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Near-IR Resonance Raman Spectroscopy of Archaelhodopsin 3: Effects of Transmembrane Potential

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Abstract

Archaelhodopsin 3 (AR3) is a light driven proton pump from *Halorubrum sodomense* that has been used as a genetically targetable neuronal silencer and an effective fluorescent sensor of transmembrane potential. Unlike the more extensively studied bacteriorhodopsin (BR) from *Halobacterium salinarum*, AR3 readily incorporates into the plasma membrane of both *E. coli* and mammalian cells. Here, we used near-IR resonance Raman confocal microscopy to study the effects of pH and membrane potential on the AR3 retinal chromophore structure. Measurements were performed both on AR3 reconstituted into *E. coli* polar lipids and *in vivo* in *E. coli* expressing AR3 in the absence and presence of a negative transmembrane potential. The retinal chromophore structure of AR3 is in an all-*trans* configuration almost identical to BR over the entire pH range from 3–11. Small changes are detected in the retinal ethylenic stretching frequency and Schiff Base (SB) hydrogen bonding strength relative to BR which may be related to a different water structure near the SB. In the case of the AR3 mutant D95N, at neutral pH an all-*trans* retinal O-like species (O^{all-trans}) is found. At higher pH a second 13-*cis* retinal N-like species (N^{13-cis}) is detected which is attributed to a slowly decaying intermediate in the red-light photocycle of D95N. However, the amount of N^{13-cis} detected is less in *E. coli* cells but is restored upon addition of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or sonication, both of which dissipate the normal negative membrane potential. We postulate that these changes are due to the effect of membrane potential on the N^{13-cis} to M^{13-cis} levels accumulated in the D95N red-light photocycle and on a molecular level by the effects of the electric field on the protonation/deprotonation of the cytoplasmic accessible SB. This mechanism also provides a possible explanation for the observed fluorescence dependence of AR3 and other microbial rhodopsins on transmembrane potential.

Keywords

optogenetics; archaelhodopsins; proton pump; resonance Raman spectroscopy; transmembrane voltage

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Supporting Information Available: Supporting figure showing alteration of the RRS of BR D85N at pH 10 with various powers of 785-nm excitation is provided. (Figure S1). These materials are available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Rapid progress in the new field of optogenetics^{1–8} has focused interest on characterizing the molecular mechanism and ultimately custom-engineering of “neurophotonic” rhodopsins. Such rhodopsins, which are normally derived from microbial rhodopsins, have the properties that they express readily in neurons, fold in a native form into the neuronal plasma membrane, and can function as light-triggered activators or silencers of neuronal electrical activity. Recently, several microbial rhodopsins have been shown to be capable of functioning in the *E. coli* and neuronal membranes as fluorescent transmembrane voltage sensors^{9,10} (for reviews on microbial rhodopsins see references^{11,12}).

In this work, we focus on Archaelhodopsin 3 (AR3), a light-driven proton pump found in *Halorubrum sodomense*. AR3 is related to bacteriorhodopsin (BR) from *Halobacterium salinarum* with approximately 75% sequence homology. Importantly, all key residues implicated in proton pumping in BR are conserved, including the transmembrane Asp, Glu and Arg residues associated with proton transport (Figure 1). Despite these similarities, differences are likely to exist between the chromophore structures, photocycle kinetics and proton pumping mechanisms of BR and AR3 which may ultimately be important for optogenetic applications. For example, Archaelhodopsin 4 (AR4, 84% homology to AR3) isolated from a salt lake in Tibet, exhibited a reverse in the order of the light-driven proton release and uptake, potentially attributable to alterations in the proton release complex (PRC)^{13,14}.

Unlike BR, which expresses poorly as a functional protein in the plasma membrane of *E. coli*¹⁵, archaelhodopsin from *Halobrubrum* expressed well in a functional form in the plasma membrane of both *E. coli* and mammalian cells^{4,16}. This might be due to significant differences in the cytoplasmic (CP) and extracellular (EC) loop regions of BR and AR3 (see Figure 1). Furthermore, x-ray crystallographic structures^{17,18} of the closely related archaelhodopsin 1 (AR1) and archaelhodopsin 2 (AR2), reveal significant structural differences despite the close homology (90%)¹⁹.

Recent studies have established that AR3 can serve both as a high-performance genetically targetable optical neural silencer⁴ and as a sensitive fluorescent sensor of transmembrane potential^{9,10}. Activating/silencing neurons and simultaneously measuring membrane potential using genetically expressed proteins is particularly attractive since it opens the door to a complete optical “toolkit” for studying complex neural circuitry and even brain function, a goal first envisioned by Francis Crick in his seminal 1979 paper²⁰. However, future progress in this new field will depend in large part on characterization of the molecular basis of optogenetic rhodopsin function in order to facilitate bottom-up bioengineering of new and improved optogenetic rhodopsins. This includes elucidating the retinal chromophore structure and protein interactions which determine the visible absorption, molecular determinants of photocycle dynamics, and molecular basis of voltage response.

Near-infrared resonance Raman spectroscopy (RRS) was used to study AR3 because the vibrations of the unphotolyzed chromophore are enhanced without significant contributions from vibrations from the protein, lipids or photocycle intermediates. This makes a spinning cell or flow apparatus, which avoids interference from photointermediates excited by a visible probe beam, unnecessary and confocal microscopy allows a small amount of the sample (several microliters) to be measured in a stationary capillary. The Raman excitation wavelength (785 nm) is significantly red-shifted relative to the visible absorption maximum (~565 nm in the case of AR3) but still in pre-resonance with the chromophore vibrational modes. This effect has also been demonstrated with excitation wavelength out to 1060

nm^{21–23}. In addition, with confocal microscopy, as we demonstrate here, near-IR RRS can be obtained from AR3 expressed in *E. coli* cells allowing the effects of membrane voltage to be studied.

Our results show that AR3 reconstituted in *E. coli* polar lipids (ECPL) has a chromophore structure almost identical to BR over a wide pH range from 3–11. In addition, the AR3 mutant Asp95→Asn95 (D95N), where the Schiff base counterion is neutralized, is very similar to the analogous mutant D85N in BR; it exhibits a red-shifted and predominantly all-*trans* O-like chromophore. At pH above 7, an N-like species is detected in both D95N AR3 and D85N BR mutants which is attributed to a photointermediate of the red-light photocycle of D95N AR3. However, much less of this N-intermediate appears at pH 9 in AR3 measured *in vivo* in *E. coli* cells expressing AR3 compared to AR3 ECPL membrane fragments at pH 9. Furthermore, under conditions where the normally negative membrane potential is absent, such as by addition of the proton ionophore CCCP or sonication of the cells, the N-like species increases. In order to explain these results, we postulate that the membrane potential alters the equilibrium between a 13-*cis* N (N^{13-*cis*}) and a 13-*cis* M (M^{13-*cis*}) intermediates in the red-light photocycle due to the effects of electric field on the protonation state of the SB which is accessible to the cytoplasmic channel.

MATERIALS AND METHODS

AR3 expression in *E. coli* and reconstitution in *E. coli* polar lipids

The methods for expression, purification, and reconstitution of WT AR3 in *E. coli* polar lipids is similar to a previous report²⁴ with some modifications. Unlike typical expression of BR or archaerhodopsins in halobacterial strains¹³, which endogenously express all-*trans* retinal, *E. coli* does not express retinal and thus expression of functional archaerhodopsin requires supplementation¹⁶. Briefly, *E. coli* (strain BL21, pet28b plasmid) was grown in 1 L of LB medium with 50 mg/L kanamycin, to an O.D of 0.4 at 600 nm at 37 °C. All-*trans* retinal (5 μM) and inducer (IPTG 0.5 mM) were added and cells were grown for an additional 3.5 hours in the dark at 32°C. Cells were then harvested by centrifugation, resuspended in sonication buffer (50 mM Tris, 5 mM MgCl₂ at pH 7.0), and lysed by freeze-thaw followed by sonication of the sample on ice for 2 minutes, 3 times. The lysate was then centrifuged and the pellet resuspended in binding buffer (50 mM K₂HPO₄, 300 mM NaCl 1.5% octylglucoside (OG) and 5 mM imidazole at pH 7.0). The mixture was homogenized with a glass Wheaton homogenizer and centrifuged again. Ni-NTA Agarose (QIAGEN) beads were added to the supernatant and washed with binding buffer and incubated 1 hour at 0°C using a rotary shaker. Ni-NTA agarose beads with bound AR3 were loaded into 3 ml disposable plastic column and washed with 2 ml of binding buffer. AR3 was eluted with 1 ml of elution buffer (50 mM K₂HPO₄, 300 mM NaCl, 1.5% OG and 250 mM imidazole at pH 7.0). Purified His-tagged AR3 was reconstituted in *E. coli* polar lipids (ECPL) (Avanti, Alabaster AL) at 1:10 protein-to-lipid (w/w) ratio. Lipids initially dissolved in chloroform were dried under argon and resuspended in dialysis buffer (50 mM K₂HPO₄, 300 mM NaCl pH 7.0) to which OG was added to the final concentration of 1.5%. The lipid solution was incubated with the OG-solubilized protein for 1 hour on ice and dialyzed against the dialysis buffer overnight at 4°C followed by a buffer change and an additional dialysis for 3 hours. The reconstituted protein was centrifuged for 10 min and resuspended in dialysis buffer 3 times. AR3 samples were stored at 4°C. Typical yields based on measurement of the OD at 570 nm for the reconstituted AR3 proteoliposomes ranged from 400 μg up to 800 μg per liter of *E. coli* culture.

BR in purple membrane

Bacteriorhodopsin in its native purple membrane was isolated from *Halobacterium salinarum* using standard procedures previously reported²⁵

Near-IR Resonance Raman Spectroscopy

Approximately 20 Ng of AR3 or AR3 D95N reconstituted in *E. coli* polar lipids as described above was pelleted in wash buffer (5mM NaH₂PO₄, 10mM NaCl, 10mM MES/TRIS) and adjusted to various pH values using HCl/NaOH titration and pelleted using a SCI Logex D2012 centrifuge spun at 15,000 rpm for 5 minutes. A portion of the pellet was inserted in the middle of a square quartz capillary (0.5 mm ID, Wale Apparatus, Hellertown PA) using a 20 μ L pipette tip and sealed on both ends with Critoseal (Leica Microsystems, Buffalo Grove IL) to avoid dehydration. The level of water in the pellet was sufficient to keep the membranes fully hydrated during the course of the measurements.

Raman spectra were obtained at room temperature on a Bruker Senterra confocal Raman microscope (Olympus BX51M) equipped with a 20 $\times$ or 50 $\times$ objectives with numerical apertures (NA) of 0.4 and 0.75, respectively, using 785-nm laser excitation. A laser power of 100 mW (40 mW at sample) was used for most measurements unless otherwise noted. This was sufficient to keep the WT AR3 samples fully light adapted as determined from the RRS spectra. The Raman scattered light was dispersed onto a CCD (Andor, iDus, South Windsor CT) giving an effective resolution of 3–5 cm⁻¹. In order to obtain high signal to noise, spectra were typically averaged by measuring for 150 seconds, the sample left in the dark 1 minute and this cycle repeated over 4–6 hours. Spectra obtained from an empty quartz capillary were subtracted to eliminate fluorescence background. For RRS measurements of AR3 in *E. coli* cells, freshly grown cells expressing protein (strain BL21(DE3)) were pelleted from the growth medium by centrifugation (10 min at 3,000 rpm, Avanti JB, Beckman Coulter, Brea CA). The pellet was resuspended in M9 buffer (Sigma Aldrich, St Louis MO) and pH adjusted to desired values. The cells were centrifuged (10 min at 14,000 rpm) and the pellet was inserted into a quartz capillary (see above). CCCP when indicated was added to the M9 buffer at a concentration of 5 μ M. Cell sonication when indicated was performed on ice in M9 buffer with a microtip sonicator (Sonifier Cell Distributor W185, Ultrasonics, Plainview NY) for 3 cycles of one minute sonication bursts.

Visible Absorption Measurements

Visible absorption measurements of the AR3 in proteolipid membranes were made on a Cary 6000 spectrophotometer equipped with an internal diffuse reflectance accessory (DRA) adapted to fit a liquid sample in order to reduce scattering effects of the membrane. The membrane suspension in dialysis buffer (300 mM NaCl, 10 mM K₂HPO₄) was inserted in a 1 cm path-length quartz cuvette (Agilent Technologies, Santa Clara, CA) and stirred with a stir-bar at room temperature. The sample was light-adapted with >500 nm illumination for 10 minutes using a Dolan-Jenner Model 180 fiber illuminator. Immediately after the light adaptation, absorption spectra were acquired from 200 to 800 nm with 1 nm resolution and 0.266 s integration time. Each spectrum took approximately 160 seconds to collect. The sample was left in dark for 1 hour prior to measuring a dark-adapted spectrum under identical conditions. Baseline corrections were performed using either a 5 point quartic or a 2 point linear fit with GRAMS/AI (v 7.02 Thermo Fisher) Spectroscopy Suite.

RESULTS

AR3 and BR have very similar retinal chromophore structures and Schiff base hydrogen bonding

Figure 2 shows a comparison of the near-IR resonance Raman spectra recorded for BR (in purple membrane) and AR3 (in ECPL membrane fragments) at pH 7 under identical conditions (see Materials and Methods). Almost all of the bands in the RRS of BR have previously been assigned to vibrational normal modes of the retinylidene chromophore on the basis of comparison with the RRS of isotope labeled retinal model compounds and BR regenerated with these retinals^{26–28}. The most intense band for AR3 appears at 1527 cm⁻¹, very close to the frequency of a similar band in BR assigned to the symmetric C=C ethylenic stretch mode^{29,30}. The slight upshift in frequency ($\nu_{C=C}$) agrees with time-resolved and low-temperature FTIR difference measurements of AR3²⁴ and indicates a slight blue-shift in visible absorption maximum (λ_{max}) of AR3 of 3–4 nm based on an empirical inverse relationship observed between λ_{max} and $\nu_{C=C}$ for both microbial and animal rhodopsins^{31–33}.

In agreement, we measured the visible absorption of light-adapted AR3 in EPCL and found a λ_{max} of 565 nm, slightly below the normally reported λ_{max} of light-adapted BR at 568 nm (Figure 3). A slight blue shift in λ_{max} relative to BR was also reported for AR4 in claret membrane isolated from the native *H. sp. Xz515* archaeobacteria. This blue shift relative to BR was attributed to a Met-145 (BR) → Phe (AR4) substitution¹³. Unlike in AR4, such a replacement does not occur in the AR3 sequence (see Figure 1, residue Met-155) relative to BR and hence could not be the cause of the apparent blue shift in AR3. However, the adjacent residue, Cys154 replaces homologous residue Ala144 in BR which is located close to the β -ionone ring of the retinal³⁴, thus possibly contributing to this observed shift.

A blue-shift in the λ_{max} of AR3 to 557nm was observed (Figure 3) after several hours of dark adaptation which in BR causes a shift to 560 nm. Similar to BR, this most likely reflects a partial isomerization of the retinal to a 13-*cis*/C=N *syn* configuration³⁵. Incorporation of AR3 in detergent micelles formed from OG or dodecylmaltoside (DM) also resulted in a distinct blue-shift of the λ_{max} to near 560nm even after light-adaptation and most likely reflects an increased 13-*cis* retinal content of the protein which is associated with a monomer relative to trimer organization of the protein^{36,37}. For example, a downshifted absorbance of both light- and dark-adapted BR to 554 and 549 nm, respectively, is observed in BR incorporated into nanolipoprotein particles (NLPs) containing dimyristoylphosphatidylcholine (DMPC) lipids³⁸.

An alternate explanation for the blue shift of AR3 compared to BR comes from focus on the Schiff base which attaches the retinal chromophore to the protein at Lys 226 (see Figure 1). The 1641 cm⁻¹ band in the RRS of AR3 (Figure 2) appears near the frequency of the band assigned to the protonated Schiff base (SB) C=N stretching mode in BR, indicating the AR3 SB is protonated and has a similar *but not identical* environment. This is confirmed by hydrogen/deuterium (H/D) exchange which causes a 20 cm⁻¹ downshift (Figure 2) in the SB C=N frequency of AR3 compared to 17 cm⁻¹ for BR. Since the magnitude of the H/D exchange downshift correlates with hydrogen bond strength of the Schiff base proton^{29,39}, this result indicates that the hydrogen bonding strength of the SB in AR3 may be slightly stronger than in BR, potentially accounting for the blue-shifted λ_{max} . In comparison, a previous study²¹ revealed a 23 cm⁻¹ H/D downshift in green proteorhodopsin (GPR) (λ_{max} =523 nm) compared to the red-shift observed in BR of 17 cm⁻¹. It is possible that the differences in hydrogen bond strength of the SB are related to a different structure of water molecules near the SB in AR3 as revealed by earlier FTIR difference measurements²⁴.

The 1150–1300 cm^{-1} fingerprint region reflects the C-C stretching modes of the retinylidene chromophore and is highly sensitive to the configuration around double and single bonds in a conjugated polyene^{26–28}. In the case of BR, analysis of this region led to the conclusion that the retinal exists in an all-*trans* C=N anti (*trans*) configuration^{35,40}. The fact the AR3 fingerprint region is almost identical in both H₂O and D₂O provides strong evidence *that the chromophore structure in the light-adapted form is almost identical*. This conclusion is further supported by the almost identical frequencies of the C-CH₃ methyl rock mode (1006 cm^{-1}) and the hydrogen-out-of-plane mode (HOOP) at 958 cm^{-1} in AR3 and BR.

We also find that the AR3 retinal chromophore structure remains the same over a wide pH range from 3–10 as indicated by almost identical RRS (see Figure 4). For example, the SB C=N frequency is constant at 1641 cm^{-1} over this range indicating the protonation state and hydrogen bonding strength of the SB is not affected by changes in external environment of the protein. However, below pH 3 a downshift in the ethylenic $\nu_{\text{C}=\text{C}}$ frequency is observed from 1527 to 1515 cm^{-1} similar to the BR acid purple-to-blue transition. This change is caused by protonation of Asp95 SB counterion (Asp85 in BR sequence) which induces a red-shift in the visible absorption as predicted by the point charge model (see discussion and reference 60). The fact that we only observe this shift below pH 3 in both AR3 and BR indicates that the pK_a of the Schiff base counterions (Asp95/Asp85) are similar. In contrast, in AR2 this transition has been reported to be near pH 5⁴¹ and in AR4 close to pH 3.5¹³.

N-like intermediate with 13-*cis* retinal configuration detected in the photocycle of the mutant D95N

The visible absorption of the mutant AR3 D95N exhibited a red-shifted $\lambda_{\text{max}} \sim 590$ nm at neutral pH (Figure 3) consistent with earlier studies on the homologous mutant BR D85N^{42–46}. In agreement the RRS of D95N at pH 7 exhibited a $\nu_{\text{C}=\text{C}}$ at 1518 cm^{-1} (Figure 5), downshifted 9 cm^{-1} from WT AR3 as expected on the basis of the previous RRS measurements on BR D85N⁴² and the empirical inverse relationship between λ_{max} and $\nu_{\text{C}=\text{C}}$ discussed above^{31–33}.

The presence of a second species at pH above 7 was also indicated by the appearance of an ethylenic band with a $\nu_{\text{C}=\text{C}}$ at 1527 cm^{-1} near the native AR3 (Figure 5). However, this high pH species is not the same as WT AR3 with a similar $\nu_{\text{C}=\text{C}}$ since its RRS was similar to the N intermediate in the native BR photocycle^{47,48}. For example, the fingerprint region displayed a band at 1185 cm^{-1} , characteristic of the N intermediate's 13-*cis* retinal chromophore, along with a shoulder at 1547 cm^{-1} assigned to a second C=C stretch mode in N⁴⁷. An additional band near 1565 cm^{-1} also appeared above pH 10 which is highly characteristic of the $\nu_{\text{C}=\text{C}}$ of the M₄₁₂ chromophore^{49,50}. Since the 785-nm excitation is not expected to resonantly enhance this intermediate as much as N and O, it is most likely present at a much higher concentration relative to these other species at high pH.

Although these results are consistent with the existence of an N-like species in equilibrium the O species at pH above 7, as previously reported for the mutant BR D85N⁵¹, a second possibility we considered is that excitation of the O-like dark species by the 785 nm excitation produces an N^{13-*cis*} intermediate. In support of this hypothesis, it has been reported that at high pH red-light excitation of BR D85N produces a photocycle which involves a long-lived N^{13-*cis*} intermediate^{47,52} (see also Discussion section).

In order to test this possibility, the RRS of AR3 D95N at pH 10 was recorded at both 1 and 100 mW of 785 nm excitation power. As seen in Figure 6, bands at 1527 cm^{-1} and 1185 cm^{-1} characteristic of N^{13-*cis*} are reduced significantly in intensity relative to the spectrum recorded at 100 mW. Hence we conclude that the N^{13-*cis*} species detected at high pH in D95N is predominantly due to accumulation of a red-light driven D95N photocycle that is

excited by absorption of 785-nm photons. Note a similar result was also obtained for RRS measurement on BR D85N (see supplementary data, Figure 1).

Effect of transmembrane potential on the N-like 13-cis retinal containing intermediate in AR3 D95N

In order to investigate the effects of transmembrane potential on AR3 D95N, RRS were recorded of intact *E. coli* cells expressing AR3. *E. coli* cells with elevated pH in the extracellular medium are able to maintain a negative transmembrane potential or a normal pH⁵³. Surprisingly, the accumulation of the N-like intermediate observed in reconstituted membranes at pH 9 at 100 mW was significantly reduced in cells recorded under similar conditions (Figure 7, top two traces). The 1527 and 1185 cm⁻¹ bands associated with the N^{13-cis} intermediate were much weaker relative to the bands associated with the O-like dark state at 1518 and 1168 cm⁻¹. In contrast, eliminating the transmembrane potential by either addition of CCCP or by sonication partially restored the bands associated with N at 1527 and 1185 cm⁻¹ (Figure 7, 3rd and 4th trace from top). In order to test if this effect was fully reproducible and not due to uncontrolled variations in power, pH or buffer, 4 separate samples were measured for three different conditions: i) *E. coli* untreated, ii) treated by sonication and iii) addition of CCCP (see Materials and Methods). As seen in Figure 8 and Table I, the 1185/1168 cm⁻¹ and 1527/1517 cm⁻¹ ratios were found to be statistically different under these three conditions (see Table 1). Specifically, both the 1185/1168 cm⁻¹ and 1527/1517 cm⁻¹ ratios are higher for conditions where the membrane potential is dissipated and at least for the limited cases measured appear to reflect the changes in the membrane potential. However, more detailed measurements will be necessary in order to establish a quantitative correlation.

Note that at lower pH (pH 7) no significant differences were observed between AR3 in *E. coli* and reconstituted in membrane except those noted below presumably because the latter does not accumulate significant N intermediate. All of the new bands which appeared in the RRS of *E. coli* expressing AR3 compared to reconstituted AR3 can be attributed to non-resonant contributions which are detected in the spectrum of *E. coli* that does not express AR3 (see Figure 7, bottom trace). For example, the band at 1003 cm⁻¹ caused an increase in the intensity and downshift in frequency of the band assigned in reconstituted AR3 D95N membrane to the retinal methyl rock mode at 1006 cm⁻¹. Additional non-resonant contribution occurred near 1006, 1157, 1461 and 1575 cm⁻¹. Note the 1575 cm⁻¹ band overlaps with the band at 1567 cm⁻¹ assigned to the M intermediate discussed above and thus in addition to weak resonance enhancement, this overlap made it difficult to detect any build-up of the M intermediate in *E. coli* expressed AR3.

DISCUSSION

This study focused on the retinal chromophore structure in AR3 and the mutant D95N. AR3 and the closely related ArchT from *Halorubrum* strain TP009⁵⁴, whose amino acid sequence differs by only 8 amino acid substitutions with AR3, exhibit attractive features for optogenetics. Unlike the related BR proton pump, both incorporate into the plasma membrane of mammalian cells, such as neurons and HEK cells, and can function as high-light sensitivity optical neural silencers^{4,54}.

AR3 and the mutant AR3 D95N have also been used as genetically encoded fluorescent transmembrane voltage sensors with ten-fold increased sensitivity and speed over existing protein-based voltage indicators in both *E. coli* and mammalian neurons^{9,10}. The mutant AR3 D95N is particularly useful as a voltage sensor since it does not function as a proton pump and hence does not produce light-induced hyperpolarization like WT AR3. However, this mutant exhibits a slowed response to voltage change compared to WT AR3⁹.

Elucidating the detailed molecular properties and photocycle of AR3, AR3 mutants such as D95N, and in general other rhodopsins which exhibit useful optogenetic properties promises to lead to new and improved bioengineered versions.

A second motivation for these studies is to further explore the molecular mechanism of ion transport and light-sensing in microbial rhodopsins. Since the initial discovery of BR, thousands of proton pumping and light-sensing microbial-like rhodopsins have been discovered in all of the major domains of life including archaea, eubacteria, fungus, and algae^{11,55,56}. While extensive biophysical studies have led to a detailed model of the BR pump mechanism, other proton pumping rhodopsins have not been studied as thoroughly. For example, AR4 from the *H. sp. Xy515* found in a Tibetan salt lake appears to reverse the order of proton release and uptake, possibly due to an altered proton release complex¹³ but is restored to normal in Trixton X100⁵⁷. In the case of proteorhodopsin, which are ubiquitous in marine bacteria, the two different green and blue types have been found to have significant differences in their mechanisms compared to BR²¹. In general, the application of techniques such as resonance Raman and FTIR-difference spectroscopy will be useful to characterize and classify the molecular mechanisms of various microbial rhodopsins across many kingdoms of life.

Similarity of Retinal Chromophore Structures in BR and AR3

Although WT BR does not readily incorporate like AR3 into the *E. coli* plasma membrane, they share a 75% sequence homology. This includes almost all of the major residues which have been implicated in the BR proton pump mechanism (Figure 1) including the transmembrane residues (AR3 sequence numbering used in this paper) Asp95 (counterion and proton acceptor from SB), Asp106 (proton donor to SB), Asp125 (located near β -ionone ring and sensitive to retinal isomerization), Asp222 (part of charge groups near SB), Glu204 and Glu214 (part of proton release complex) and Arg92 which serves as part of the proton ejection mechanism (see Figure 1 of reference¹⁹). Note, however, that in contrast to the extensive investigations of the effects of mutagenesis on BR function, especially point mutations of Asp and Glu residues^{43–47}, such studies have not yet been performed for AR3.

Differences in the sequence of AR3 relative to BR, however, are found which might alter protein-chromophore interactions and chromophore structure including substitution of Cys154 (AR3) in place of an Ala144 (BR), which, in the BR crystal structure, is located close to the β -ionone ring⁵⁸. In addition, several Asp/Glu residues are found in the cytoplasmic and extracellular loops of AR3 not present in BR¹⁹. Differences in the hydrogen bonding and structural changes of internal water molecules in BR and AR3 have also been detected using low-temperature and time-resolved FTIR difference spectroscopy including a weakly hydrogen bonded water (W401) which is part of a hydrogen-bonded pentagonal cluster located near the retinal Schiff base²⁴. Small differences in the structure of the protein, retinal chromophore and internal water molecules have also been found between BR, AR1 and AR2 on the basis of x-ray crystallography⁵⁹. This suggests that some differences may exist between the structure of AR3, which has not yet been solved, and the well-studied structure of BR which could alter the proton pump mechanism.

The similarity of RRS spectra of BR and AR3 indicate that the respective retinal chromophore structures are very similar. However, differences detected in the retinal C=C and C=N stretch frequency, the $\lambda_{\max}$ of visible absorption, and the H/D isotope shift demonstrate that differences still exist in the retinal-protein interactions. In the context of the simple point charge model of the retinal visible absorption⁶⁰, moving a negative charged residue such as Asp95 closer to the positively charged SB is expected to blue-shift the $\lambda_{\max}$. One possibility is that the arrangement of water molecules and charged residues near the AR3 SB are different from BR (as previously surmised from FTIR measurements²⁴) and act

to favor the counterion-SB interaction. A change in the interaction of the chromophore with specific residues may also play a role in color regulation. For example, Thr99 (Thr89 in BR) can influence the $\lambda_{\max}$ ⁶¹.

A Molecular Model to Account for Effects of Membrane Potential on AR3 D95N

An important property of AR3 and the mutant D95N is the sensitivity of its intrinsic fluorescence to changes in transmembrane voltage⁹. This voltage sensing has been attributed to the effect of the electrochemical potential on the SB protonation state in agreement with earlier electro-chromic studies of D85N BR films⁶². However, only a SB accessible to the cytoplasmic (CP) channel is expected to deprotonate with increased membrane depolarization (*i.e.* negative potential and electric field directed towards the cytoplasm) (see Figure 1a of reference⁹). In contrast, the standard model of the BR proton pump mechanism, based on a variety of biophysical studies, envisions the light-adapted all-*trans* conformation to have an extracellular (EC) accessible SB^{52,58,63–64}.

The observed voltage modulated fluorescence and RRS of AR3 are likely to arise from the same mechanism. We propose this involves voltage modulation of an equilibrium between a 13-*cis* retinal containing N-like and M-like intermediates (N^{13-cis} and M^{13-cis} , respectively) that are part of the D95N red-light photocycle. As shown in Figure 9, adapted from a proposed photocycle for BR D85N⁵², the absorption of red-light by the O-like all-*trans* dark form of D95N ($O^{all-trans}$) results in a phototransition from $O^{all-trans}$ to N^{13-cis} followed by establishment of an equilibrium between N^{13-cis} and M^{13-cis} . Both intermediates have CP accessible SBs similar to the late M and N intermediates in the wild-type BR photocycle⁵². Note, however, that the D95N red-light photocycle involves essentially the reverse steps as occurs in the late BR photocycle, *e.g.* $O^{all-trans} \rightarrow N^{13-cis} \rightarrow M^{13-cis}$. Furthermore, the photocycle is completed by the thermal decay of N^{13-cis} back to $O^{all-trans}$ resulting in no net proton transport⁵². However, absorption of a second blue photon (blue arrow, Figure 9) by M^{13-cis} (at least in case of BR D85N) results in retinal isomerization to $M^{all-trans}$ and reformation of $O^{all-trans}$ by reprotonation of the SB from the extracellular (EC) side of the membrane. This two photon photocycle leading to inverted two-photon driven proton pumping has been observed at least for the case of BR D85N⁵².

We postulate that an increase in the level of N^{13-cis} intermediate relative to $O^{all-trans}$ occurs when the normally negative membrane potential in *E. coli* is dissipated (depolarized). This effect arises from a shift in the equilibrium between M^{13-cis} and N^{13-cis} due to electric field effects on the protonation state of the SB. As shown in Figure 9, a negative potential favors deprotonation of the SB into the cytoplasmic channel and possibly onto the SB donor group Asp106. By disrupting this potential with CCCP or sonication, the membrane potential is dissipated and an increase in SB protonation and N^{13-cis} formation occurs. In the case of the membrane fragments, no membrane potential is present and hence the N^{13-cis} form increases in concentration relative to M^{13-cis} .

In support of this model, a theoretical analysis of the effects of membrane potential on proton transfer reactions in BR photocycle⁶⁵ predicts the M^{13-cis} to N^{13-cis} transition should be highly sensitive to membrane potential since it involves a proton transfer from Asp96 (homologous to AR3 Asp106) to the SB through a network of water molecules^{63,66}. In the case of AR3, recent FTIR studies²⁴ indicate D106 deprotonates during N formation, although other residues in the cytoplasmic loops region appear to also be involved in SB reprotonation (unpublished results). It also should be noted that other transitions may be voltage dependent. For example, the $O^{all-trans}$ to $M^{all-trans}$ equilibrium which is pH dependent involves deprotonation through an extracellular channel and is thus likely to favor $M^{all-trans}$.

A similar model also provides a potential explanation for the observed voltage dependent fluorescence of AR3 D95N. It has been reported that absorption of a photon by the N-intermediate of the native BR photocycle leads to formation of a “Q” intermediate which exhibits fluorescence emission above $>700\text{ nm}^{67}$. Hence, the fluorescence observed for D95N is expected to be a highly non-linear 3-photon process involving a first photon absorbed by $O^{alltrans}$, a second photon absorbed by N^{13-cis} and third photon which excites the Q intermediate to emit a fluorescent photon (this is consistent with recent studies (A. Cohen and J. Kralj, private communication)).

It is also likely that a similar mechanism involving voltage modulation of the M^{13-cis} to N^{13-cis} equilibrium is active in the photocycles of microbial rhodopsins in general. For example, previous studies of voltage effects on the BR photocycle has revealed that the M to N transition is slower with higher negative potential^{68,69}. Voltage modulation of fluorescence is also observed in WT AR3 as well as green absorbing proteorhodopsin (GPR)¹⁰, which can be accounted for by effects of membrane potential on the M^{13-cis} to N^{13-cis} equilibrium. Importantly, further understanding of the mechanism of voltage regulation in microbial rhodopsins can be important for understanding the molecular basis of their function and for bottom-up bioengineering of improved optogenetic rhodopsins.

CONCLUSIONS

Confocal near-IR resonance Raman spectroscopy has been used to study the retinal chromophore structure of AR3, a light driven proton pump from *Halorubrum sodomense*, reconstituted into lipid membranes along with the effects of transmembrane potential on the photocycle of the AR3 mutant D95N in intact *E. coli* cells. The all-*trans* retinal chromophore has a very similar structure to BR, although some differences may exist in the protein-chromophore interactions near the Schiff base involving internal water molecules, accounting for a small blue-shift observed in the visible absorption maximum. The RRS of the D95N mutant, which has been used as a fluorescent voltage sensor, exhibits a voltage sensitivity which is attributed to alterations in the photo-stationary accumulation of a 13-*cis* N-like intermediate. A possible explanation of this behavior is based on electric field induced deprotonation/protonation of the SB proton in the N-intermediate which has access to a cytoplasmic directed channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AR3	Archaeorhodopsin 3
BR	bacteriorhodopsin

CCCP	carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
CP	cytoplasmic
EC	extracellular
FTIR	Fourier transform infrared
OG	octylglucoside
RRS	resonance Raman spectroscopy
ECPL	<i>E. coli</i> polar lipids
SB	Schiff base
WT	wild-type.

REFERENCES

1. Deisseroth K. *Sci. Am.* 2010; 303:48–55. [PubMed: 21033283]
2. Deisseroth K. *Nat Methods.* 2011; 8:26–29. [PubMed: 21191368]
3. Diester I, Kaufman MT, Mogri M, Pashaie R, Goo W, Yizhar O, Ramakrishnan C, Deisseroth K, Shenoy KV. *Nat. Neurosci.* 2011; 14:387–397. [PubMed: 21278729]
4. Chow BY, Han X, Dobry AS, Qian X, Chuong AS, Li M, Henninger MA, Belfort GM, Lin Y, Monahan PE, et al. *Nature.* 2011; 463:98–102. [PubMed: 20054397]
5. Guo ZV, Hart AC, Ramanathan S. *Nat. Methods.* 2009; 6:891–896. [PubMed: 19898486]
6. Herlitze S, Landmesser LT. *Curr. Opin. Neurobiol.* 2007; 17:87–94. [PubMed: 17174547]
7. Kleinlogel S, Feldbauer K, Dempski RE, Fotis H, Wood PG, Bamann C, Bamberg E. *Nat. Neurosci.* 2010; 14:513–518. [PubMed: 21399632]
8. Yizhar O, Fenno LE, Davidson TJ, Mogri M, Diesseroth K. *Neuron.* 2011; 71:9–34. [PubMed: 21745635]
9. Kralj JM, Douglass AD, Hochbaum DR, Maclaurin D, Cohen AE. *Nat. Methods.* 2012; 9:90–95. [PubMed: 22120467]
10. Kralj JM, Hochbaum DR, Douglass AD, Cohen AE. *Science.* 2011; 333:345–348. [PubMed: 21764748]
11. Jung, K-H.; Spudich, JL. ch. 124. In: Horspool, WM.; Lenci, F., editors. *CRC Hand book of Organic Photochemistry and Photobiology*. London: CRC Press; 2004. p. 1-12.
12. Lanyi JK, Luecke H. *Curr Opin Struct Biol.* 2001; 11:415–419. [PubMed: 11495732]
13. Ming M, Lu M, Balashov SP, Ebrey TG, Li Q, Ding J. *Biophys. J.* 2006; 90:3322–3332. [PubMed: 16473896]
14. Wu J, Ma D, Wang Y, Ming M, Balashov SP, Ding J. *J. Phys. Chem. B.* 2009; 113:4482–4491. [PubMed: 19281200]
15. Dunn RJ, Hackett NR, McCoy JM, Chao BH, Kimura K, Khorana HG. *J. Biol. Chem.* 1987; 262:9246–9254. [PubMed: 3298252]
16. Feng J, Liu HC, Chu JF, Zhou PJ, Tang JA, Liu SJ. *Extremophiles.* 2006; 10:29–33. [PubMed: 16075162]
17. Lanyi JK. *Mol. Membr. Biol.* 2004; 21:143–150. [PubMed: 15204622]
18. Lanyi JK. *Annu. Rev. Physiol.* 2004; 66:665–688. [PubMed: 14977418]
19. Ihara K, Umemura T, Katagiri I, Kitajima-Ihara T, Sugiyama Y, Kimura Y, Mukohata Y. *J. Mol. Biol.* 1999; 285:163–174. [PubMed: 9878396]
20. Crick FH. *Sci. Am.* 1979; 241:219–232. [PubMed: 115087]
21. Kralj JM, Spudich EN, Spudich JL, Rothschild KJ. *J. Phys. Chem. B.* 2008; 112:11770–11776. [PubMed: 18717545]

22. Rath P, Krebs MP, He Y, Khorana HG, Rothschild KJ. *Biochemistry*. 1993; 32:2272–2281. [PubMed: 8443170]
23. Dioumaev AK, Brown LS, Shih J, Spudich EN, Spudich JL, Lanyi JK. *Biochemistry*. 2002; 41:5348–5358. [PubMed: 11969395]
24. Saint Clair EC, Ogren JI, Mamaev S, Kralj JM, Rothschild KJ. *J. Biol. Phys.* 2012; 38:153–168. [PubMed: 23277676]
25. Becher BM, Cassim JY. *Prepr. Biochem.* 1975; 5:161–178.
26. Smith SO, Braiman MS, Myers AB, Pardo JA, Courtin JML, Winkel C, Lugtenburg J, Mathies RA. *J. Am. Chem. Soc.* 1987; 109:3108–3125.
27. Smith SO, Hornung I, Van der Steen R, Pardo JA, Braiman MS, Lugtenburg J, Mathies RA. *Proc. Nat. Acad. Sci.* 1986; 83:967–971. [PubMed: 3006035]
28. Smith SO, Lugtenburg J, Mathies RA. *J. Membr. Biol.* 1985; 85:95–109. [PubMed: 4009698]
29. Lewis A, Spoonhower J, Bogomolni RA, Lozier RH, Stoeckenius W. *Proc. Natl. Acad. Sci.* 1974; 71:4462–4466. [PubMed: 4530995]
30. Eyring G, Mathies R. *Proc. Natl. Acad. Sci.* 1979; 76:33–37. [PubMed: 284349]
31. Bergo V, Amsden JJ, Spudich EN, Spudich JL, Rothschild KJ. *Biochemistry*. 2004; 43:9075–9083. [PubMed: 15248764]
32. Bergo V, Spudich EN, Spudich JL, Rothschild KJ. *Photochem. Photobiol.* 2002; 76:341–349. [PubMed: 12403457]
33. Bergo V, Spudich EN, Spudich JL, Rothschild KJ. *J. Biol. Chem.* 2003; 278:36556–36562. [PubMed: 12821665]
34. Luecke H. *Biochim. Biophys. Acta*. 2000; 1460:133–156. [PubMed: 10984596]
35. Smith SO, Pardo JA, Lugtenburg J, Mathies RA. *J. Phys. Chem.* 1987; 91:804–819.
36. Dencher NA, Kohl KD, Heyn MP. *Biochemistry*. 1983; 22:1323–1334. [PubMed: 6838856]
37. Wang J, Link S, Heyes CD, El-Sayed MA. *Biophys. J.* 2002; 83:1557–1566. [PubMed: 12202380]
38. Cappuccio JA, Blanchette CD, Sulchek TA, Arroyo ES, Kralj JM, Hinz AK, Kuhn EA, Chromy BA, Segelke BW, Rothschild KJ, Fletcher JE, Katzen F, Peterson TC, Kudlicki WA, Bench G, Hoepflich PD, et al. *Mol. Cell. Proteomics*. 2008; 7:2246–2253. [PubMed: 18603642]
39. Baasov T, Friedman N, Sheves M. *Biochemistry*. 1987; 26:3210–3217. [PubMed: 3607019]
40. Smith SO, Braiman MS, Myers AB, Pardo JA, Courtin JML, Winkel C, Lugtenburg J, Mathies RA. *J. Am. Chem. Soc.* 1987; 109:3108–3125.
41. Mukohata Y, Ihara K, Uegaki K, Miyashita Y, Sugiyama Y. *Photochem. Photobiol.* 1991; 54:1039–1045. [PubMed: 1723208]
42. Rath P, Marti T, Sonar S, Khorana HG, Rothschild KJ. *J. Biol. Chem.* 1993; 268:17742–17749. [PubMed: 8349659]
43. Marti T, Rosselet SJ, Otto H, Heyn MP, Khorana HG. *J. Biol. Chem.* 1991; 266:18674–1883.
44. Mogi T, Stern LJ, Marti T, Chao BH, Khorana HG. *Proc. Natl. Acad. Sci.* 1988; 85:4148–41452. [PubMed: 3288985]
45. Otto H, Marti T, Holz M, Mogi T, Stern LJ, Engel F, Khorana HG, Heyn MP. *Proc. Natl. Acad. Sci.* 1990; 87:1018–1022. [PubMed: 2153966]
46. Thorgeirsson TE, Milder SJ, Miercke LJ, Betlach MC, Shand RF, Stroud RM, Kliger DS. *Biochemistry*. 1991; 30:9133–9142. [PubMed: 1892824]
47. Nilsson A, Rath P, Olejnik J, Coleman M, Rothschild KJ. *J. Biol. Chem.* 1995; 270:29746–29751. [PubMed: 8530365]
48. Fodor SP, Ames JB, Gebhard R, van den Berg EM, Stoeckenius W, Lugtenburg J, Mathies RA. *Biochemistry*. 1988; 27:7097–7101. [PubMed: 2848578]
49. Deng H, Pande C, Callender RH, Ebrey TG. *Photochem. Photobiol.* 1985; 41:467–4670. [PubMed: 4011703]
50. Nakagawa M, Ogura T, Maeda A, Kitagawa T. *Biochemistry*. 1989; 28:1347–1352.
51. Turner GJ, Miercke LJ, Thorgeirsson TE, Kliger DS, Betlach MC, Stroud RM. *Biochemistry*. 1993; 32:1332–1337. [PubMed: 8448142]

52. Tittor J, Schweiger U, Oesterhelt D, Bamberg E. *Biophys. J.* 1994; 67:1682–1690. [PubMed: 7819500]
53. Zilberstein D, Agmon V, Schuldiner S, Padan E. *J. Bacteriol.* 1984; 158:246–252. [PubMed: 6325389]
54. Han X, Chow BY, Zhou H, Klapoetke NC, Chuong A, Rajimehr R, Yang A, Baratta MV, Winkle J, Desimone R, et al. *Front Syst Neurosci.* 2011; 5:1–8. [PubMed: 21347218]
55. Spudich JL. *Trends in microbiology.* 2006; 14:480–487. [PubMed: 17005405]
56. Jung KH, Trivedi VD, Spudich JL. *Mol Microbiol.* 2003; 47:1513–1522. [PubMed: 12622809]
57. Ming M, Wang Y, Wu J, Ma D, Li Q, Ding J. *FEBS Lett.* 2006; 580:6749–6753. [PubMed: 17134701]
58. Luecke H, Schobert B, Richter HT, Cartailler JP, Lanyi JK. *J. Mol. Biol.* 1999; 291:899–911. [PubMed: 10452895]
59. Enami N, Yoshimura K, Murakami M, Okumura H, Ihara K, Kouyama T. *J. Mol. Biol.* 2006; 358:675–685. [PubMed: 16540121]
60. Honig B, Dinur U, Nakanishi K, Balogh-Nair V, Gawinowicz MA, Arnaboldi M, Motto MG. *J. Am. Chem. Soc.* 1979; 101:7084–7086.
61. Russell TS, Coleman M, Rath P, Nilsson A, Rothschild KJ. *Biochemistry.* 1997; 36:7490–7497. [PubMed: 9200698]
62. Kolodner P, Lukashev EP, Ching YC, Rousseau DL. *Proc. Natl. Acad. Sci.* 1996; 93:11618–11621. [PubMed: 8876185]
63. Bousche O, Braiman M, He YW, Marti T, Khorana HG, Rothschild KJ. *J. Biol. Chem.* 1991; 266:11063–11067. [PubMed: 2040618]
64. Otto H, Marti T, Holz M, Mogi T, Lindau M, Khorana HG, Heyn MP. *Proc. Natl. Acad. Sci.* 1989; 86:9228–9232. [PubMed: 2556706]
65. Bombarda E, Becker T, Ullmann GM. *J. Am. Chem. Soc.* 2006; 128:12129–12139. [PubMed: 16967962]
66. Freier E, Wolf S, Gerwert K. *Proc. Natl. Acad. Sci.* 2011; 108:11435–11439. [PubMed: 21709261]
67. Ohtani H, Tsukamoto Y, Sakoda Y, Hamaguchi H. *FEBS Lett.* 1995; 359:65–68. [PubMed: 7851532]
68. Groma GI, Helgerson SL, Wolber PK, Beece D, Dancsházy Z, Keszthelyi L, Stoeckenius W. *Biophys. J.* 1984; 45:985–992. [PubMed: 6329348]
79. Manor D, Hasselbacher CA, Spudich JL. *Biochemistry.* 1988; 27:5843–5848. [PubMed: 3191096]

Figure 1. Sequence of AR3 and predicted folding pattern in membrane based on earlier models of archaerhodopsins and other microbial rhodopsins (see for example reference¹³). Red highlighted residues are involved in the BR proton transport mechanism (see text). D95 in AR3 (yellow), is homologous to the D85 Schiff base proton acceptor in BR and was replaced by an asparagine to form mutant D95N described in this paper. M155 and C154 are highlighted in blue and orange, respectively.

Figure 2. RRS of the BR purple membrane and AR3 reconstituted into *E. coli* polar lipids recorded in H₂O and D₂O at pH 7

Data was recorded at room temperature using a 785-nm probe laser with 100 mW power (40mW measured at the sample). A background spectrum of the quartz capillary and buffer was subtracted from the sample. The spectra were scaled using the intensity of the ethylenic band at 1526 cm⁻¹. Additional details given in Materials and Methods. Small negative or positive peaks in spectra are denoted by “*” (see also other Figures) and are due to artifacts caused by cosmic radiation entering detector during measurement.

Figure 3. Visible absorption of AR3 reconstituted into *E. coli* lipids measured after dark adaptation (dashed line) and light adaptation (solid line) at room temperature
The mutant D95N recorded in dark is shown for comparison (dotted line) (see text). Spectra were baseline corrected as described in Materials and Methods. Tick marks on Y-axis for the light and dark adapted spectra are both in 100 mOD intervals and 50 mOD intervals for the D95N spectrum. All spectra were measured in pH7 buffer identical to that used for RRS measurements, see Materials and Methods.

Figure 4. RRS of AR3 reconstituted into *E. coli* polar lipids measured from pH 2 to 10
All data was recorded under same conditions as described in Materials and Methods. Small artifact (negative peaks) in spectra are indicated by “*”.

Figure 5. RRS of AR3 D95N reconstituted into *E. coli* polar lipids measured at various pHs ranging from 1.5 to 11

All spectra were recorded using 100 mW, 785nm excitation. In addition to the 1518 cm^{-1} band seen at pH 7 arising from the red-shifted $\text{O}^{\text{all-trans}}$ species a second band appears at 1527 cm^{-1} at pH > 7 indicative of $\text{N}^{13\text{-cis}}$.

Figure 6. Variation of RRS of AR3 D95N at pH 10 with power of 785-nm excitation at 100 mW and 1 mW intensity

The contributions from the N¹³-*cis* species drops significantly in AR3 D95N relative to O^{all}-*trans* as based on drop in intensity of 1527 and 1185 cm⁻¹ bands.

Figure 7. Comparison of RRS of AR3 D95N in membrane fragments and *in vivo* in *E. coli* at pH 9 under various conditions

A. Membrane fragments reconstituted in *E. coli* polar lipids. B. In *E. coli* cells. C. In *E. coli* cells with CCCP added. D. In sonicated *E. coli* cells. E. *E. coli* cell not expressing AR3 D95N. All spectra were recorded using 100 mW 785nm excitation. Solid vertical lines with arrows indicate assignment of major bands in AR3 RRS spectra that arise from non-resonant contribution present in non-expressing *E. coli* (lower trace).

Figure 8. Reproducibility of RRS of AR3 D95N at pH 9 recorded *in vivo* in *E. coli* under different conditions

Top set (red) not treated, middle set (blue) sonicated and bottom set (green) treated with CCCP (see Materials and Methods).

Figure 9. Model of AR3 D95N red-light photocycles adapted from Tittor *et al.*⁵² for BR
 Absorption of a photon by $O^{all-trans}$ (red-light photocycle) results in transition to N^{13-cis} and switch from extracellular (EC) to cytoplasmic (CP) accessible SB followed by establishment of an equilibrium between N^{13-cis} and M^{13-cis} (red arrows) which is postulated here to be voltage sensitive and favor M^{13-cis} for normal negative cell potential.

Table 1Effects of various treatments to *E. coli* expressing AR3 D95N on Raman peak ratios.

		1185/1168	1527/1517
CCCP+	ratio [§]	0.91	0.91
	SD [§]	±0.05	±0.03
Untreated	ratio	0.72	0.78
	SD	±0.09	±0.09
SON	ratio	1.11	1.11
	SD	±0.2	±0.19
Untreated	ratio	0.74	0.72
	SD	±0.05	±0.05

[§]Peak ratios and standard deviation (SD) were calculated for *E. coli* cells expressing AR3 D95N under different conditions described in text. Ratios listed in table were calculated in case of 1185/1168 by measuring integrated intensity around each peak (1185: 1193.5-1179, 1168: 1179-1160) and for 1527/1517 ratio by using just the measured intensity at the peak frequency (1527 and 1517). Four different samples were measured for each set shown. In case of untreated cells, two different sets of 4 spectra for samples were measured. SON: sonicated; CCCP+: addition of CCCP to cells as described in Materials and Methods.