Supporting Information:

Red-shifting Mutation of Light-driven Sodium Pump Rhodopsin

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Supplementary Table 1. **Observed vs computed vertical excitation energies $\Delta E_{S1\text{-}S0}$ (kcal/mol) and maximum absorption wavelengths (λ_{max}) for KR2 WT and its mutants P219G, P219T, S254A, and P219T/S254A; ARM models computed at CASPT2//CASSCF(12,12)/6-31G*/AMBER level of theory.** The oscillator strength (f_{Osc}) for $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions and standard deviation (DEV.ST) are also shown.

Sample	Obs. $\Delta E_{S1\text{-}S0}$ (λ_{max})	Comp. $\Delta E_{S1\text{-}S0}$ (λ_{max})	$f_{Osc\ S0\text{-}S1}$	$f_{Osc\ S0\text{-}S2}$	Error	DES.ST
KR2 WT	54.4 (525)	55.2 (517)	1.06	0.43	0.8	0.2415
P219G	53.4 (535)	54.3 (526)	1.01	0.40	0.9	0.1838
P219T	52.7 (542)	53.5 (534)	1.05	0.42	0.8	0.4423
S254A	52.4 (545)	53.1 (538)	1.00	0.41	0.7	0.3838
P219T/S254A	50.6 (565)	51.5 (555)	1.12	0.46	0.9	0.5638

Supplementary Table 2. Computed 3-root CASPT2//CASSCF(12,12)/6-31G*/AMBER vertical excitation energies $\Delta E_{S1\text{-}S0}$ (kcal/mol) of KR2 WT and P219G, P219T, S254A, P219T/S254A mutants, computed when the amino acids where turned off individually and also concurrently.

Sample	Computed E_{S0} (a.u.)	Computed E_{S1} (a.u.)	$\Delta E_{S1\text{-}S0}$ (kcal/mol)	ΔE_{off} (kcal/mol)	$\Delta\Delta E = \Delta E_{S1\text{-}S0} - \Delta E_{off}$ (kcal/mol)
KR2 WT	-872.002324	-871.914423	55.2		
P219 OFF	-872.005518	-871.919671		53.9	+1.3
S254 OFF	-871.993035	-871.909239		52.6	+2.6
Both OFF	-871.984483	-871.901750		51.9	+3.2
P219G	-872.004081	-871.917494	54.3		
G219 OFF	-872.000409	-871.910690		56.3	-2.0
S254 OFF	-872.001467	-871.918690		51.9	+2.4
Both OFF	-871.987028	-871.896631		56.7	-2.4
P219T	-872.004267	-871.919068	53.5		
T219 OFF	-872.000397	-871.911704		55.7	-2.2
S254 OFF	-872.001308	-871.920153		50.9	+2.5
Both OFF	-871.986901	-871.897137		56.3	-2.9
S254A	-871.998014	-871.913387	53.1		
P219 OFF	-872.010538	-871.930712		50.1	+3.0
A254 OFF	-872.019812	-871.928719		57.2	-4.1
Both OFF	-872.020781	-871.929141		57.5	-4.4
P219T/S254A	-871.999028	-871.917009	51.5		
T219 OFF	-871.990471	-871.904659		53.8	-2.4
A254 OFF	-872.045650	-871.959600		54.0	-2.5
Both OFF	-872.045994	-871.953091		58.3	-6.8

Supplementary Table 3. Double and single bond lengths (Å) of QM/MM models built with ARM protocol at CASSCF(12,12)/6-31G*/AMBER level of theory of KR2 WT and its mutants.

Bond/Sample	WT	P219T	S254A	P219T/S254A
C5=C6	1.37	1.371	1.371	1.375
C6-C7	1.486	1.484	1.483	1.481
C7=C8	1.355	1.356	1.355	1.356
C8-C9	1.475	1.473	1.472	1.473
C9=C10	1.362	1.362	1.362	1.365
C10-C11	1.456	1.450	1.453	1.451
C11=C12	1.355	1.357	1.357	1.360
C12-C13	1.462	1.461	1.464	1.465
C13=C14	1.362	1.364	1.368	1.369
C14-C15	1.445	1.442	1.439	1.439
C15=N	1.289	1.288	1.288	1.290

Supplementary Table 4. Double and single bond lengths (Å) of QM/MM models built with ARM protocol at CASSCF(12,12)/6-31G*/AMBER level of theory of KR2 WT and its mutants.

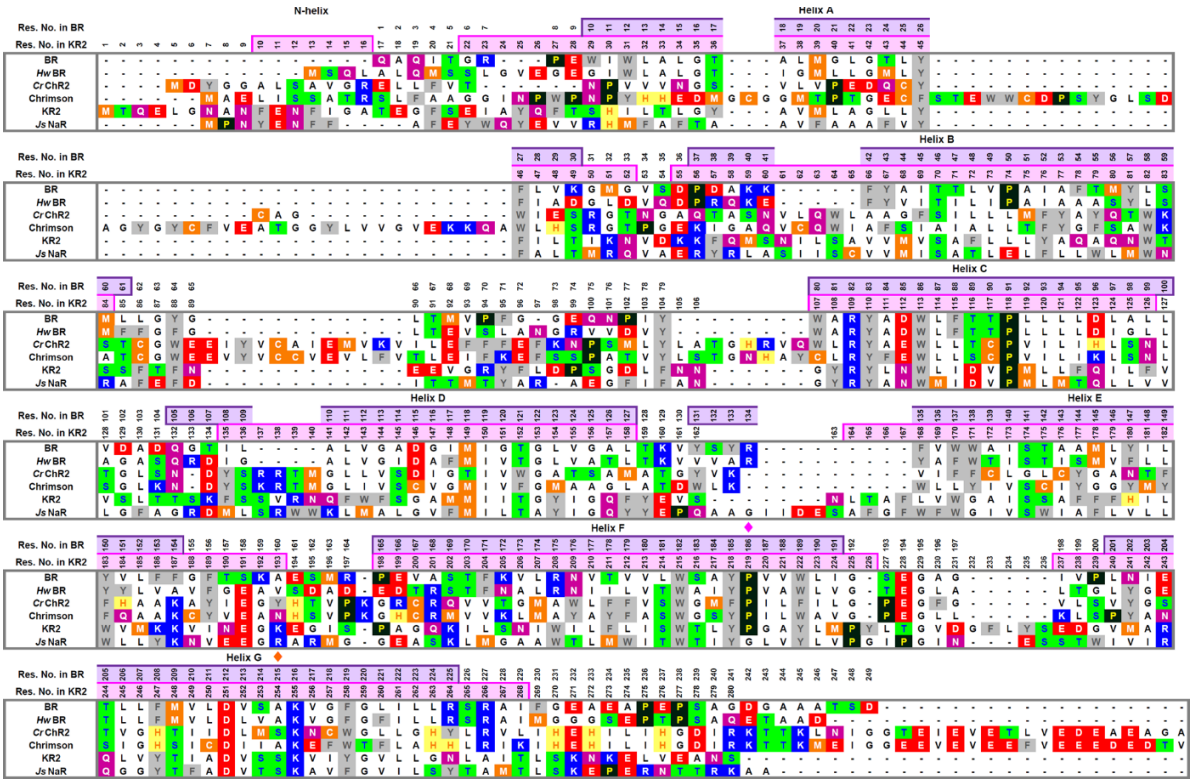
Dihedral/Sample	WT	P219T	S254A	P219T/S254A
C4-C5=C6-C7	172.46	171.92	172.50	171.49
C5=C6-C7=C8	-175.74	-174.40	-174.26	-175.44
C6-C7=C8-C9	173.97	173.45	173.98	173.14
C7=C8-C9=C10	-172.54	-173.27	-172.04	-173.40
C8-C9=C10-C11	170.81	170.76	171.61	170.12
C9=C10-C11=C12	-178.55	-177.45	-178.93	-177.27
C10-C11=C12-C13	162.81	162.35	161.32	160.18
C11=C12-C13=C14	-171.66	-172.00	-170.36	-169.90
C12-C13=C14-C15	151.20	151.39	150.02	149.41
C13=C14-C15=N	-178.92	-177.98	-176.10	-174.46
C14-C15=N-C_δ	159.55	159.86	157.89	152.10

Supplementary Table 5. **Total positive charge on the C11H-C12H-C13(Me)-C14H-C15H-NHR moiety (i.e., relevant to the C11=C12 isomerization).** In *parenthesis* we also give the charges corresponding to the shorter C13(Me)-C14H-C15H-NHR moiety (i.e., relevant to the C13=C14 isomerization).

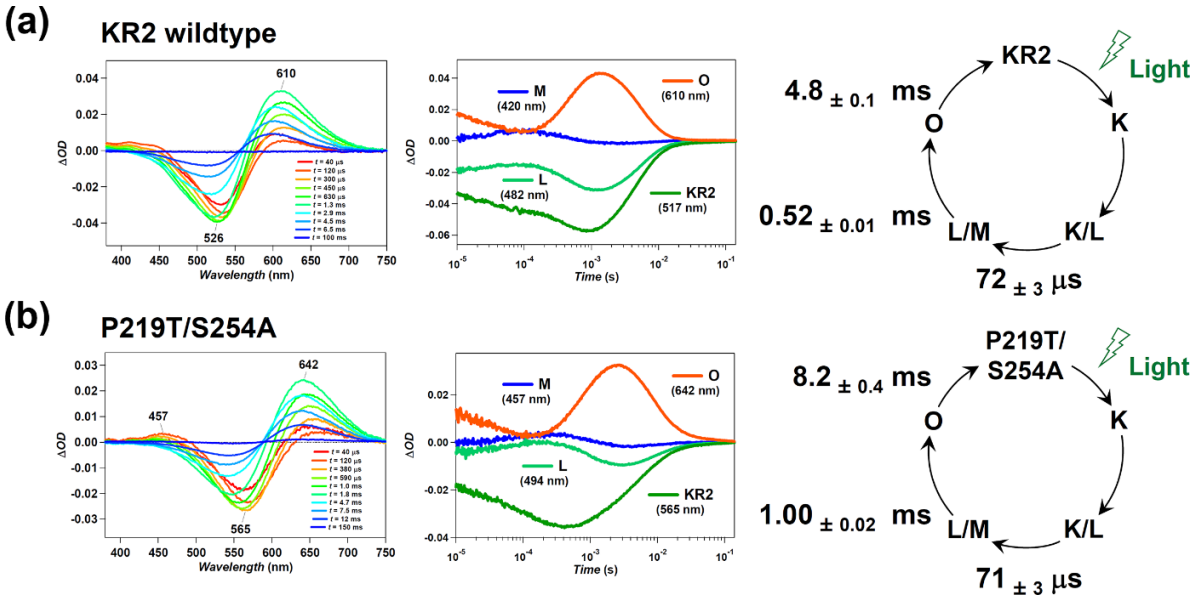
Protein	S_0 Charge (*e*)	S_1 Charge (*e*)	S_2 Charge (*e*)
KR2 WT	0.83 *(0.78)*	0.45 *(0.42)*	0.74 *(0.70)*
P219G	0.85 *(0.80)*	0.42 *(0.39)*	0.76 *(0.72)*
P219T	0.84 *(0.78)*	0.44 *(0.40)*	0.74 *(0.70)*
S254A	0.87 *(0.81)*	0.41 *(0.37)*	0.79 *(0.73)*
P219T/S254A	0.81 *(0.77)*	0.47 *(0.45)*	0.72 *(0.69)*

Supplementary Table 6. **List of primers used for the site-directed mutations.**

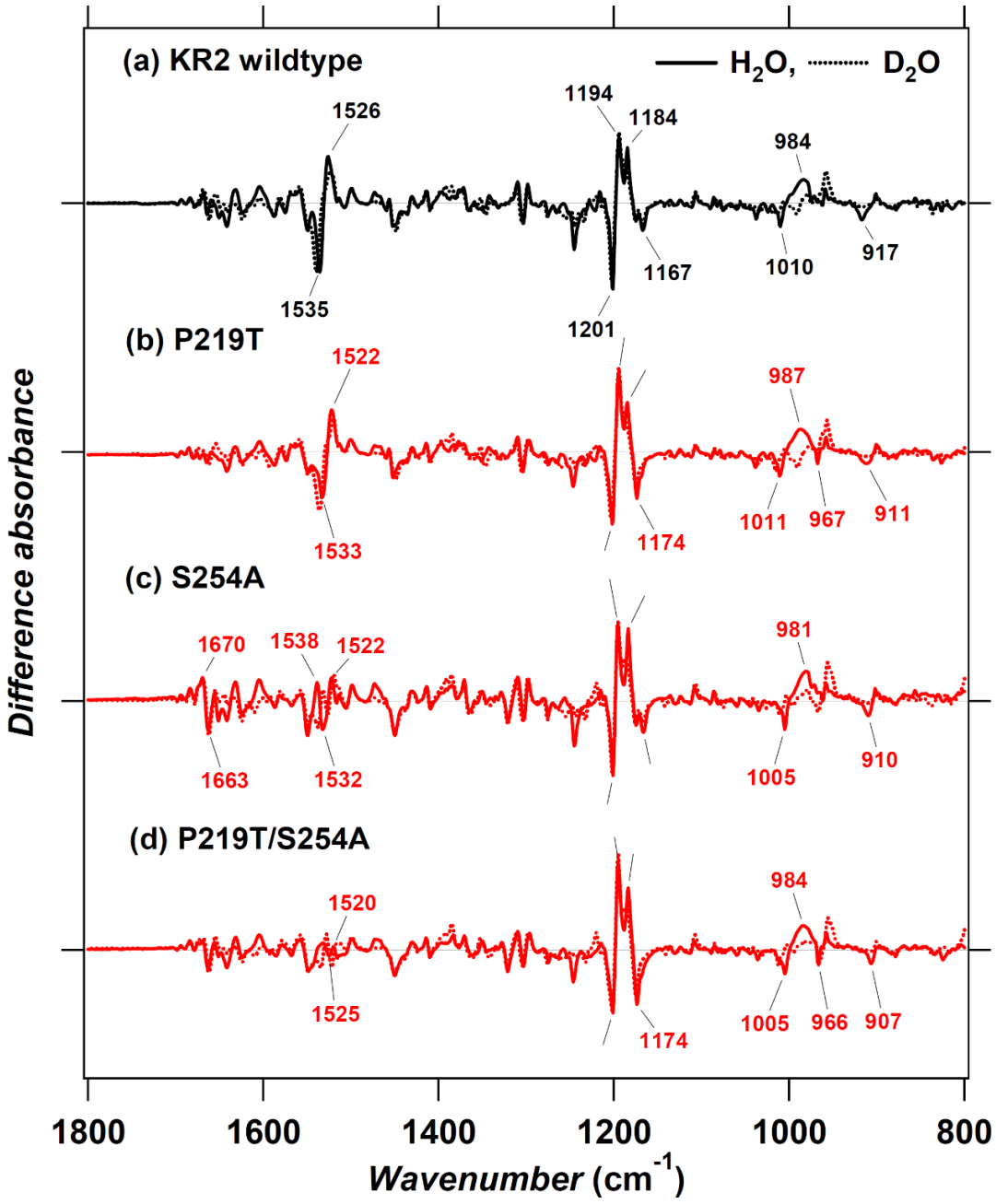
Mutation	Sense primer	Anti-sense primer
KR2 L168Y	CCTGACTGCGTTTTATGTCTGGGGAGCCATTAG	CTAATGGCTCCCCAGACATAAAACGCAGTCAGG
KR2 G171S	GCGTTTCTGGTCTGGAGCGCCATTAGCAGTGCG	CGCACTGCTAATGGCGCTCCAGACCAGAAACGC
KR2 F178Y	CATTAGCAGTGCGTTCTATTTTCACATTCTCTGG	CCAGAGAATGTGAAAATAGAACGCACTGCTAATG
KR2 G146S	CAGTTTTGGTTCTCAAGCGCCATGATGATCATTAC	GTAATGATCATCATGGCGCTTGAGAACCAAAACTG
KR2 M149S	GTTCTCAGGTGCCATGAGCATCATTACAGGC	GCCTGTAATGATGCTCATGGCACCTGAGAAC
KR2 I150T	CTCAGGTGCCATGATGACCATTACAGGCTATATC	GATATAGCCTGTAATGGTCATCATGGCACCTGAG
KR2 G153S	GCCATGATGATCATTACAAGCTATATCGGACAG	CTGTCCGATATAGCTTGTAATGATCATCATGGC
KR2 G156S	CATTACAGGCTATATCAGCCAGTTTTACGAAGTG	CACTTCGTAAAACTGGCTGATATAGCCTGTAATG
KR2 I181T	GCGTTCTTCTTTCACACCCTCTGGGTTATGAAG	CTTCATAACCCAGAGGGTGTGAAAGAAGAACGC
KR2 F211Y	CAACATCTGGATTCTGTATCTGATCTCTTGGACG	CGTCCAAGAGATCAGATACAGAATCCAGATGTTG
KR2 W215Y	CTGTTTCTGATCTCTTATACGTTGTACCCAGGTG	CACCTGGGTACAACGTATAAGAGATCAGAAACAG
KR2 P219T	CTCTTGGACGTTGTACACCGGTGCGTATTTAATG	CATTAAATACGCACCGGTGTACAACGTCCAAGAG
KR2 P219G	CTCTTGGACGTTGTACGGCGGTGCGTATTTAATG	CATTAAATACGCACCGCCGTACAACGTCCAAGAG
KR2 S254A	TGCAGATGTGTCGGCGAAAGTCATTTATG	CATAAATGACTTTCGCCGACACATCTGCA
JsNaR G216P	CACCTGGACCATTTACCCGCTGGTGTATCTGGTTC	GAACCAGATACACCAGCGGGTAAATGGTCCAGGTG
JsNaR S247A	CTTTGCGGACGTTACCGCGAAAGCGGTGTTCGGC	GCCGAACACCGCTTTCGCGGTAACGTCCGCAAAG
NpHR P226T	CATGTGGCTCGGCTACACCATCGTGTGGGCACTC	GAGTGCCCACACGATGGTGTAGCCGAGCCACATG
PoXeR P179T	GTTTTGGGTTAGCTATACCCTGGTTTGGCTGATC	GATCAGCCAAACCAGGGTATAGCTAACCCAAAAC



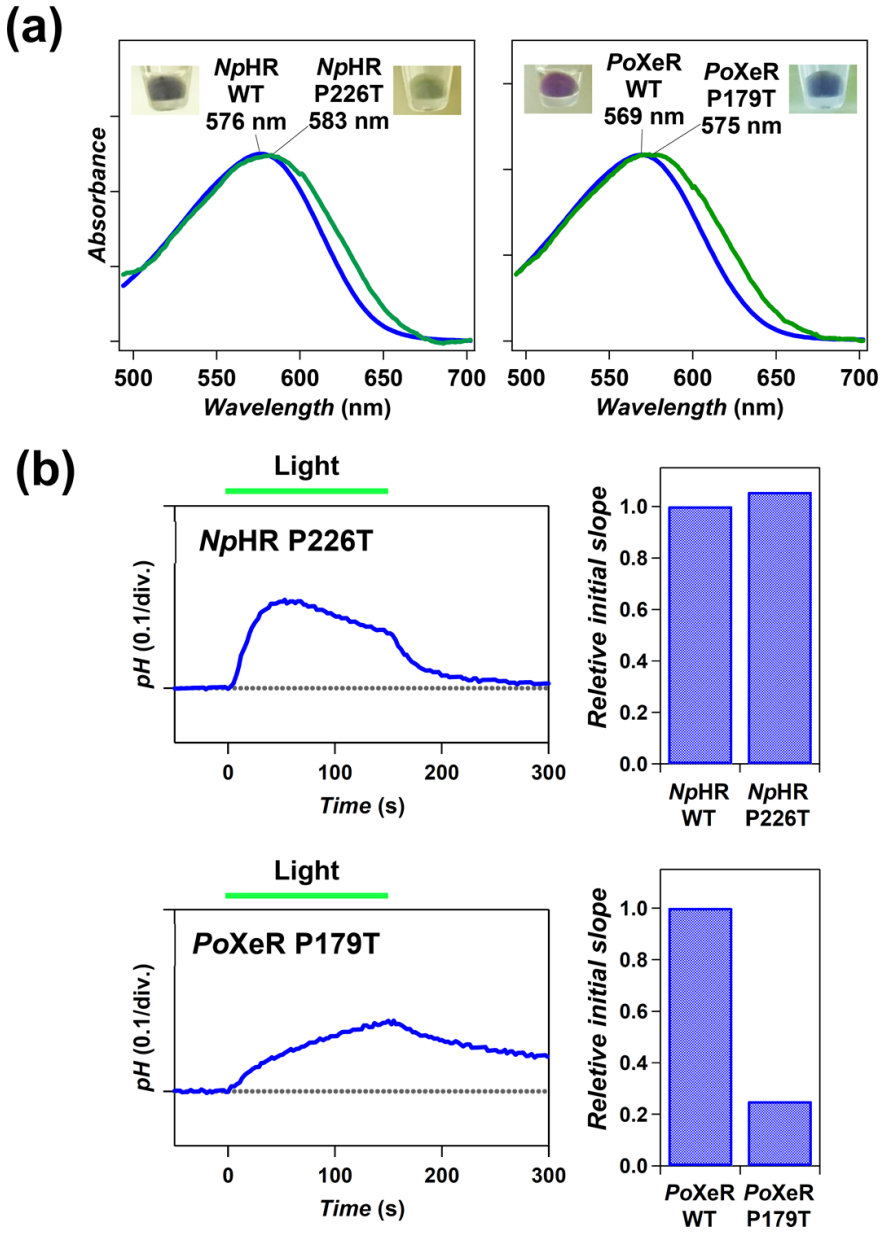
Supplementary Figure 1. Multiple alignment of amino acid sequences of microbial rhodopsins. The amino acid sequences were aligned by ClustalW[1] for BR, BR of *Haloquadratum walsbyi* (*Hw*BR), ChR2 without the C-terminal sequence, Chrimson, KR2 and *Js*NaR. The residue numbers (Res. No.) in BR and KR2 are shown in the first and second row, respectively. Transmembrane helices of BR and KR2 were indicated by purple and pink rectangles, respectively, according to their X-ray crystallographic structures ((PDB code: 1M0L (BR)[2] and 3X3C (KR2)[3]). The positions of KR2 Pro219 and Ser254 are indicated by pink and orange diamonds, respectively.



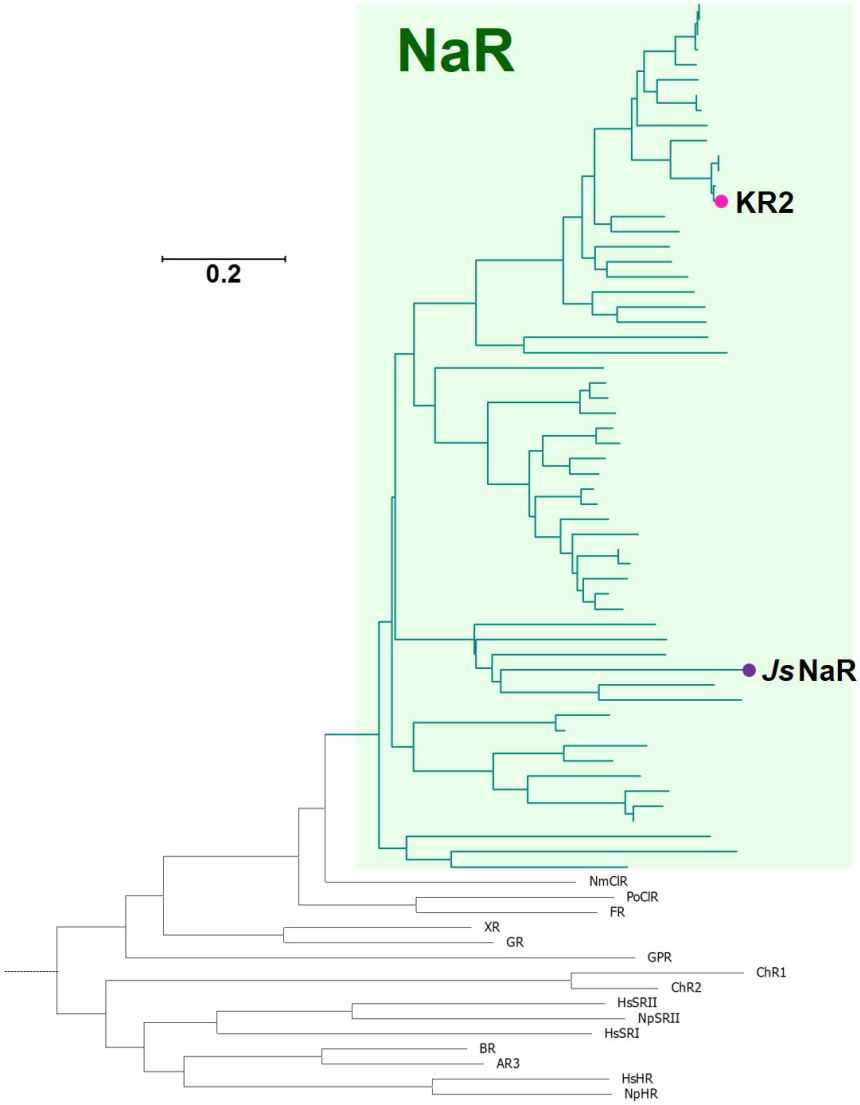
Supplementary Figure 2. The photocycles of KR2 mutants. Transient absorption spectra (left), the time evolutions of transient absorption change at specific wavelengths (middle) and the photocycles determined by the curve fitting with multi-exponential functions (right) of KR2 WT (a) and P219T/S254A (b), in POPE/POPG liposome (molar ratio = 3 : 1) with a protein-to-lipid molar ratio of 1:50. The lifetimes and their standard deviations of the photo-intermediates in are indicated in the photocycles. The green, light-green, blue, and orange lines in (b) show the photo-bleaching of the initial sate, accumulations of L-, M- and O-intermediates, respectively. Source data are provided as a Source Data file



Supplementary Figure 3. Light-induced infrared absorption changes of KR2 mutants. Light-induced FTIR difference spectra of (a) KR2 WT (black) and the mutants (red), (b) P219T, (c) S254A and (d) P219T/S254A, in the 1800-800 cm^{-1} region at T = 77 K and pH 8.0. Solid and dotted lines represent the samples hydrated with H_2O and D_2O, respectively. Source data are provided as a Source Data file



Supplementary Figure 4. The effect of mutation at the proline corresponding to KR2 Pro219 in the chloride pump (*Np*HR) and inward proton pump (*Po*XeR) rhodopsins. (a) Absorption spectra of *Np*HR WT, P226T, *Po*XeR WT and P179T. The wildtype and mutant proteins are indicated in blue and green, respectively. (b) Ion pump activity assays by monitoring pH changes in the external media of the suspension of *E. coli* cells expressing *Po*XeR P179T and *Np*HR P226T (left) and the relative initial slopes of each to the wildtype (right). The light was illuminated at t = 0-150 s indicated by light-green bars. Source data are provided as a Source Data file



Supplementary Figure 5. Phylogenetic relationship of KR2 and *Js*NaR in the microbial rhodopsin sub-family. NaRs are indicated with green branches. The evolutionary history was inferred using the Neighbor-Joining method[4]. The optimal tree with the sum of branch length = 17.62164409 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method[5] and are in the units of the number of amino acid substitutions per site. The analysis involved 73 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 393 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[6]. KR2 and *Js*NaR are indicated by pink and purple circles, respectively.

Supplementary References

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