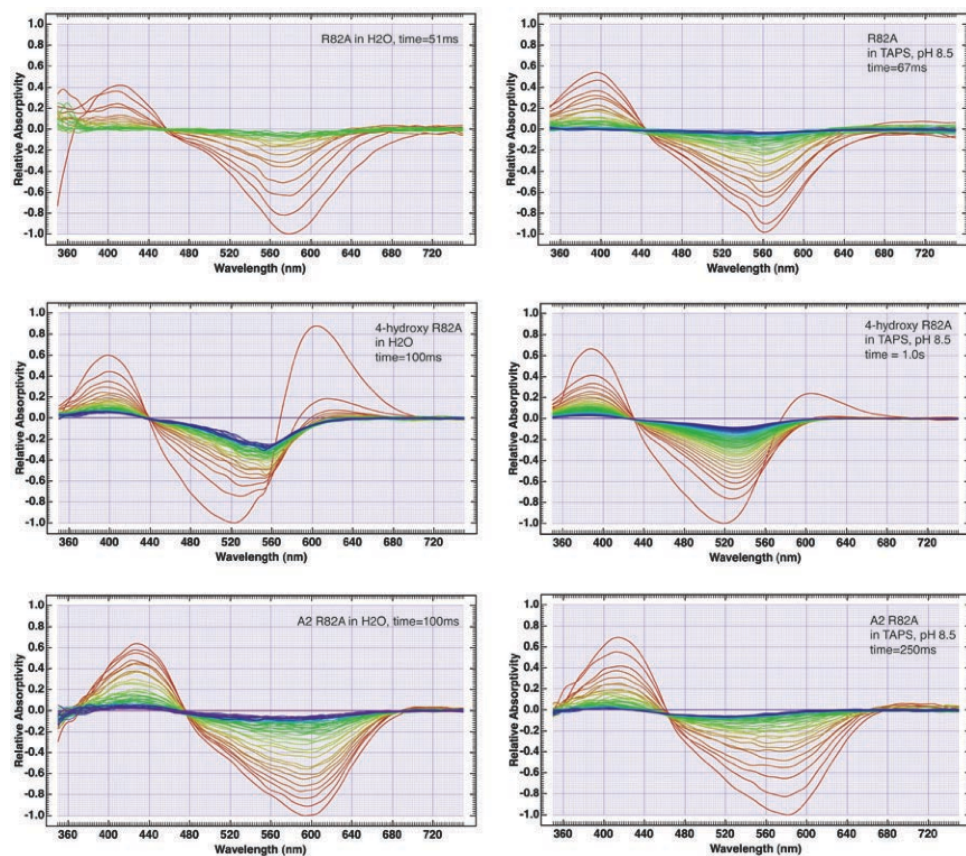


Figure 7. Time resolved difference spectra of R82A (top), 4-hydroxy R82A (center) and A2 R82A (bottom). The measurements were recorded on samples in H₂O (left column) and pH = 8.5 (right column) at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

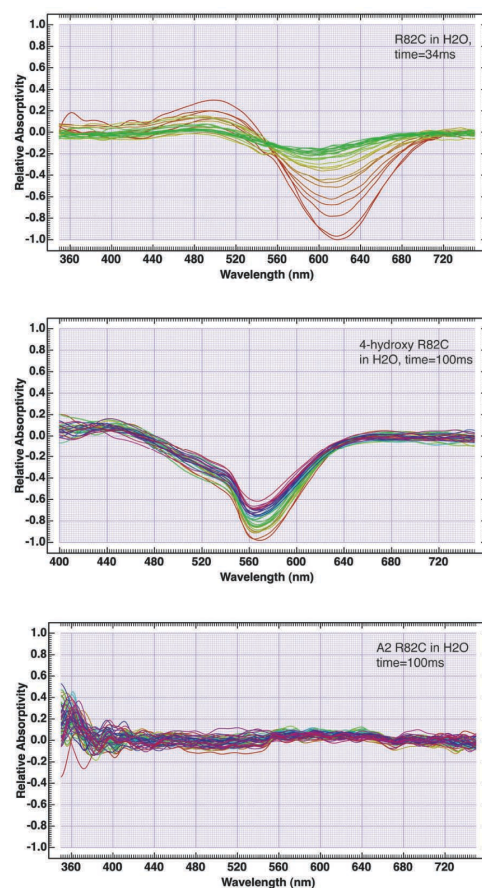


Figure 8. Time resolved difference spectra of R82C (top), 4-hydroxy R82C (center) and A2 R82C (bottom). The measurements were recorded on samples in H₂O at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

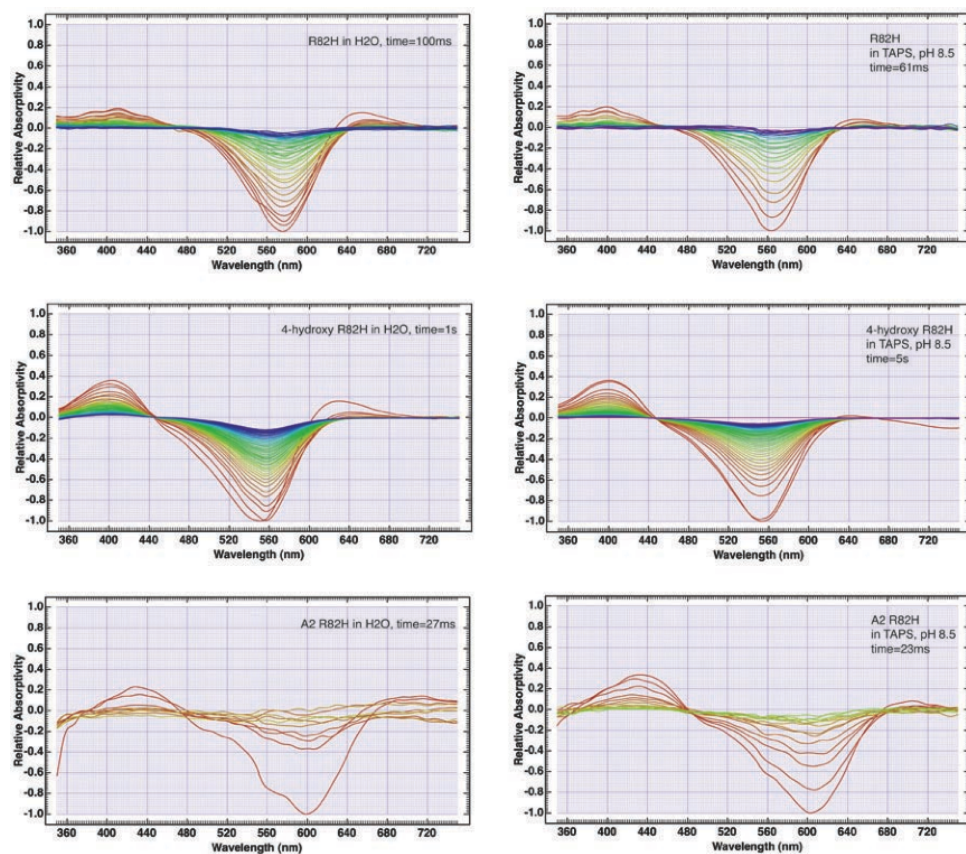


Figure 9. Time resolved difference spectra of R82H (top), 4-hydroxy R82H (center) and A2 R82H (bottom). The measurements were recorded on samples in H₂O (left column) and pH = 8.5 (right column) at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

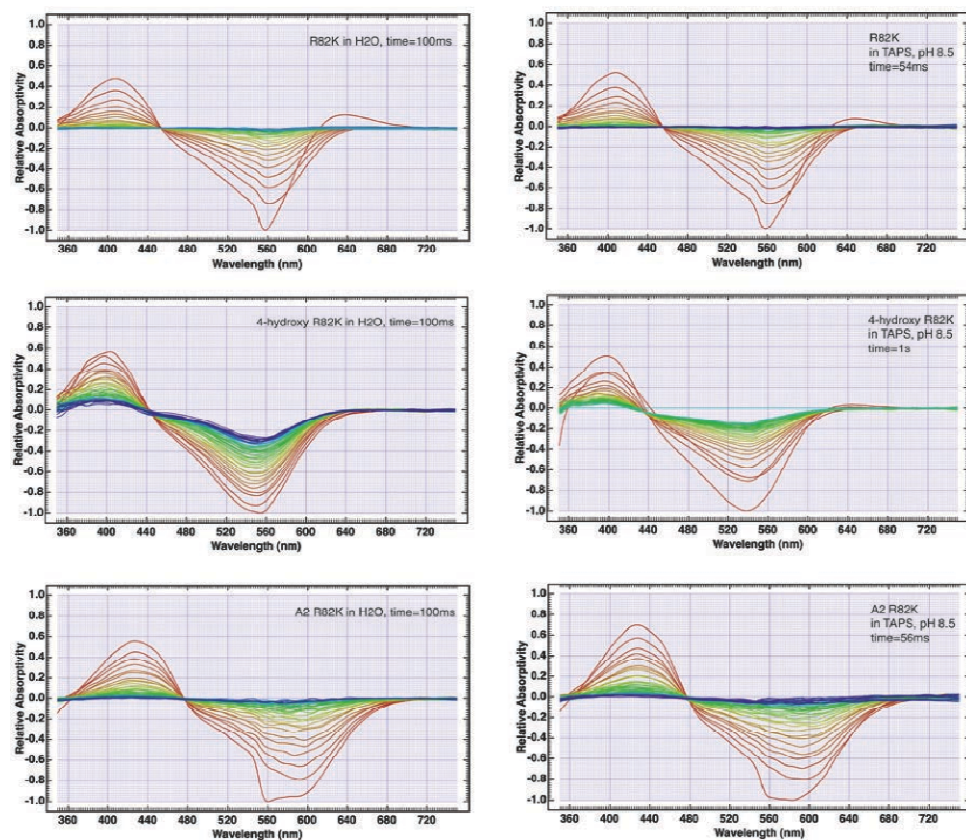


Figure 10. Time resolved difference spectra of R82K (top), 4-hydroxy R82K (center) and A2 R82K (bottom). The measurements were recorded on samples in H₂O (left column) and pH = 8.5 (right column) at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

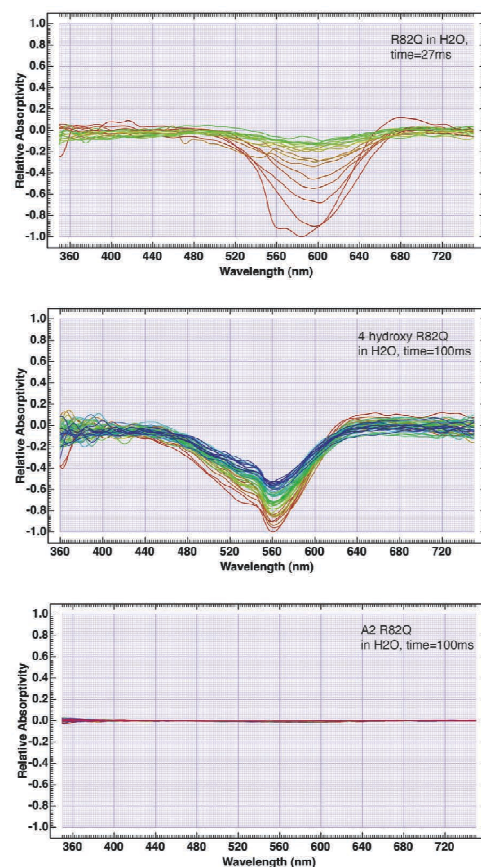


Figure 11. Time resolved difference spectra of R82Q (top), 4-hydroxy R82Q (center) and A2 R82Q (bottom). The measurements were recorded on samples in H₂O at 25°C showing the formation of M-state (~400nm) and O-state (~640nm).

Comment [H15]: This is actually part of the Results section.

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Wild-Type BR: The λ_{\max} of WT-BR was measured in both distilled water (pH 6.8) and 10 mM TAPS buffer (pH 8.5) in both light and dark adaptations. Amongst the WT-BR analogs, the most red-shifted was A2 BR. As observed in the time-resolved difference spectra data for only the WT-BR analogs, the O State that is most red-shifted is also seen in A2 BR in both distilled water and TAPS. Normally, the O State is present at around 640 nm, but in the case of A2 BR, the O State was observed around 660 nm. Its M State was also red-shifted by about 20 nm. In comparison to WT-BR, the A2 analog had a shorter O state, as seen in both distilled water and TAPS, while the 4-hydroxy BR analog barely showed any O state at all. The 4-hydroxy BR presented a blue-shifted λ_{\max} , as well as a blue-shifted M and O state.

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Comment [H17]: Please be more precise.

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R82 Analogs: Looking at Table 2, the λ_{\max} 's, of the R82 mutants and their A2 counterparts were all red-shifted. The A2 mutants were more red-shifted than the unmodified R82 analogs. Meanwhile, all of the 4-hydroxy mutants of the R82 analogs were blue-shifted in comparison to WT-BR. Furthermore, the greater red-shift and blue-shift occurred red in TAPS as opposed to distilled water. The greatest red-shift occurred red in A2 R82H in TAPS (40 nm) while the greatest blue-shift occurred red in 4-Hydroxy R82A (30 nm).

Comment [H18]: Use the past tense when describing experimental results.

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Most of the analogs presented a very small amount of O state. If there was an O State, it was fairly short-lived, such as with the 4-Hydroxy R82A sample. A minimal amount of short-lived O state was also observed in R82K, R82H, and 4-hydroxy R82H (the R82H analogs were only seen to have an O State in distilled water). Besides the 4-hydroxy R82A sample being significantly blue-shifted, the O States for the samples that presented it had the O State occur at its usual wavelength around 640 nm.

Almost all of the samples displayed an M State in both distilled water and TAPS, although some more than others and some for a longer amount of times than others. The

exception is R82C, R82Q and its respective mutants. This is because these analogs tend to die quickly and the modified retinals did not incorporate properly. The M State occurred at its normal wavelength of 400 nm for all the samples, except for A2 R82K and A2 R82A, which were both slightly red-shifted by about 20 nm. The longest-lived M States occurred in the 4-Hydroxy R82 samples, especially 4-Hydroxy R82K, which appears to have the longest M state. The 4-Hydroxy R82 analogs presented longer M states than the M states of the 4-Hydroxy BR.

Conclusion

Deleted: Conclusions/Future Work

Although more research needs to be done, it appears that despite the minimal amount of O State, the protein is photocycling in some of the R82 analogs. This means that there is a possibility that the samples are actively pumping protons. However, there is also a good chance that protein returns to its resting state after the M state, which explains the lack of O state formation. In terms of device application, it appears that these analogs would not be an effective candidate for three-dimensional paged memory, since the O State is not very enhanced and would probably not go into the branched P1 and P2 states. Furthermore, two of the criteria for three-dimensional paged memory are a longer life span and a greater yield of the O State, which none of the analogs seem to express.

The analogs that displayed a slightly enhanced M State could potentially be an effective candidate for holographic memory, since the criteria require both a greater M State life span and a greater yield of the M State.

Future work for this project includes retrieving more time-resolved spectroscopy and UV-Vis spectroscopy data, including the analogs that are missing from the results (such as time-resolved spectra data on R82N). Furthermore, time-resolved spectra difference data and UV-Vis light/dark adaptation data on samples suspended in both a phosphate buffer (pH 7.0) and a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) would be useful to have. Finally, data on samples with an incorporated 4-keto retinal would be beneficial in adding any new insight. These two additions to the research could possibly display some enhanced M States and O States that would be useful for device application.

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