Near-IR Resonance Raman Characterization of an Anion Channelrhodopsin

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Abstract

Optogenetics relies on the expression of specific microbial rhodopsins in the neuronal plasma membrane. Most notably, this includes channelrhodopsins (ChRs), which when heterologously expressed in neurons functions as light-gated cation channels. Recently, a new class of microbial rhodopsins, termed anion channel rhodopsins (ACRs), has been discovered. These proteins function as efficient light-activated channels strictly selective for anions. They exclude the flow of protons and other cations and cause hyperpolarization of the membrane potential in neurons by allowing the inward flow of chloride ions. In this study, confocal near-infrared resonance Raman spectroscopy (RRS) along with hydrogen/deuterium exchange, retinal analog substitution, and site-directed mutagenesis were used to study the retinal structure as well as its interactions with the protein in the unphotolyzed state of an ACR from *Guillardia theta* (*Gt*ACR1). These measurements reveal that: *i*) the retinal chromophore exists as an all-*trans* configuration with a protonated Schiff base (PSB) very similar to that of bacteriorhodopsin (BR); *ii*) the chromophore RRS spectrum is insensitive to changes in pH from 3 to 11, whereas above this pH the Schiff base (SB) deprotonates; *iii*) when Ser97, the homolog to Asp85 in BR, is replaced with a Glu, it remains in a neutral form (i.e. as a carboxylic acid) but deprotonates at higher pH to form a blue-shifted species; iv) Asp234, the homolog of the protonated retinylidene Schiff base (SB) counterion Asp212 in BR, does not serve as the primary counteranion for the protonated SB; *iv*) substitution of Glu68 with an Gln increases the pH at which SB deprotonation is observed. These results suggest that Glu68 and Asp234 located near the SB exist in a neutral state in unphotolyzed *Gt*ACR1 and indicate that other unidentified negative charge(s) stabilize the protonated state of the *Gt*ACR1 SB.

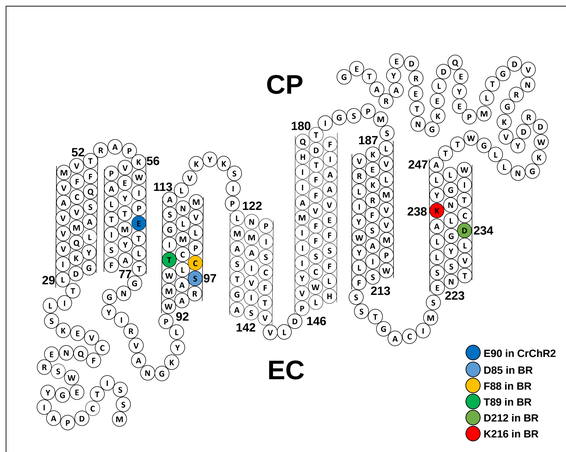

Figure 1. Sequence of *Gt*ACR1 and predicted folding pattern in the membrane based on earlier models of other microbial rhodopsins. Highlighted residues include Ser97 and Asp234, which are homologs of Asp85 and Asp212 in BR which form, along with a water molecule, the complex counterion to the SB. In most cation pumps and cation channelrhodopsins, the BR Asp85 homolog is an Asp or Glu residue. However, in *Gt*ACR1, it is replaced by Ser, a neutral residue similar to halorhodopsin anion pumps (Thr in halorhodopsin) and other anion channelrhodopsins (Ala in *Psu*ACR1).

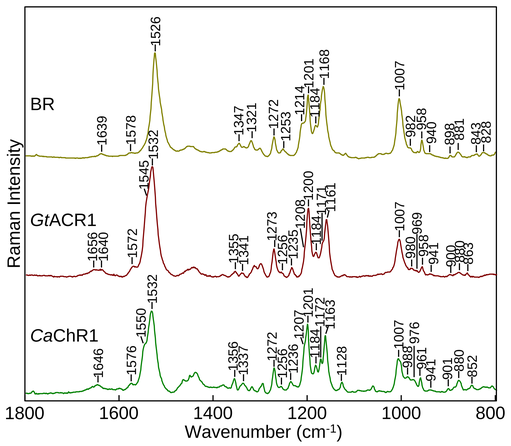

Figure 2. RRS of the BR (in purple membrane), *Gt*ACR1, and *Ca*ChR1 (reconstituted into *E. coli* polar lipids). Data was recorded at room temperature using a 785-nm probe laser with 100 mW power (40mW measured at the sample) for BR and *Ca*ChR1, and with 300mW (70mW measured at the sample) *for* GtACR1. A background spectrum of the borosilicate capillary and buffer was subtracted from the sample. The spectrum of *Gt*ACR1 is very similar to spectra of BR and *Ca*ChR1, especially in the C-C stretch fingerprint region, indicating *Gt*ACR1 has an all-*trans* retinal configuration like BR and *Ca*ChR1.

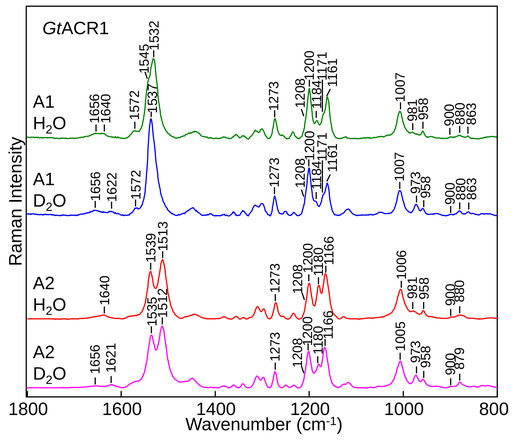

Figure 3. Comparison of resonance Raman spectra of *Gt*ACR1 with A1 and A2 retinal (3,4-dehydroretinal) recorded in H_2O and D_2O. Experimental conditions are the same as described in Figure 2. The 18cm^{-1} downshift of the 1640cm^{-1} band in D_2O assigns the band to Schiff base vibrational mode (also confirms protonation of the SB). The magnitude of the downshift is similar to BR, indicating similar hydrogen bonding strength, whereas it's much larger in *Ca*ChR1 (26cm^{-1}), indicating *Ca*ChR1 has greater hydrogen bonding strength. The peak at 1656 cm^{-1} is most likely an amide I vibration. A2 retinal substitution causes a red-shift in the visible absorption, corresponding to downshift of the ethylenic mode to 1513 cm^{-1}. The substitution affects the two components of the ethylenic peaks (1532 cm^{-1} and 1545 cm^{-1}) differently.

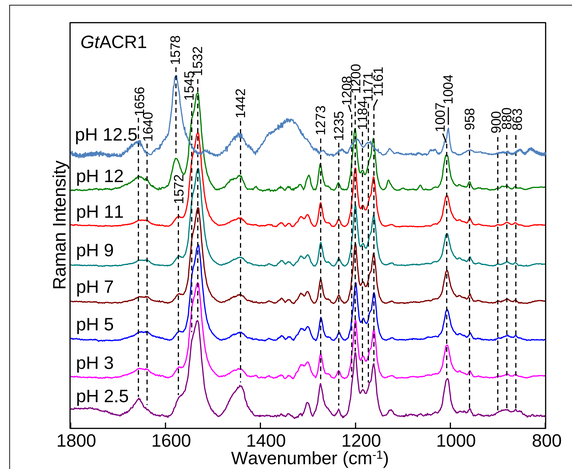

Figure 4. Resonance Raman spectra of *Gt*ACR1 recorded at various pH values ranging from 2.5 to 12.5. Experimental conditions are the same as described in Figure 2. The RRS of *Gt*ACR1 is insensitive to pH changes over the range 3-11. At pH above 11, the 1578cm^{-1} band gradually increases until 1532cm^{-1} band completely disappears at pH 12.5. This most likely corresponds to SB deprotonation which occurs in the BR M intermediate. The pK_a for SB deprotonation may actually be lower, because the blue-shifted visible absorption of this M-like intermediate is not expected to be strongly resonantly enhanced by 785-nm laser.

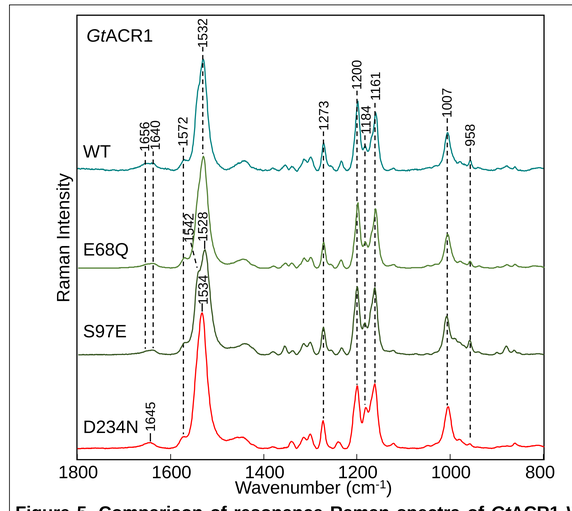

Figure 5. Comparison of resonance Raman spectra of *Gt*ACR1 WT and its mutants E68Q, S97E, and D234N. Experimental conditions are the same as described in Figure 2. None of the three mutants dramatically affect the RRS. S97E downshifts the ethylenic band slightly, whereas a negatively charged E97 is expected to cause an upshift of the ethylenic band. This suggests that E97 is still neutral at pH 7. The upshift of the ethylenic in D234N is also inconsistent with a negatively charged D234. A similar conclusion was also reached for E68 which does not shift substantially when substituted with Q68. These results suggest that E68, S97, and D234 are all neutral in the unphotolyzed state of *Gt*ACR1 and do not act as primary counterion to the SB.

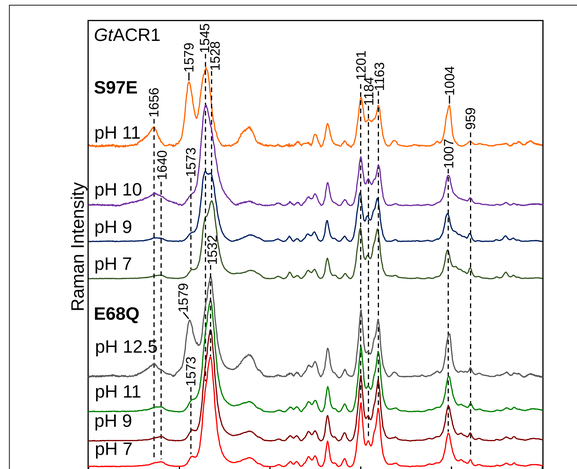

Figure 6. Resonance Raman spectra of *Gt*ACR1 mutant S97E and E68Q recorded at various pH values. Experimental conditions are the same as described in Figure 2. The gradual transition from 1528 cm^{-1} to 1545 cm^{-1} for the ethylenic band >pH 7 in S97E suggests that unlike S97, E97 deprotonates at high pH values (providing a negative charge near the SB). At even higher pH ~11 it acts as the proton acceptor for the SB allowing formation of blue-shifted form. E68Q exhibits a higher pH value for the SB deprotonation than WT, which suggests E68 may serve as the SB proton acceptor at high pH.

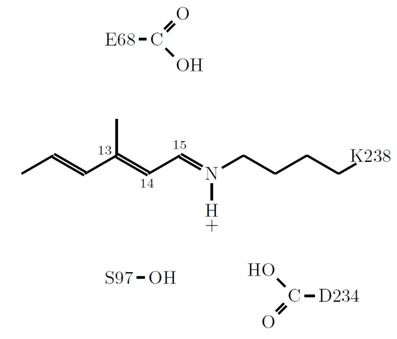

Figure 7. Schematic showing key residues and their ionization state in the photoactive site of unphotolyzed *Gt*ACR1. The RRS results indicate that the unphotolyzed *Gt*ACR1 has a pure all-*trans* retinal configuration with a protonated SB. In addition, the data suggests that E68, S97, and D234 located near the SB exist in a neutral state and do not serve as counterion for the protonated SB.

References

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