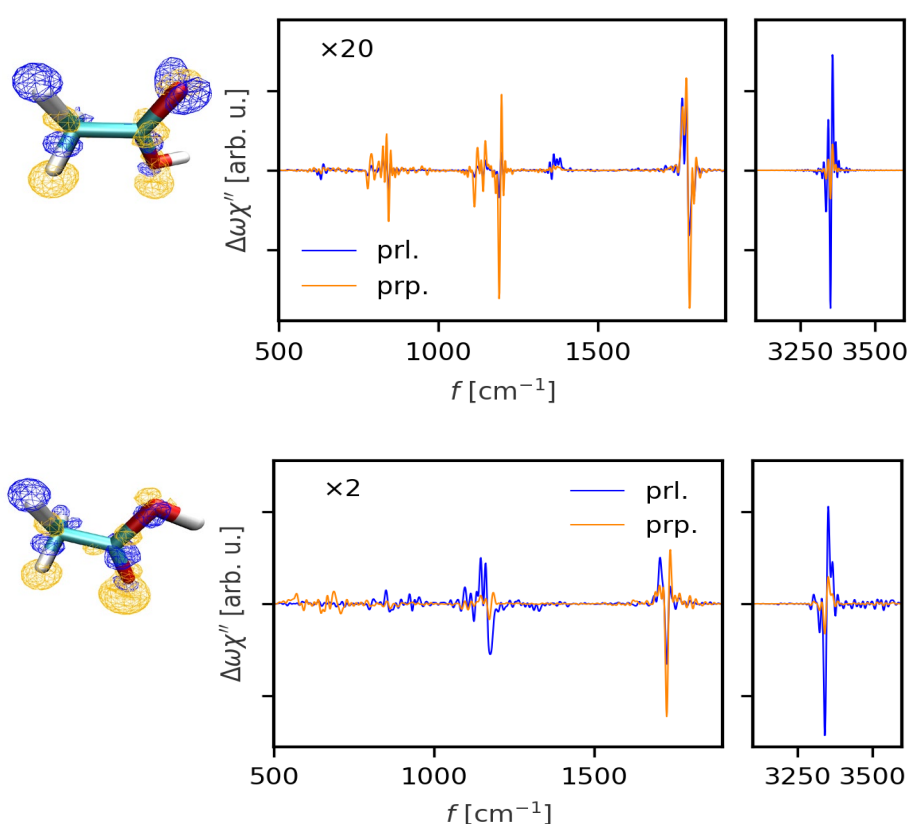


Extended Data Figure 7: Simulated IR spectra of Asp96

To simulate the effect of the difference electric field resulting from the charge separation at the retinal upon photoexcitation on the Asp96 residue in different protonation states, QM/MM simulations using DFT on the BLYP-D3BJ/def2-TZVPPD level are performed as described in the methods section.

For each protonation state the entire preparation and equilibration protocol is followed. Then, for each setup, two simulation series are performed, one with the partial charges of the excited state applied to the retinal and one with the partial charges of the ground state.

Infrared difference spectra are computed from 20 independent simulations of 2 ps length each, by subtracting the spectra with the group state partial charges on the retinal from the spectra with the excited state partial charges. The difference spectra are shown below as projected on the axis of the retinal dipole moment in blue and on the orthogonal plane in orange. Two conformations are investigated with protonated oxygen closer to the retinal, Asp96 i) (upper panel, Figure 3b i), and with protonated oxygen closer to the cytoplasmic side, Asp96 ii) (lower panel, Figure 3b ii). Snapshots of ED differences at Asp96 are indicated by blue (increased ED) and yellow (decreased ED) mesh.



Assuming similar signal strength of $\nu(\text{C}=\text{O})$ the carbonyl stretching vibrations ($\sim 1700 \text{ cm}^{-1}$) for both conformations, the OH stretching vibration ($\sim 3350 \text{ cm}^{-1}$) is reduced for Asp96 ii) (lower panel). This is probably due to its OH vector being almost perpendicular to the transient electric field change. For both conformations the negative parent signal shows stronger signals for perpendicular polarization, i.e. perpendicular to retinal's tdm. Upon

electric field change conformation Asp96 i) (upper panel) shows a red-shifted (positive) signal with similar strength for both polarizations. Conformation Asp96 ii) (lower panel) exhibits blue-shifted signal with stronger strength for perpendicular polarization and red-shifted signal with stronger strength for parallel polarization. The change in polarization dependence can be explained by a distribution of conformations. Since we expect a blue-shifted contribution with stronger perpendicular polarization from the $\text{C}=\text{O}$ stretching vibration of PRG (Figure 5a,b) the parallel polarized signal contribution around 1720 cm^{-1} is probably reduced in our measurement (Figure 5c). Thus, we assume the Asp96 ii) conformation to be present in *HsBR* ground state. This is supported by the simulated relative angle of $\nu(\text{C}=\text{O})$ Asp96 ii) conformation to be around 90° in the ground state, matching our experimental observations. In contrast, for Asp96 i) conformation the averaged simulated relative angle is about 40° (see EDF 8).