Resonance Raman and Low-temperature FTIR-Difference Spectroscopy of Channelrhodopsin-1 from *Chlamydomonas Augustae*

Adrian Yi[1], John I. Ogren[1], Sergey Mamaev[1], Hai Li[2], Johan Lugtenburg[3], Willem J. DeGrip[3], John Spudich[2], Kenneth J. Rothschild[1]

[1]Department of Physics, Photonics Center and Molecular Biophysics Laboratory, Boston University, Boston, MA 02215, USA

[2]Center for Membrane Biology, Department of Biochemistry and Molecular Biology, The University of Texas Health Science Center, Houston, TX 77030, USA

[3]Department of Biophysical Organic Chemistry, Leiden Institute of Chemistry, Leiden University, 2300 AR Leiden, The Netherlands

BU MBL · The Boston University photonics center · Universiteit Leiden The Netherlands · BOSTON UNIVERSITY · The University of Texas Health Science Center at Houston

Abstract: Channelrhodopsins (ChRs) from green algae function as light-gated ion channels when expressed heterologously in mammalian cells. Considerable interest has focused on understanding the molecular mechanisms of ChRs in order to bioengineer their properties for specific optogenetic applications. Thus far, most studies have focused on channelrhodopsin-2 from *Chlamydomonas reinhardtii* (*Cr*ChR2).

Here, resonance Raman and low-temperature FTIR-difference spectroscopy are applied to study protein conformational changes occurring during the photocycle of the red-shifted channelrhodopsin-1 from *Chlamydomonas augustae* (*Ca*ChR1). Substitutions with isotope-labeled retinals or the retinal analog A2, site-directed mutagenesis, hydrogen-deuterium exchange and $H_2{}^{18}O$ exchange are used to assign bands to the retinal chromophore and the protein.

The primary phototransition of *Ca*ChR1 at 80 K involves, in contrast to *Cr*ChR2, almost exclusively all-*trans* to 13-*cis* isomerization of the retinal chromophore, similar to the primary phototransition of bacteriorhodopsin (BR). A negative amide II band is identified in the retinal ethylenic stretch region of *Ca*ChR1 which, along with amide I bands, reflects alterations in protein backbone structure early in the photocycle. A decrease in the hydrogen bond strength of a weakly hydrogen bonded internal water is detected in both *Ca*ChR1 and *Cr*ChR2, but the bands are much broader in *Cr*ChR2 indicating a more heterogeneous environment.

Based on the low-temperature FTIR-difference data from mutants involving the residues Glu169 and Asp299 (homologs of the Asp85 and Asp212 Schiff base counterions in BR), we propose a model for protonation changes occurring near the Schiff base during the P_1 and P_2 steps of the photocycle.

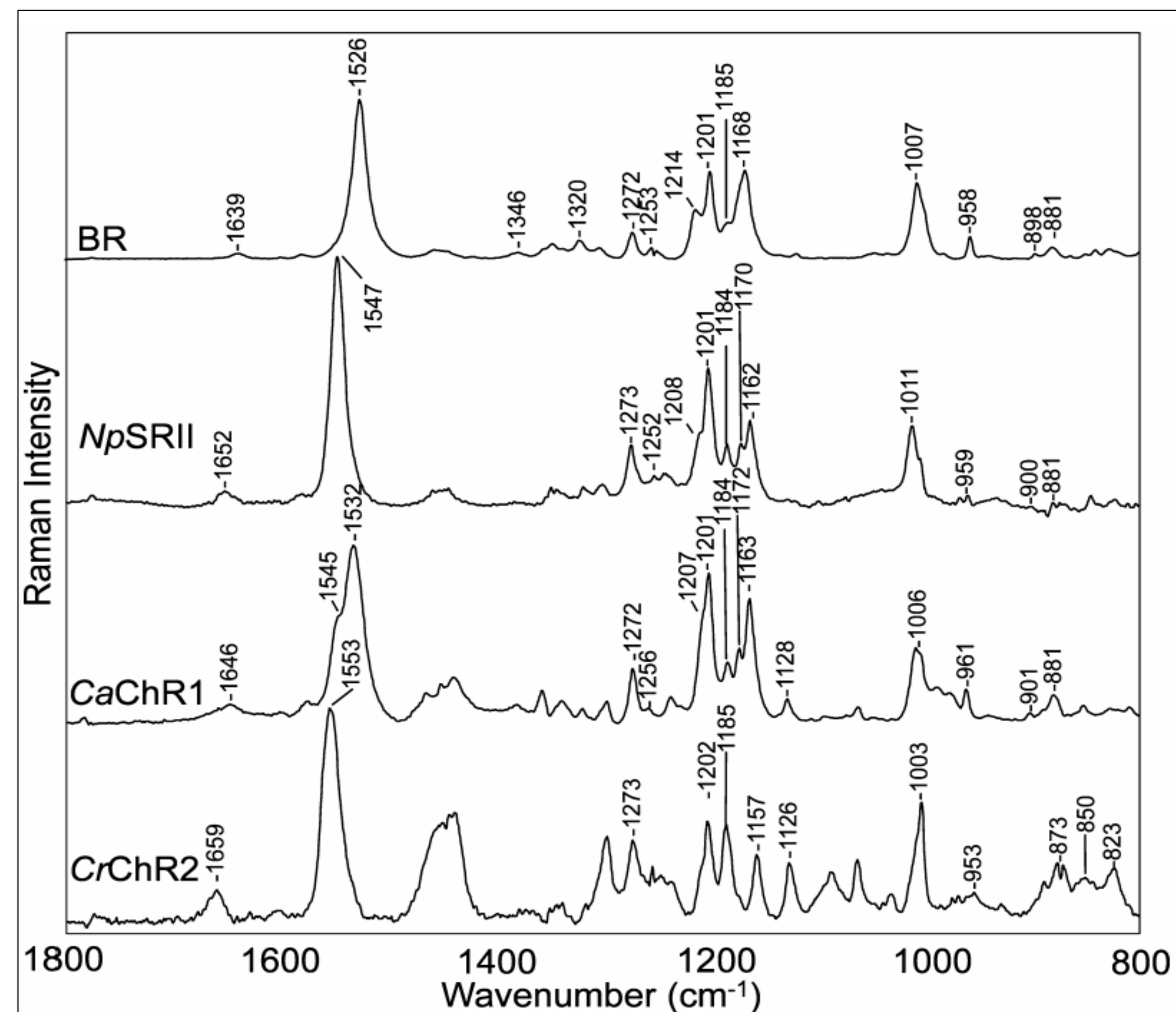

Figure 1. Resonance Raman spectra (RRS) of bacteriorhodopsin (BR), sensory rhodopsin II (*Np*SRII), *Ca*ChR1, and *Cr*ChR2.
Bands in the fingerprint region near 1200 cm^{-1} are due to the C-C single bond vibrations, which are very sensitive to the retinal conformation. The RRS for BR, *Np*SRII, and *Ca*ChR1 are very similar, especially in the fingerprint region; peaks near 1168 cm^{-1} and 1201 cm^{-1}, with a shoulder at 1214 cm^{-1}, and a small peak at 1185 cm^{-1}. The small peak near 1170 cm^{-1} that appear in the spectra of *Np*SRII and *Ca*ChR1 may be due to the downshift of the bigger peak at 1168 cm^{-1}. Earlier studies of BR and *Np*SRII determined the all-*trans* ground state retinal conformation, using techniques such as isotope labeling, molecular dynamics, and x-ray crystallography. We conclude that *Ca*ChR1 an all-*trans* unphotolyzed retinal conformation, unlike the mixed 13-*cis* and all-*trans* state present in *Cr*ChR2.

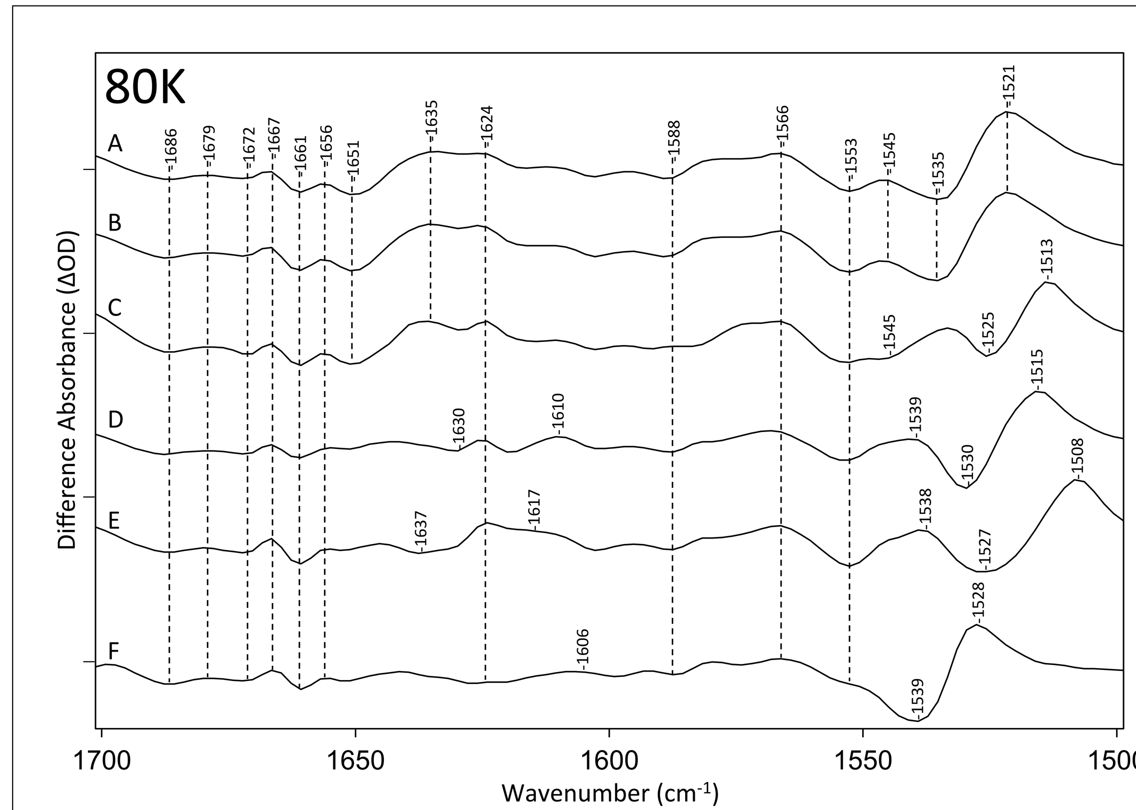

Figure 2. FTIR-difference spectra showing P_1 minus *Ca*ChR1 at 80K: (A) *Ca*ChR1 WT, bleached *Ca*ChR1 regenerated with (B) A1 retinal, (C) A2 retinal, (D) [15-^{13}C, 15-^{2}H] retinal, (E) [14-^{2}H, 15-^{2}H] retinal, and (F) *Ca*ChR1 WT in D_2O.
RRS of *Ca*ChR1 shows the ethylenic C=C peak at 1532 cm^{-1}, with a shoulder at 1545 cm^{-1}. Ethylenic band should downshift due to isotope labeling with ^{13}C and ^2H, because of the increase in mass, and with A2 retinal, because of the increased level of electron delocalization due to the extra double bond in the β-ionone ring. Here, we assigned the negative 1535 cm^{-1} band and positive 1521 cm^{-1} band as the ethylenic mode in the ground and P_1 state, respectively. We assign the negative 1553 cm^{-1} band an amide II mode. Similarly, negative 1651 cm^{-1} and positive 1635 cm^{-1} bands are assigned to the Schiff base C=N stretch mode in the ground and P_1 state, respectively. Increased mass by $^{13}C/^2H$ isotope labeling or H/D exchange causes these bands to downshift by different amounts. Interestingly, the downshift of these bands due to H/D exchange confirms that the SB is protonated in both the ground and P_1 states, similar to BR. Other bands that do not exhibit band shifts are most likely amide I bands.

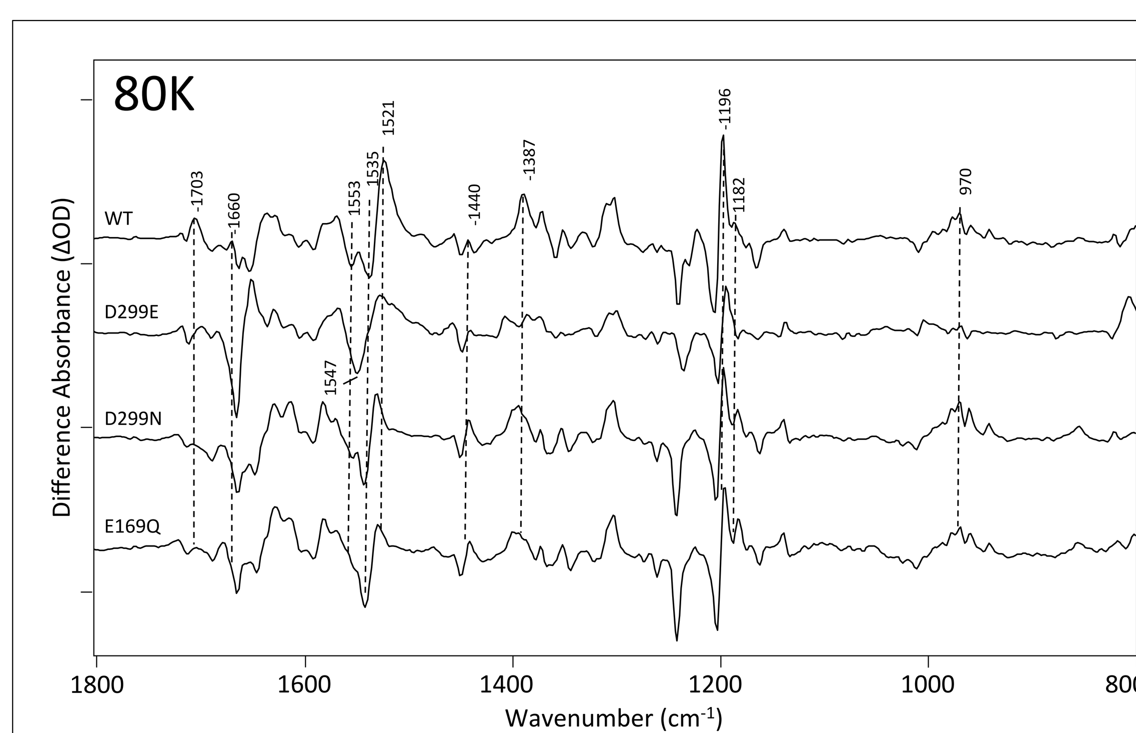

Figure 3. FTIR-difference spectra of *Ca*ChR1 and its mutants at 80K, representing the spectrum of the ground state subtracted from the spectrum of the P_1 state.
The fingerprint region, which is sensitive to the retinal configuration, is similar to BR and other rhodopsin proteins that exhibit pure all-*trans* to 13-*cis* transition in its early primary phototransition with two negative bands near 1200 and 1165-1170 cm^{-1} and a positive band near 1195 cm^{-1}. The mutants D299E, D299N, and E169Q all exhibit this feature, confirming that *Ca*ChR1 wild-type and its mutants all exhibit all-*trans* to 13-*cis* transition.

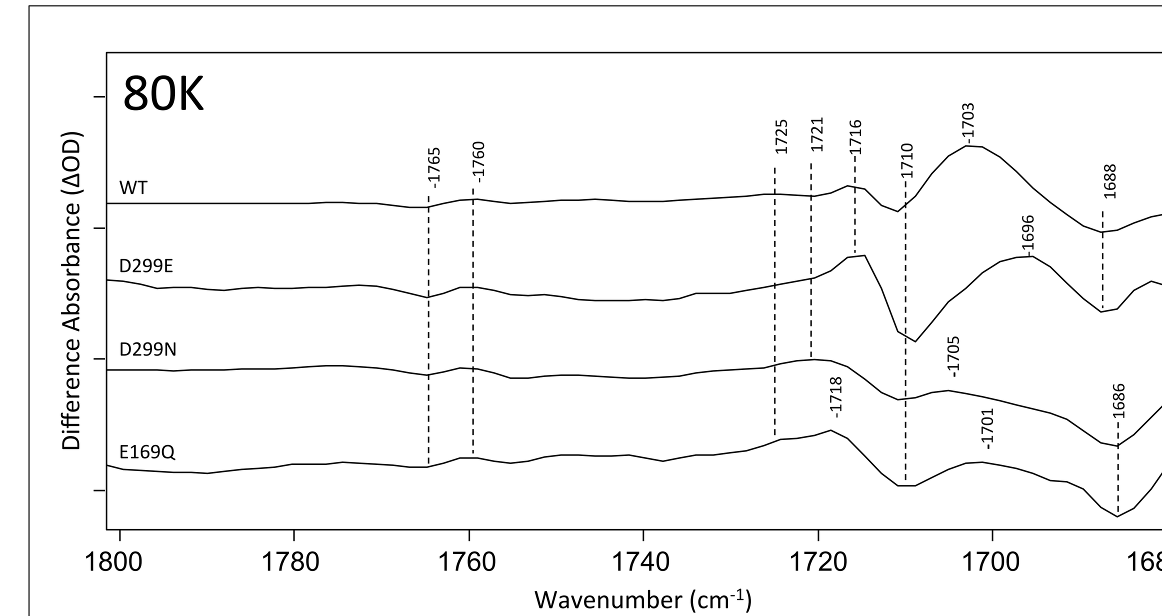

Figure 4. FTIR-difference spectra of *Ca*ChR1 from Figure 3 over the carboxylic acid region from 1680 to 1800 cm^{-1}.
The largest difference between the spectrum of *Ca*ChR1 D299E and that of *Ca*ChR1 WT is the shift of the positive band at 1703 cm^{-1} to 1696 cm^{-1}, indicating the assignment of this band to Asp299. However, there is no negative band associated with this residue, confirming that the Asp299 is in an ionized state in the ground state at neutral pH, as previously reported by Li et. al. and Ogren et. al., and that it becomes protonated during its transition to the P_1 state. The shift of the large positive 1703 cm^{-1} band also reveals the larger 1710 cm^{-1} band which appears smaller in WT. In the spectra of D299N and E169E mutants, the size of the 1710 cm^{-1} band is reduced despite the disappearance of the positive 1703 cm^{-1} band, indicating the disappearance of a negative band near 1710 cm^{-1}. We assign this band to Glu169, because this peak does not contribute to the D299E mutant. We suggest that the Glu169 donates its proton to Asp299 during the *Ca*ChR1→ P_1 transition. Other bands are relatively unchanged, indicating their assignment to other residues.

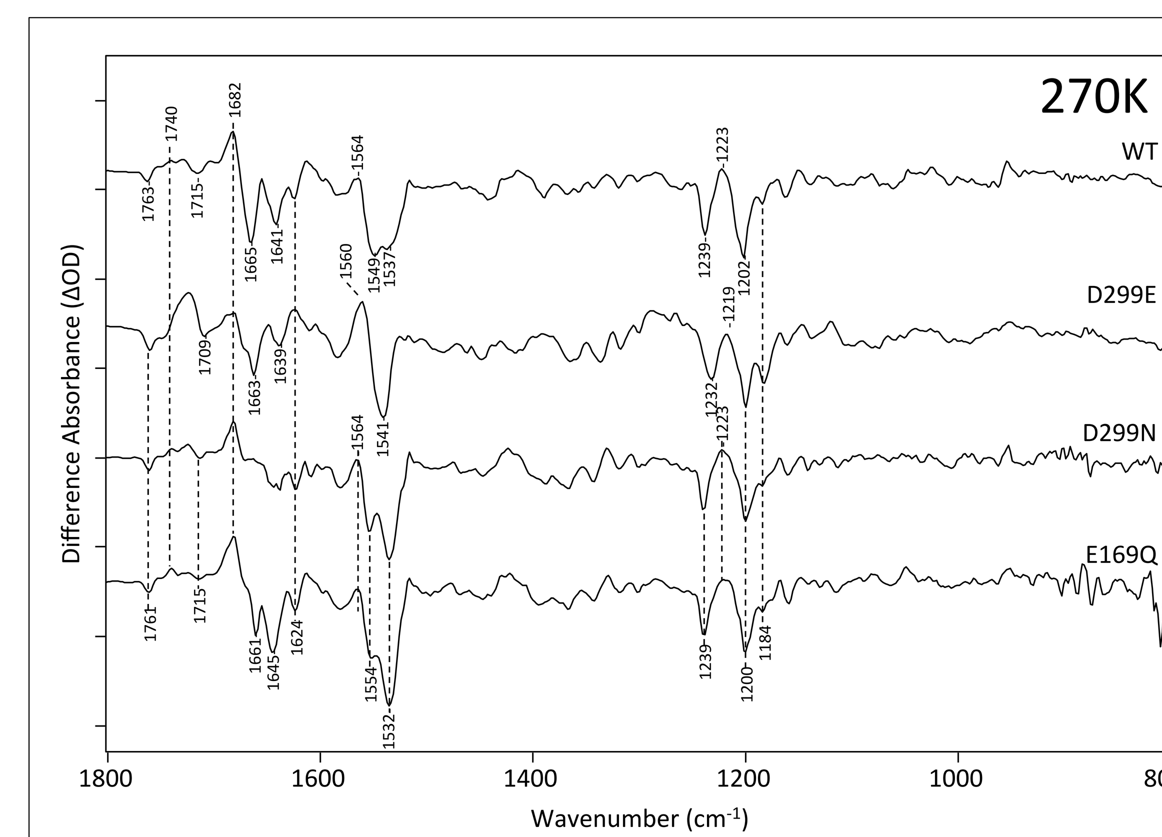

Figure 5. FTIR-difference spectra of *Ca*ChR1 and its mutants at 270K, representing the spectrum of the state unphotolyzed state subtracted from the spectrum of the P_2 state.
The negative bands at 1549 cm^{-1} and 1532 cm^{-1} have already been identified as amide II and ethylenic vibrations, respectively. The positive band at 1564 cm^{-1} is the only positive peak in the ethylenic region, indicating it's likely the ethylenic stretch mode associated with the P_2 state. The fingerprint region is similar to BR and other rhodopsin proteins that have pure all-*trans* retinal configurations with a protonated SB to a 13-*cis* retinal with unprotonated SB. This indicates the P2 state of *Ca*ChR1 has an unprotonated SB. These features do not significantly change in the spectra of the mutants D299E, D299N, or E169Q, confirming that the wild-type and these mutants all exhibit the same changes in retinal conformation and SB protonation state.

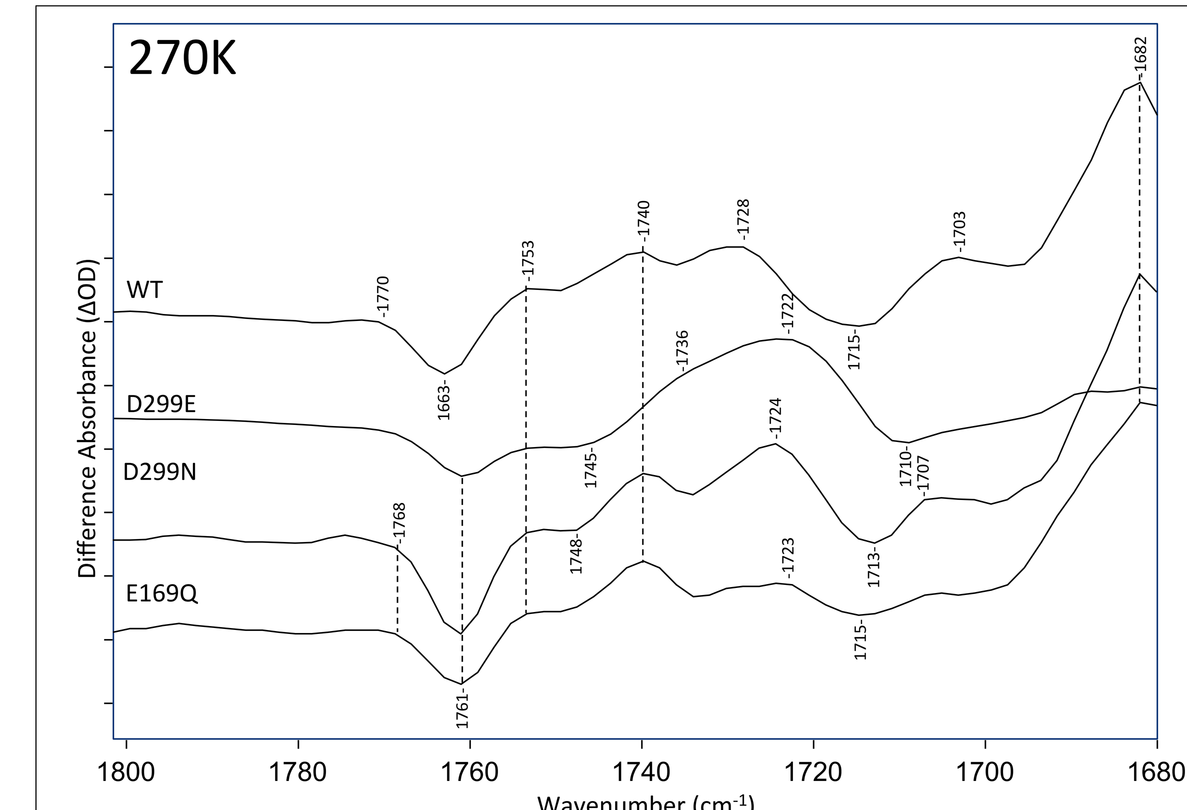

Figure 6. FTIR-difference spectra of *Ca*ChR1 from Figure 5 over the carboxylic acid region from 1680 to 1800 cm^{-1}.
The disappearance of the positive band at 1728 cm^{-1} and the broad negative band near 1715-1720 cm^{-1} in the E169Q mutant spectrum are assigned to Glu169, which indicates weakening of the hydrogen bonding of Glu169 during the *Ca*ChR1 → P_2 transition. In the spectra of D299E and D299N mutants, region near 1740-1753 cm-1 drops in intensity, indicating a positive band associated with Asp299 in this region. The above observations indicate a change in hydrogen bonding strength of the protonated E169 and the protonation of an unprotonated Asp299, which agrees with the ground state suggested by previous RRS and 80K difference-FTIR data. We conclude that P_2 state of *Ca*ChR1 has both a neutral Glu169 and Asp299.

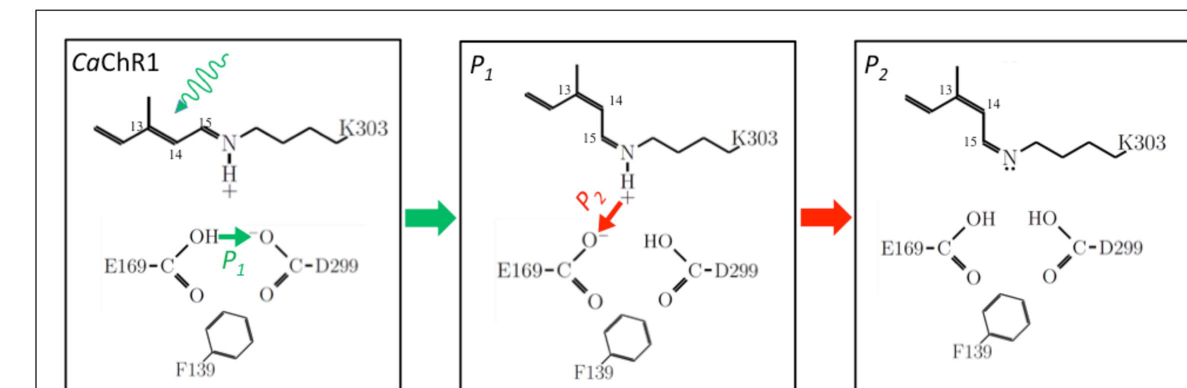

Figure 7. Suggested model of protonation changes near the Schiff base during the P_1 and P_2 steps of the photocycle of *Ca*ChR1.
The data from RRS and low-temperature FTIR-difference spectroscopy (80K and 270K) experiments presented here suggest a model for the proton transfers occurring in the early photocycle of *Ca*ChR1. The changes include the proton transfer from the Glu169 COOH to the Asp299 COO^- which is triggered by absorption of a photon by retinal (green arrow) causing the primary phototransition to P_1. During the thermal transition from P_1 to P_2, the proton moves from the SB to the now negative Glu169 COO^- group causing its neutralization. Thus, in the P_2 state Glu169, Asp299 and the SB are all neutral. Interestingly, in *Ca*ChR1 the cation channel appears to open at the P_2 state. One possibility is that this channel directly involves Glu169 Asp299 and the SB which act as a gate and when neutral allows the flow of cations but when charged prevents this flow. The involvement of these groups is consistent with the high resolution x-ray determined structure of the C1-C2 chimeric channel rhodopsin (Kato et al., 2012).

References

Li, H., Govorunova, E. G., Sineshchekov, O. A., and Spudich, J. L., Role of a Helix B Lysine Reisude in the Photoactive Site in Channelrhodopsin. *Biophys. J.* 106, 1607-1617 (2014).

Ogren, J. I., Mamaev, S., Russano, D., Li, H., Spudich, J. L., and Rothschild, K. J., Retinal Chromophore Structure and Schiff Base Interactions in Red-shifted Channelrhodopsin-1 from *Chlamydomonas augustae*. *Biochemistry* 53, 3961– 3970 (2014).

Ogren, J. I., Yi, A., Mamaev, S., Li, H., Lugtenburg, J., DeGrip, W. J., Spudich, J. L., and Rothschild, K. J., Comparison of the Structural Changes Occurring during the Primary Phototransition of Two Different Channelrhodopsins from *Chlamydomonas* Algae*. Biochemistry* 54, 377-388 (2015).

Kato, H. E. et. al, Crystal structure of the channelrhodopsin light-gated cation channel. *Nature*. 2012 Jan 22;482(7385): 369-74.