Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin-2

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Although channelrhodopsin (ChR) is a widely applied light-activated ion channel, important properties such as light adaptation, photocurrent inactivation, and alteration of the ion selectivity during continuous illumination are not well understood from a molecular perspective. Herein, we address these open questions using single-turnover electrophysiology, time-resolved step-scan FTIR, and Raman spectroscopy of fully dark-adapted ChR2. This yields a unifying parallel photocycle model integrating now all so far controversial discussed data. In dark-adapted ChR2, the protonated retinal Schiff base chromophore (RSBH\textsuperscript{+}) adopts an all-trans, C=Н-anti conformation only. Upon light activation, a branching reaction into either a 13-cis, C=N-anti or a 13-cis, C=N-syn retinal conformation occurs. The anti-cycle features sequential H\textsuperscript{+} and Na\textsuperscript{+} conductance in a late M-like state and an N-like open-channel state. In contrast, the 13-cis, C=N-syn isomer represents a second closed-channel state identical to the long-lived \textit{P}_{480} state, which has been previously assigned to a late intermediate in a single-photon model. Light excitation of \textit{P}_{480} induces a parallel syn-photocycle with an open-channel state of small conductance and high proton selectivity. \textit{E}\textsubscript{90} becomes deprotonated in \textit{P}_{480} and stays deprotonated in the C=N-syn cycle. Deprotonation of \textit{E}\textsubscript{90} and successive pore hydration are crucial for late proton conduction following light adaptation. Parallel anti- and syn-photocycles now explain inactivation and ion selectivity changes of ChR2 during continuous illumination, fostering the future rational design of optogenetic tools.

Significance

Understanding the mechanisms of photoactivated biological processes facilitates the development of new molecular tools, engineered for specific optogenetic applications, allowing the control of neuronal activity with light. Here, we use a variety of experimental and theoretical techniques to examine the precise nature of the light-activated ion channel in one of the most important molecular species used in optogenetics, channelrhodopsin-2. Existing models for the photochemical and photophysical pathway after light absorption by the molecule fail to explain many aspects of its observed behavior, including the inactivation of the photocurrent under continuous illumination. We resolve this by proposing a branched photocycle explaining electrical and photocchemical channel properties and establishing the structure of intermediates during channel turnover.

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C₁ to O₁ compared with that from C₁ to O₂, consistent with recent theoretical calculations (14).

Time-resolved FTIR spectroscopy was originally established as a powerful approach for the determination of the molecular reaction mechanism of BR (19). Accordingly, the dark-adapted ChR2 photocycle was recorded between 50 ns and 140 s after exposure to a light pulse by step-scan and rapid-scan FTIR. These measurements revealed an ultrafast all-trans to 13-cis isomerization and subsequent deprotonation of the RSBH⁺ in parallel with protonation of the counter-ion residues E123 and D253 (18). Deprotonation of D56 coincides with P₃₉₀ depletion, which was previously considered as indicative of RSB deprotonation (17, 18). FTIR studies paired with HPLC analysis of the slow-cycling step-function variant C₁₂₈T provided spectroscopic evidence for two distinct closed states with different retinal isomers (20). NMR-spectroscopic data of the ChR2 (WT) and WT-like variant H₁₃₄R showed that although different closed states exist, the fully dark-adapted state [called the initial dark-adapted state (IDA)] of ChR2 is composed of 100% all-trans, C = N-anti retinal (21, 22). Raman experiments on ChR2-H₁₃₄R revealed that illumination of the IDA at 80 K produced an apparent dark state (DA’app) containing a second retinal isomer (22). Following double isomerization around the C₁₃ = C₁₄ and the C = N double bonds, 13-cis, C = N-syn retinal is formed, and this was proposed as the transformation step for forming the second “metastable” dark state (22). Both retinal isomers in the DA’app were proposed to initiate distinct photocycles, with both involving homologous P₅₂₀, P₃₉₀, P₂₂₀, and P₄₈₀-like intermediates.

The central gate residue E₉₀ is one of the key determinants of proton selectivity in ChR2 (16, 18, 23) and related cation-conducting ChRs (24). During the photocycle, E₉₀, which is located in the central gate in the middle of the putative pore, is deprotonated and remains deprotonated until P₅₈₀ decays (16–18). From experiments with high laser pulse repetition frequencies preventing complete dark adaptation, a late deprotonation of E₉₀ exclusively in P₅₈₀ was proposed for ChR2 (17). In contrast, E₉₀ deprotonation within submicroseconds after light excitation was observed in single-turnover experiments on fully dark-adapted ChR2 (18). Thus, there seemed to be a controversy between fully dark-adapted and non–dark-adapted FTIR experiments on the timing of E₉₀ deprotonation in a single photocycle model.

Here, we present a unifying functional study of dark- and light-adapted ChR2 by integrating single-turnover electrical recordings and FTIR measurements on ChR2, Raman spectroscopy with ⁱ³C-labeled retinal, and molecular dynamics (MD) simulations. The controversies observed between single-turnover experiments and recordings under continuous illumination are resolved by developing an extended model, including two parallel photocycles with C = N-anti and C = N-syn retinal conformations. The light-adapted 13-cis, C = N-syn state is the P₅₂₀ intermediate, which was formerly assigned to the last intermediate of the anti-cycle in a linear photocycle model. Within the anti-cycle, ion conductance evolves in two subsequent steps, resulting in two different conductive states of distinct ion selectivity (O₁-early and O₁-late). Interestingly, E₉₀ stays protonated in the anti-cycle. In contrast, the syn-cycle initiated by photoexcitation of P₄₈₀, which represents the second C₁ in Fig. 1B, comprises a third slowly decaying O₂ of high proton selectivity but low overall ion conductance. Conductance of O₂ depends on deprotonation of E₉₀ and is completely abolished in the ChR2 E₉₀Q mutant. Our results resolve the former discrepancies. In the anti-photocycle, E₉₀ stays protonated and channel opening of O₁-early and O₁-late is observed, whereas in the syn-cycle, including P₅₂₀, E₉₀ is deprotonated and favors proton conductance of O₂.

Results

Single-Turnover Patch-Clamp Recordings Identify Three Conducting ChR2 States. To examine functional changes during light adaptation of ChR2, we recorded single-turnover photocurrents in HEK293 cells following 7-ns laser excitation before and after light adaptation. We addressed changes in ion selectivity by reducing either the extracellular sodium (110 mM → 1 mM) or extracellular proton concentration (pH 7.2 → pH 9.0) (Fig. 2A and B).

Under symmetrical sodium and proton concentrations, the dark-adapted ChR2 pore opens biexponentially with two almost voltage-independent time constants (150 μs and 2.5 ms). The photocurrents decline, with a dominant voltage-dependent time constant of 10–22 ms and a second, minor, slow time constant of 70–220 ms (Fig. 2A and B, Top), in general agreement with previous reports (11). Decreasing extracellular Na⁺ not only reduces inward-directed photocurrent amplitudes but also affects the temporal evolution of inward currents (Fig. 2B, Middle). Whereas inward currents in low extracellular Na⁺ are predominantly carried by protons (H⁺ flux), inward currents under symmetrical conditions are mediated by both H⁺ and Na⁺ ions. Subtraction of photocurrents at high and low Na⁺ at pH 7.2 allows an approximation of the pure inward Na⁺ flux (Fig. 2C). Strikingly, the proton flux peaks as early as 300 μs after excitation, significantly earlier than Na⁺ flux (2.5 ms). This observation is indicative of two open states with distinct ion selectivity following single excitation of dark-adapted ChR2.

During continuous illumination, photocurrents peak within milliseconds (dependent on the light intensity) and subsequently decline to a stationary level. Inactivation is more pronounced at positive voltages, contributing to the increased inward rectification of stationary photocurrents compared with the initial peak current (3). After light adaptation, laser pulse-induced photocurrents are significantly reduced in amplitude. The photocurrent still rises and decays biexponentially, however, reaching a maximal amplitude at 100 ms after excitation (Fig. 2D). However, notably, relative photocurrent changes differ at early time points (<200 μs) and during the slow photocurrent decline (after 50–100 ms), indicating different open-state conformations in the early and late stages of the photocycle of light- and dark-adapted ChR2. In particular, at 100 ms after the laser excitation, the photocurrents even increase in amplitude for light-adapted ChR2 (especially at pH 9.0), indicative of an additional slowly decaying open state. In summary, there are at least three different conductive states: O₁-early and O₁-late in the dark-adapted photocycle and O₂ after light adaptation.

The proton versus sodium selectivity of all three open states is analyzed at three different time points after the actinic laser pulses (0.1 ms, 5 ms, and 100 ms; Fig. 2E). Although the photocurrents at 0.1 ms and 100 ms are barely distinguishable in high and low extracellular Na⁺ in either the dark- or light-adapted
protein, reduction of the extracellular proton concentration causes a strong shift in reversal potential and an increase in outward-directed photocurrent amplitudes that is even more pronounced in the light-adapted channel than in the dark-adapted channel. In contrast, 5 ms after excitation of photocurrents, both ionic changes, a reduction in extracellular sodium or proton concentration, shift the reversal potential and decrease the inward photocurrent amplitude. We conclude that after channel opening, the short-lived highly proton-selective O1\text{early} is followed by the more Na\textsuperscript{+}-selective but still highly proton-permeable O1\text{late}. After multiphoton excitation and light adaptation, the contribution of O1\text{early} and O1\text{late} decreases in favor of the third highly proton-selective O2\text{z}, which, although small in amplitude, significantly contributes to stationary photocurrents at alkaline pH due to its long lifetime.

**Single-Turnover Time-Resolved FTIR Measurements Reveal a Splitting of the Photocycle After Light Activation of Fully Dark-Adapted ChR2.**

The single-turnover electrophysiology data recall that dark adaptation and light adaptation need to be compared thoroughly for the correct interpretation of time-resolved measurements. However, most time-resolved spectroscopy studies are performed with barely dark-adapted samples at rather high repetition rates to avoid long measurement times. To elucidate the underlying molecular mechanism of the observed channel-gating transitions and different ion conductance, we performed single-turnover time-resolved FTIR measurements of the fully dark-adapted ChR2 WT-like H134R variant with a time resolution of 50 ns over nine orders of magnitude (Fig. 3A), similar to our data from 2015 (18). The ChR2 WT-like H134R mutant shows higher protein expression in *Pichia pastoris* compared with the WT protein and has been used for the examination of light adaptation before (22). Electrical properties and photocycle kinetics are comparable, although slightly slower than those of the WT protein (25), and the same IR bands are observed in WT and in H134R. However, some crucial IR marker bands are more pronounced in H134R, which simplifies the presentation of the data set. Dark adaptation of D\textsubscript{390} was achieved by long dark periods of 140 s between pulsed excitation (temperature = 15 °C), which increased the advanced step-scan measurement time to about 4 wk (18), whereas light-adapted samples take a few hours only (17). The appearance of the marker band at 1188 cm\textsuperscript{-1} (not time-resolved) indicates the all-trans to 13-cis,C=C-N\textsuperscript{anti} isomerization because it represents the C\textsubscript{15}C\textsubscript{16} stretching vibration of 13-cis retinal as originally assigned in BR by site-specific isotopic labeling (26). The decay of the 1,188 cm\textsuperscript{-1} marker band within a microsecond (green line in Fig. 3A) indicates the formation of the M-like P\textsubscript{390} intermediate with a deprotonated RSB (18). As in other microbial rhodopsins, the subsequent rise and decay of the N-like P\textsubscript{320} intermediate with a deprotonated Schiff base can be monitored by its reappearance and the decay reflects formation of all-trans isomer on the time scale of a few milliseconds (19). Comparing the time course of this marker band (1,188 cm\textsuperscript{-1}) with the single-turnover electrical measurements, we can now assign the described conducting states O\textsubscript{1}\text{early} and O\textsubscript{1}\text{late} to the last part of P\textsubscript{390} (M\textsubscript{2}) and P\textsubscript{320} (N), respectively, which is in line with earlier reports on the WT protein (11, 18). Due to these similarities and the abundance of spectroscopic data on BR, we decided to name the ChR2 intermediates as follows: P\textsubscript{390}\textsuperscript{K}, P\textsubscript{390}\textsubscript{M\textsubscript{1}}, P\textsubscript{390}\textsubscript{M\textsubscript{2}}, and P\textsubscript{320}\textsuperscript{N}. Global fitting of the whole dataset (solid lines, Fig. 3A) describes the data adequately. The apparent rate constants of H134R are similar to those of earlier reports for the dark-adapted ChR2 WT (18).

**Light-Induced Splitting in 13-cis,C\textequiv\textsuperscript{C}=N\textsuperscript{anti} and 13-cis,C\textequiv\textsuperscript{C}=N\textsuperscript{syn} RSB\textsuperscript{H} Conformations.** Interestingly, an additional retention band at an unusual low wavenumber, 1,154 cm\textsuperscript{-1}, appears parallel to the 1,188-cm\textsuperscript{-1} 13-cis,C\textequiv\textsuperscript{C}=N\textsuperscript{anti} marker band (not time-resolved). Because...
the low-wavenumber band is more pronounced in H134R than in WT (SI Appendix, Supplementary Notes 1 and 3). We discuss the data here for the reader on the mutant, although the results are also valid for the WT. The 1,154-cm\(^{-1}\) band persists from nanoseconds to seconds after a single pulse of excitation light (Fig. 3A). The band is assigned here to the C14-C15 stretching vibration of retinal because of the characteristic 14-cm\(^{-1}\) downshift upon retinal \(1^{3}C_{14-15}\) carbon-specific labeling (Fig. 3B, upper part). The band assignment is confirmed by additional Raman experiments (SI Appendix, Fig. S6). The 22-cm\(^{-1}\) upshift of the C14-C15 band in D2O indicates a 13-cis\(\rightarrow\)N-syn conformation (Fig. 3B, lower part). In 13-cis\(\rightarrow\)N-syn retinal, the C14-C15 stretching vibration is strongly coupled to the N-H bending vibration, which is decoupled in D2O (N-D) and results in a deuteration-induced large upshift in the syn-conformation, but not in the anti-conformation (27, 28). Therefore, the band at 1,154 cm\(^{-1}\) represents a 13-cis\(\rightarrow\)N-syn marker band. The band assignments are confirmed by extended Raman experiments shown in SI Appendix in more detail for P480 (SI Appendix, Supplementary Notes 1 and 3–5, Figs. S1–S6, and Table S1).

The negative difference bands in Fig. 3B reflect vibrations of dark-adapted ChR2 WT (D470). The negative band at 1,186 cm\(^{-1}\) is also assigned to the C14-C15 stretching vibration of D470 because of the characteristic isotope downshift. From the additional analysis of the D470 Raman spectrum (SI Appendix, Supplementary Notes 3 and 4), we conclude that the retinal of dark-adapted ChR2 is in a 100% all-trans\(\rightarrow\)N-syn conformation, in agreement with NMR data (21, 22). A detailed band assignment of the P480 and D470 vibrational spectra and retinal conformations is provided in SI Appendix, Supplementary Notes 1 and 3–5, Figs. S1–S7, and Table S1.

The parallel but temporally unresolved appearance of the bands at 1,188 cm\(^{-1}\) and 1,154 cm\(^{-1}\) in single-turnover experiments in Fig. 3A indicates that light absorption induces parallel isomerization of all-trans\(\rightarrow\)N-anti retinal in D470 into either a 13-cis\(\rightarrow\)N-anti or a 13-cis\(\rightarrow\)N-syn conformation. The splitting ratio into parallel syn- and anti-pathways can be estimated as 1:1 under our measurement conditions (SI Appendix, Fig. S3).

Considering that the 13-cis\(\rightarrow\)N-syn isomerization occurs in parallel to the 13-cis\(\rightarrow\)N-anti isomerization, we conclude that the 13-cis\(\rightarrow\)N-syn retinal conformation observed in P480 is therefore not the last intermediate of the 13-cis\(\rightarrow\)N-anti photocycle, as proposed in the single-cycle model. P480 reflects a long-lived 13-cis\(\rightarrow\)N-syn state, which appears in parallel to 13-cis\(\rightarrow\)N-anti state instantaneously.

The conclusion that the P480 is not the last intermediate of the 13-cis\(\rightarrow\)N-anti single photocycle but appears in parallel to the light-activated state is furthermore strongly supported by detailed Raman experiments, as described in SI Appendix. The Raman results are in agreement with former Raman studies on light adaptation (22). Upon complete light adaptation of ChR2 due to long illumination periods, a P480 state evolves. It is composed of a 40:60 mixture of the all-trans\(\rightarrow\)N-anti species in D470 and the 13-cis\(\rightarrow\)N-syn species in P480 (SI Appendix, Fig. S3). The bands observed in the Raman spectra of D470 and P480 correlate with retinal bands seen in the IR difference spectra in Fig. 3B and are in agreement with the published Raman spectra of the all-trans\(\rightarrow\)N-anti and 13-cis\(\rightarrow\)N-syn bands of the D470 state (22) (SI Appendix, Supplementary Note 3). The Raman data confirm the ultrafast C\(\rightarrow\)N-syn formation in P480 as seen in Fig. 3A at 1,154 cm\(^{-1}\).

**E90 Deprotonates upon 13-cis\(\rightarrow\)N-syn Formation.** The E90-deprotonation marker band (1,718 cm\(^{-1}\)) (18) and the C\(\rightarrow\)N-syn marker band (1,154 cm\(^{-1}\)) (Fig. 3A) appear instantaneously, and are not time-resolved. Both marker bands persist alongside the dark-adapted anti-cycle intermediates (P\(\rightarrow\)K\(\rightarrow\)P(E90\(\rightarrow\)K)\(\rightarrow\)P) in D2O (N-D) and are not time-resolved. Both marker bands persist alongside the dark-adapted anti-cycle intermediates (P\(\rightarrow\)K\(\rightarrow\)P(E90\(\rightarrow\)K)\(\rightarrow\)P) in D2O.
FTIR Amplitude Spectra Indicate Structural Differences of O₁ and O₂.

The O₁ and O₂ FTIR amplitude spectra of the 30-ms and 250-ms apparent rates are shown in Fig. 4C. Both decay-associated amplitude spectra exhibit negative D₁70 marker bands (Fig. 4C and SI Appendix, Figs. S8 and S10), indicating a direct transition from the anti- and syn-photocycles into the all-trans,C=N-anti configuration of D₁70.

The t₁/2 = 30-ms FTIR decay-associated amplitude spectrum of the C=N-anti cycle exhibits carbonyl bands at 1,760 cm⁻¹ (+)/1,736 cm⁻¹ (−) and 1,728 cm⁻¹ and 1,695 cm⁻¹, which were assigned to protonation of the counter-ion D₂53 (18, 29) (1,728 cm⁻¹) and deprotonation of D₁56 (1,736 cm⁻¹). Furthermore, the helix hydration marker bands at 1,662 cm⁻¹ (−)/1,650 cm⁻¹ are present, which are now assigned to both O₁cuddy and O₁late. In the t₁/2 = 250-ms decay-associated amplitude spectrum, all carbonyl bands are strongly reduced, including the bands of the Schiff base proton acceptor D₂53 (at 1,728 cm⁻¹), as well as D₁56 (1,736 cm⁻¹), which has been proposed to be the RSB reprotonation donor (17). Because the Schiff base deprotonation is not observed, the corresponding counter-ion D₂53 protonation is not seen either. Also, reprotonation of D₁56 base deprotonation is not observed, the corresponding counter-ion is designated the P*520 intermediate to distinguish it from the P520. Because the Schiff base isomerization was changed from the dark-adapted all-trans,C=N-anti conformation (Fig. 5C, Left) to either a 13-cis,C=N-anti single isomerization (SI Appendix, Supplementary Note 7 and Fig. S13 A and B) or a 13-cis,C=N-syn double isomerization (Fig. 5C, Right and SI Appendix, Fig. S13C). The observed changes in hydrogen bond interaction and water distribution are shown in Figs. S B and C and SI Appendix, Figs. S12–S14. It is noteworthy that the single isomerization induces an upward orientation of the RSB proton, whereas the position of the RSB proton is only slightly changed in the double isomerization (16, 22) (Fig. 5A). Starting from the WT structure, E90 keeps its initial downward orientation in the dark-adapted state (Fig. 5 B and C). Very recently, a more advanced method to perform such isomerization simulations was introduced by Ardevol and Hummer (31). They simulated a homology model of ChR2 based on the C121 chimera crystal structure (PDB ID code 3UG9) (32) and obtained a downward flip of the initially upward-oriented E90. We have already observed the same downward movement in our model based on the same crystal structure using a classical approach (18). This proves that even our classical approach correctly predicts alterations of the hydrogen bond pattern of E90 due to retinal isomerization. It seems that E90 is trapped in a local minimum in both models but finds the correct position for ChR2 [as observed in the PDB ID code 6eid crystal structure (30)] after disturbance by isomerization.

Following 13-cis,C=N-syn double isomerization, helices 2 and 7 stay connected via E90 and D253 as long as E90 remains protonated (SI Appendix, Fig. S12). Deprotonation of E90 leads to an alternative contact between E90 and K93 (Fig. 5 B and C and SI Appendix, Fig. S12) that opens the central gate and results in an influx of water molecules into the pore (Fig. 5C). This influx is in agreement with the channel opening due to E90 deprotonation proposed formerly in the E90-Helix2-tilt (EHT) model in the 13-cis,C=N-anti conformation (18). We now attribute E90 deprotonation and pore hydration to the light-adapted closed-state Pₘₐₓ. In this light-adapted state, the inner gate still remains closed and ion permeation is hindered, in agreement with the electrophysiology results (SI Appendix, Fig. S14). As 13-cis,C=N-syn isomerization accumulates during light adaptation, we could attribute E90 deprotonation and pore hydration to the light-adapted closed-state Pₘₐₓ.
previous results of steady-state measurements (16, 33), the E90Q mutation reduces proton conductance of $O_{1\text{dark}}$ of the anti-cycle. Accordingly, upon reduction of extracellular sodium, photocurrent amplitudes are more decreased compared with the WT (Fig. 6B), and the reversal potential shifts are larger 2 ms after excitation (Fig. 6C and SI Appendix, Fig. S11C). In addition to the effect on the anti-cycle, the E90Q mutation completely abolishes the late photocurrent increase upon light adaptation, which was observed in the WT channel (SI Appendix, Fig. S11D). Instead, slow photocurrents are reduced in the E90Q mutant following continuous illumination (Fig. 6 D and E). This indicates that E90 facilitates proton conductance of $O_2$ by deprotonation, rendering it completely impermeable in the E90Q mutant. The results on the E90Q mutation validate our photocycle model with a parallel syn-cycle that involves E90 deprotonation and populates during light adaption.

**Discussion**

Microbial rhodopsins are excellent optogenetic tools (1–4). Understanding of their detailed mechanisms is catalyzed by earlier extended studies on BR providing detailed insight on how tiny light-induced protein alterations induce a proton transfer by an interplay of catalytic key residues and clusters of protein-bound water molecules along the proton transfer pathway (5–7). This detailed understanding paved the way for studies of several microbial rhodopsins, like halorhodopsins, sensory rhodopsins, and especially ChRs. In the present work, we combined single laser pulses and continuous or repetitive illumination in an advanced biophysical approach to analyze the fully dark- and light-adapted ChR2 in single-turnover electrophysiological recordings, time-resolved FTIR, and resonance Raman spectroscopic measurements, complemented by MD simulations. We verified early branching into two parallel photocycles with distinct retinal isomerization and alternative configurations of the central gate, and elaborated a unifying photocycle model shown in Fig. 7 that addresses light adaptation and temporal changes in cation conductance on a functional and molecular level.

Following longer dark periods, the IDA comprises only $D_{250}$ containing 100% all-trans, C=C=N-anti retinal, which is in agreement with the findings in earlier studies.

**Fig. 5.** Retinal conformations and formation of $P_{ \text{abs}}$. (A) Modeled representation of the calculated retinal configurations. The Schiff base orientations in the $D_{250}$ structure all-trans, C=C=N-anti (gray) and in the modeled 13-cis, C=C=N-anti (green) and 13-cis, C=C=N-syn (blue) retinal structures are shown. (B) Overview of $E_90$ hydrogen bond pattern for five independent simulations, with two monomers forming one dimer based on the ChR2 WT crystal structure [PDB ID code 6EID (30)]. Bars indicate the frequency of the respective hydrogen bond (percentage) during the 100-ns simulation. (C) Representative structure of the simulations is depicted. (Left) $D_{250}$ dark state. (Right) Structure after all-trans, C=N-anti $\rightarrow$ 13-cis, C=C=N-syn double isomerization and $E_90$ deprotonation ($P_{480}$). After deprotonation of $E_90$, the central gate opens and water invades.

**Fig. 6.** Proton and sodium conductance of the dark- and light-adapted ChR2 mutant E90Q. (A) Representative photocurrents of ChR2-E90Q with intracellular 110 mM NaCl and pH 7.2 and extracellular 110 mM Na$^+$ and pH 7.2 (Top), 1 mM Na$^+$ and pH 7.2 (Middle), 110 mM Na$^+$ and pH 9.0 (Bottom) at different holding voltages as indicated. Photocurrents were excited before and after light adaptation by 7-nm laser pulses of 470-nm wavelength light. For light adaptation, cells were illuminated for 500 ms with continuous 470-nm light. (B) Time evolution of estimated proton and sodium fluxes in the dark-adapted protein at $-60$ mV either directly measured in extracellular 1 mM Na$^+$ and pH 7.2 ("$H^+$ current") or calculated by subtraction of proton fluxes from combined inward flux of sodium and protons measured in symmetrical conditions ("Na$^+$ current") ($\nu$ [110 mM Na$^+$ (pH 7.2)] $\rightarrow$ $1$ [1 mM Na$^+$ (pH 7.2)]), mean $\pm$ SEM; WT: $n = 7$, E90Q: $n = 6$). (C) Reversal potential shift ($\Delta E_{\text{rev}}$) 2 ms after laser light excitation of the dark-adapted protein upon reduction of extracellular sodium (110 mM NaCl $\rightarrow$ 1 mM NaCl) or proton (pH, 7.2 $\rightarrow$ pH 9.0) concentration (mean $\pm$ SD; E90Q: $n = 5$–6; WT: $n = 6$–7; corrected for liquid junction potentials). (D) Equally scaled representative photocurrents of ChR2 WT and E90Q at $+30$ mV and extracellular 110 mM Na$^+$ and pH 9.0. (E) Normalized, log-binned, and averaged photocurrents of the dark-adapted (DA) or light-adapted (LA) WT and E90Q at $+30$ mV and extracellular 110 mM Na$^+$ and pH 9.0 (mean $\pm$ SEM; WT: $n = 6$, E90Q: $n = 5$).
with previous reports (21, 22). The inner gate and the closed gate are closed, and interhelical hydrogen bonding of D253 with the RSBH\(^+\) and the protonated E90 (31) prevents the invasion of water molecules from the extracellular bulk phase. After illumination of \(D_{270}\) (C\(_1\)), early branching of the photocycle due to an alternative retinal single or double isomerization occurs.

In the classical reaction path starting from \(D_{270}\) (designated the anti-cycle) all-trans, C=N=N-anti retinal isomerizes to 13-cis, C=N=N-anti, leading to P\(_{500}\) \(^K\), deprotonation of the RSBH\(^+\) in P\(_{390}\) \(^M\) and reprotonation of the RSB in P\(_{390}\) \(^N\), and direct monoeponential recovery of \(D_{270}\). Channel opening occurs at the UV/VIS silent transition from P\(_{390}\) \(^M1\) to P\(_{900}\) \(^M2\) in two subsequent steps (11) that we can now attribute to different ion selectivities pinpointing different pore conformations. Whereas during P\(_{900}\) \(^M2\), the short-lived O\(_1\)-early conducts almost exclusively protons, photocurrents of O\(_1\)-late, which evolve upon reprotonation of the RSB and formation of P\(_{520}\) \(^N\), are also carried by cations. Curiously, we observe a small positive charge displacement during P\(_{520}\) \(^N\) at 0 mV and under symmetrical ionic conditions (Fig. 2) that could result from an outward-directed proton displacement following reprotonation of the RSB. This might reflect the earlier proposed residual proton transfer in ChR2 (34) that was later associated with protonation changes of D156 (17). We note that the observed small charge transfer is barely visible after light adaptation or any applied membrane potential. It occurs more than one order of magnitude later than the peak displacement current in the proton pump BR (35) and differs from fast charge transfer observed in other ChRs (36).

In the second reaction path, illumination of \(D_{270}\) (C\(_1\)) results in all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn isomerization and direct formation of P\(_{480}\) (C\(_2\)), which is also photoactive. Alternative photoreactions have been considered before to explain the biexponential decay of the conductive-state P\(_{520}\) (17, 37, 38), and were assumed to involve an early all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn isomerization based on NMR and low-temperature Raman measurements (22). Using isotopically labeled retinal and vibrational spectroscopy, we proved that the early all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn double isomerization (also at ambient temperatures) causes early formation of P\(_{480}\) (C\(_2\)). Consequently, the slowly decaying P\(_{480}\) does not represent a late photocycle intermediate of the anti-cycle, as proposed in several previous publications (9, 11, 17, 37), but is the result of a reaction branching that occurs directly after photoexcitation of D\(_{270}\) (C\(_1\)), possibly already during the excited-state lifetime. As shown by MD simulations, P\(_{480}\) features a preopening of the central gate, allowing water influx, but remains nonconductive because the inner gate is still closed as previously proposed for the E90R chloride-conducting mutant (23).

In a third reaction path, photoactivation of P\(_{480}\) (C\(_2\)) initiates the syn-cycle, here, we identified P\(_{520}\) \(^N\) as the conductive-state O\(_2\). Under continuous illumination, P\(_{520}\) \(^N\) accumulates due to its slow decay rate and significantly contributes to the stationary photocurrent, especially at high pH. Consequently, the parallel formation of P\(_{520}\) \(^N\) and P\(_{520}\) \(^N\) accumulation accounts for the biexponential channel-closing kinetics and the evolution of proton conductance during continuous illumination (3, 39). Comparing P\(_{520}\) \(^N\) accumulation in our FTIR measurements with the small photocurrent amplitude of O\(_2\) in our electro-physiological recordings indicates a significantly reduced conductance of O\(_2\) (P\(_{520}\) \(^N\)) compared with O\(_1\)-early (P\(_{900}\) \(^M2\)) and O\(_1\)-late (P\(_{520}\) \(^N\)), as previously predicted (12, 13). This now explains the ChR2 photocurrent inactivation during continuous illumination. It also explains the remarkably small shifts of the action spectra of the dark-adapted (D\(_{270}\)) and light-adapted (P\(_{480}\)) protein (40). The reduced sodium selectivity of P\(_{520}\) \(^N\) (O\(_2\)) compared with P\(_{520}\) \(^N\) (O\(_{1\text{-late}}\)) indicates substantial differences in the open-pore structures of both conducting states that are further supported by distinct FTIR spectra for P\(_{520}\) \(^N\) and P\(_{520}\) \(^N\). In summary, the slower decay of the syn-cycle with reduced conductance leads to the accumulation of P\(_{520}\) \(^N\) and now explains photocurrent inactivation.

Multiple dark states and different retinal isomers have been observed in other microbial rhodopsins, such as BR (41) and anabaena sensory rhodopsin (ASR) (42), before. Whereas we herein confirm photoactive all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn as an alternative photoisomerization and an important step for light adaptation in ChRs, such a photoreaction has not been described for BR. What has been described for BR is a thermal photocycle model. D\(_{470}\) and P\(_{480}\) represent the closed states C\(_1\) and C\(_2\) of Fig. 18. D\(_{470}\) is the state with all-trans C=N=N-anti retinal, which is populated to almost 100% in fully dark-adapted ChR2. Upon light activation, two pathways are observed. In path 1, all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-anti isomerization initiates the anti- photocycle, with the K-like P\(_{500}\) that converts into M-like P\(_{390}\) and N-like P\(_{520}\) assigned to the open states (O\(_1\)-early) and (O\(_1\)-late). In path 2, all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn isomerization leads to P\(_{480}\) formation with deprotonated E90. The nonconducting state P\(_{480}\) slowly relaxes back to D\(_{470}\). At high flash frequencies or under continuous illumination, P\(_{480}\) (C\(_2\)) accumulates. The light-adapted D\(_{470}\) represents a mixture of D\(_{470}\) and P\(_{480}\) (C\(_1\) + C\(_2\)). The photoproduction of the photoactive P\(_{480}\) (C\(_2\)) is the N-like P\(_{520}\) \(^N\), which is regarded as the open state O\(_2\). Due to its relatively long decay time P\(_{520}\) \(^N\) accumulates under continuous illumination with bright light as commonly used in electrophysiology. During such conditions, both P\(_{480}\) and P\(_{520}\) \(^N\) contribute to the inactivation of ChR2 as they accumulate at the expense of the highly conductive O\(_2\), and its parent state D\(_{470}\). E90 remains protonated during the anti-cycle and deprotonated during the syn-cycle.

Fig. 7. Unifying photocycle model. D\(_{470}\) and P\(_{480}\) represent the closed states C\(_1\) and C\(_2\) of Fig. 18. D\(_{470}\) is the state with all-trans C=N=N-anti retinal, which is populated to almost 100% in fully dark-adapted ChR2. Upon light activation, two pathways are observed. In path 1, all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-anti isomerization initiates the anti-photocycle, with the K-like P\(_{500}\). In path 2, all-trans, C=N=N-anti \(\rightarrow\) 13-cis, C=N=N-syn isomerization leads to P\(_{480}\) formation with deprotonated E90. The nonconducting state P\(_{480}\) slowly relaxes back to D\(_{470}\). At high flash frequencies or under continuous illumination, P\(_{480}\) (C\(_2\)) accumulates. The light-adapted D\(_{470}\) represents a mixture of D\(_{470}\) and P\(_{480}\) (C\(_1\) + C\(_2\)). The photoproduction of the photoactive P\(_{480}\) (C\(_2\)) is the N-like P\(_{520}\) \(^N\), which is regarded as the open state O\(_2\). Due to its relatively long decay time P\(_{520}\) \(^N\) accumulates under continuous illumination with bright light as commonly used in electrophysiology. During such conditions, both P\(_{480}\) and P\(_{520}\) \(^N\) contribute to the inactivation of ChR2 as they accumulate at the expense of the highly conductive O\(_2\), and its parent state D\(_{470}\). E90 remains protonated during the anti-cycle and deprotonated during the syn-cycle.
equilibration between all-trans,C=N-anti (λ_{max} = 568 nm) and 13-cis,C=N-syn (λ_{max} = 548 nm) toward a ratio of roughly 6:4. Photoactivation of BR$_{540}$ initiates the syn-cycle that branches either early or late to the all-trans,C=N-anti state, accumulating all molecules in the BR$_{568}$ isomeron (43, 44). The situation is different in ASR. Here, the fully dark-adapted state again contains an all-trans,C=N-anti chromophore. Photoactivation is thought to first cause a classic 13-trans to 13-cis isomerization and then a thermal obligatory C=N isomerization late during the photocycle, ending up with a 13-cis,C=N-syn light-adapted second dark state (45). Illumination of this second dark state is thought to cause 13-cis to trans-isomerization and, at the end of the syn-cycle, a thermal C=N syn- to anti-isomerization. In summary, in microbial rhodopsins, photochemical as well as thermal single isomerization around the C$_{13}$ = C$_{14}$ bond and photochemical and thermal double isomerization around C$_{13}$ = C$_{14}$ and C$_{15}$ = N are possible, but the efficiency of both reactions in light and in darkness, as well as the preferences of the directions, vary substantially within the diverse family.

Finally, we show that E90 is crucial for proton conductance in both photocycles and constitutes one key determinant for ion selectivity changes during continuous illumination with an intriguing double function depending on its protonation state before and after light adaptation. In the anti-cycle, E90 might be directly involved in proton transport as a proton shuttle or in the organization of water molecules, both of which bridge the distinct water-filled cavities seen in the dark-state crystal structure of ChR2 (31). In this scenario, E90 would favor proton selectivity, forming either a direct or indirect shortcut for protons that cannot be taken by larger cations at a similar efficiency. By similar means, the outer pore glutamates E139 and E143 in the highly proton-selective ChR Chrimson (46) or D112 in the voltage-gated proton channel Hv1 (47) were also shown to contribute to proton conductance and selectivity. Although the selectivity filter of ChR2, localized in the central gate, might be more permissive for larger cations than that of Chrimson, localized in the outer gate (48), in both cases, substitution of essential glutamates (E90 in ChR2 and E139 in Chrimson) by equally titratable histidines preserved proton selectivity, whereas substitution with the nontitratable glutamine or alanine impaired proton conductance (16, 46). In the anti-cycle, channel opening occurs with E90 staying protonated for the entire gating process. Accordingly, recent 4-ns molecular mechanics (MM) simulations on an ChR2 homology model based on the C122 chimaera structure [PDB ID code 3ug9 (32)] showed impressively that minor hydrogen bond changes of E90 due to protonation of the selectivity filter of ChR2, localized in the central gate, might be sufficient to promote water influx at the longer microsecond simulation times (30).

In a combined study of single-turnover electrophysiology and FTIR and Raman spectroscopy with isotopic retinal labeling, site-directed mutagenesis, and MD simulations, we developed a unifying two-photocycle model that simplifies and embraces previous kinetic models and completely resolves the channel gating, light adaptation, and temporal changes in ion selectivity. Identifying the corresponding molecular transitions, we may facilitate future protein engineering of ChR variants with reduced or improved photocurrent inactivation for optogenetic applications, requiring either a stable response to continuous illumination or a transient response to light switching. Early photocycle branching by alternative retinal isomerization and the corresponding large conformational protein changes that do not directly lead to channel opening will need careful consideration for the interpretation of molecular gating transitions observed in time-resolved spectroscopy, crystallography, and optogenetic experiments.

It turns out that only tiny light-induced alterations are crucial for the specific protein function. The elucidation of the molecular reaction mechanisms of proteins therefore deserves vibrational spectroscopic techniques like time-resolved FTIR with nanoscale spatiotemporal resolution.

Materials and Methods

Yeast Culture. *P. pastoris* strain SMD1163 cells (kindly gifted by C. Bamann, Max Planck Institute of Biophysics, Frankfurt) containing the pPIC9KChR2his10 construct were precultured in buffered glycerol complex (BMGY) medium (50). Expression of ChR2 was induced in buffered methanol complex (BMMY) medium containing 2.5 μM all-trans retinal (either 12C, 13C$_{14}$,13C$_{15}$-labeled or 13C$_{10}$,13C$_{11}$-labeled) and 0.0004% biotin at an initial OD$_{600}$ of 1 and at 30 °C and 120 rpm. Cells were harvested at an OD$_{600}$ of 20 by centrifugation.

Membrane Preparation and Protein Purification. Cells were disrupted using a BeadBeater (Biospec Products), and membranes were isolated by ultracentrifugation. Homogenized membranes were solubilized with 1% decamyltoide overnight. ChR2 purification was done by nickel-nitriotriacetic acid affinity chromatography and subsequent gel filtration using a HiLoad 16/600 Superdex 200-20 pg column (General Electric).

Reconstitution of ChR2 into DPPC or EggPC. The purified ChR2 was reconstituted into DPPC (Avanti Polar Lipids) or EggPC (Avanti Polar Lipids). The lipids were solubilized with 2% cholate in 20 mM HEPES (pH 7.5), 100 mM NaCl, and 10 mM MgCl$_2$, by sonication at 50 °C for 10 min. Solubilized lipids and purified ChR2 were mixed at a 2:1 ratio (lipid/protein (wt/wt)) and incubated for 20 min. Detergent was removed overnight either by adsorption on Bio-Beads SM 2 (BioRad) or by dialysis.
The resulting suspension containing proteoliposomes and buffer was ultracentrifuged at 200,000 g for 2 h, and the pellet was then transferred and centrifuged between two CaF₂ slides to obtain an optical path length between 5 μm and 10 μm. This sample was then placed in a vacuum-tight cuvette.

**Preparation of HEK Cells.** Electrophysiological recordings were performed on stably expressing the ChR2-mVenus fusion construct HEK cell line (34), as previously described in detail (51). Briefly, HEK cells were cultured at 5% CO₂ and 37 °C in DMEM supplemented with 10% FBS, 100 μg/mL penicillin/streptomycin (Biochrom), 200 μg/mL zeocin, and 50 μg/mL blasticidin (Thermo Fisher Scientific). Cells were seeded onto polylysine-coated glass coverslips at a concentration of 1 × 10⁶ cells per milliliter and supplemented with a final concentration of 1 μM all-trans retinal (Sigma–Aldrich). Induction of ChR2-mVenus expression was induced by addition of 0.1 μM tetraacyclene (Thermo Fisher Scientific).

**Patch-Clamp Experiments in HEK293 Cells.** Patch pipettes were pulled using a P1000 micropipette puller (Sutter Instruments) and fire-polished. Pipette resistance was 1.5–2.5 MΩ. A 140 mM NaCl agar bridge served as a reference (bath) electrode. In whole-cell recordings, membrane resistance was typically >1 GΩ, while access resistance was below 10 MΩ. Pipette capacity, series resistance, and cell capacity compensation were applied. All experiments were conducted at an ambient temperature of approximately 22°C. Leaky signals were analyzed with DigiStim (DigiStim1400), and acquired using Clampex 10.4 software (all from Molecular Devices). Holding potentials were varied in 15-mV steps between −60 and +30 mV. Extracellular buffer exchange was performed manually by adding at least 5 mL of the respective buffer to the recording chamber (500-μL chamber volume), while a Ringer Bath Handler MPCU (Lorenz Messgerätebau) maintained a constant bath level. Standard bath solutions contained 110 mM NaCl, 1 mM KCl, 1 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂, and 310 mOsm. Standard pipette solutions contained 110 mM NaCl, 1 mM KCl, 1 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM Hapes at extracellular pH (pH₄) 7.2 (with glucose added up to 310 mM). Standard pipette solutions contained 110 mM NaCl, 1 mM KCl, 1 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, and 10 mM Hapes at pH 7.2 or 10 mM Tris at intracellular pH (pH₅) 9.0 (glucose was added up to 290 mM). For ion selectivity measurements, either NaCl was replaced by 140 mM LiCl or extracellular calcium was adjusted to pH 9.0, by buffering with 10 mM Tris instead of Hapes.

Continuous light was generated using a Polychrome V light source (TILL Photonics) set to 470 ± 7 nm. Light exposure was controlled with a programmable shutter system (VS25 and VCM-D1; Vincent Associates). The Polychrome V light intensity was 3.4 mW/mm² in the sample plane, measured with a calibrated optometer (P9710; Gigahertz Optik). Light intensities were calculated for the illuminated field of the W Plan-Apochromat 40x/1.0 dry/130°/0.17 numerical aperture (N.A.) (Carl Zeiss) with delivery of 470-nm ns−1 laser pulses, an Opellette polette HE:NY:DY:GCR laser/OPO system (OPOTEK) was coupled/decoupled into a M37L02 multimode fiber patch cable with a modified KT110/M free space-to-fiber coupler using AC127 019 A ML achromatic doublets (Thorlabs). Single pulses were selected using a LS62M2 shutter (Vincent Associates). Laser intensity was set to 5% using a motorized variable attenuator, resulting in a pulse energy of 100 ± 20 μJ/mm². Pulse energies were measured with a calibrated S470C thermal power sensor and a PM100D power and energy meter (Thorlabs) after passing through all of the optics. Actinic light was coupled into an Axiosvert 100 microscope (Carl Zeiss) and delivered to the sample using a 90/10 beam splitter (Chroma). To toggle between activation with the laser and the Polychrome V light source, a BBI E02 broadband dielectric mirror mounted on an MFF101/M motorized filter flip mount (Thorlabs) was used. Data were filtered at 100 KHz and sampled at 250 KHz. Due to minimal timing uncertainties, each acquired sweep was time-shifted after measurements to align it with the rising edges of the Q-switch signals of the activating laser pulses. Photocurrents were binned to 50 logarithmically spaced data points per temporal decade with custom-written MATLAB script (MathWorks).

**FTIR Experiments.** To gain insight into the changes upon illumination, we performed time-resolved FTIR difference spectroscopy at 15 °C. For the continuous light experiments, the sample was illuminated with a blue LED (λmax = 465 nm) for 5 s. Spectra were recorded before switching on the light (reference), during illumination (accumulation of the stationary photo-product (P520stat) for 5 s) and after switching off the light (decay of P520stat for 50 s) using the conventional rapid scan mode of the spectrometer. Difference spectra were calculated using the Beer–Lambert law, which results in positive photo product bands and negative educt bands in the difference spectra. For the single-turnover measurements, the sample was illuminated with a short laser pulse of an excimer laser-driven dye laser (Coumarin 102 dye; λmax = 475 nm, pulse width = 50 ns). Conventional rapid scan experiments were performed 10 ms after the laser pulse. Spectral resolution = 4 cm⁻¹ were performed with a sufficient relaxation time (trelax = 200 s) and flash frequency (frelax = 0.005 Hz) between the flashes to allow the D470 to significantly repopulate (D470relax became ∼96%). For a comparison with the photocycle under the “shortcut condition,” the flash frequency was increased (frelax = 5 s, fflash = 0.2 Hz). Using this approach, equilibrium between D470 and P520 emerges and the ChR2 molecules start the photocycle from both states. The datasets were then analyzed by a global-fitting routine as presented previously (16, 18, 52, 53) to isolate the decay-associated amplitude spectra of the transitions involved in D470 recovery. To get access to the earlier intermediates of the dark-adapted (D470relax ~ 91%) photocycle of H134R (Fig. 3A), step-scan measurements were performed with a light pulse repetition rate of 0.007 Hz (frelax = 140-s, detector rise time = 50 ns, resolution = 8 cm⁻¹, wavenumber range: 0–1,974 cm⁻¹) already published for the WT (18). One measurement was completed after 22 h, and ~15 measurements were averaged to give the final result. H134R used for the step scan was expressed in COS (abbreviation for CV-1 in Origin with SV40 genes) cells and prepared as described in our earlier publication (18).

**Raman Experiments.** The Raman experiments were performed with samples that were the same as those for the rapid scan FTIR experiments, but with a higher optical path length (20–50 μm). The room temperature was ~18 °C. We used the Raman microscope XPloRA One (HORIBA Scientific) to scan the sample. To prevent sample degradation due to long illumination, we performed measurements at a 785-nm excitation wavelength to ensure the lowest possible photoexcitation of the sample and a sufficient enhancement of the Raman signal due to the preresonant Raman effect. Laser power at the sample position was 28 mW. A 50× objective (Olympus LCPLN-IR) was used, resulting in a confocal volume of ∼1 μm² in the sample plane.

To excite the D470 state of the sample and create a photoproduct with a high P520 fraction, the sample was illuminated with an external blue-light source (100-W halogen lamp filtered with a 470-nm filter coupled to the observation beam path of the microscope). Sample illumination was controlled by a shutter between the lamp and sample.

To ensure that the measured photoproduct spectra are free of contamination by their preceding intermediates (P520stat and P520relax) under continuous illumination, a controlled illumination/excitation experiment was performed:

1. Laser on + illumination on (formation of P520stat)
2. Wait 0.5 s
3. Acquisition of spectrum (tintegration = 2 s; P520stat is measured)
4. Laser on + illumination off (slow relaxation of P520stat; trelax = ~40 s)
5. Wait 0.5 s
6. Acquisition of spectrum (tintegration = 2 s; high fraction of P520 free of illumination artifacts is measured (P520relax))

Go to next position on the sample

This procedure was repeated for a 15 × 15 spot matrix (pixel spacing = 5 μm) of the sample, and the acquired spectra were averaged for each illumination condition.

Next, the illumination was stopped and a relaxation phase of at least 5 min was commenced to allow full relaxation of the generated P520. The dark-state D470 was measured for the same spot matrix (tintegration = 2 s).

To obtain the pure lamp artifact, the same area was measured without the laser with only illumination of the sample. This spectrum was later subtracted from the spectra measured under continuous illumination.

The complete protocol was performed for three spectral regions (center wavenumbers: 900 cm⁻¹, 1,250 cm⁻¹, and 1,550 cm⁻¹), which were then combined to obtain the complete spectrum ranging from 650 cm⁻¹ to 1,700 cm⁻¹ with a wavenumber spacing of ~0.2 cm⁻¹ and a nominal resolution of 0.6 cm⁻¹.

**MD Simulations.** The MD simulations were performed according to our previous reports (16, 18), except for the force field and the GROMACS version used. We used the Optimized Potentials for Liquid Simulations/All-Atom (OPLS/AA) force field and GROMACS version 2016.3. A series of 5 × 100-ns independent and unrestrained MD simulations was performed for each protonation state of E90 with the respective chromophore configuration. The MD simulations were performed consecutively using the resulting structures of dark-adapted ChR2 (all-trans; C=N-ant + with protonated E90)
given by MD simulations as a starting point for the isomerization (discussed below). Each MD simulation was initiated using a different temperature seed number to generate the random distribution of starting velocities.

Water Dynamic and Run-Average Structure. The water dynamic and run-average structures were calculated according to our previous report (18).

Retinal Isomerization. Retinal all-trans-C13=C= anti- to 13-cis,C=anti isomerization was performed as described earlier (18), achieved via the following scheme. The torsion angles of the C13–C14 and C=C–N double bonds were tilted counterclockwise in 20° steps, starting in the range 0–180°. For each tilting step, the retinal + K257 (without backbone) atoms were maintained as a freeze group and the rest of the simulation system was allowed to relax in a 10 ns unrestrained MD simulation as described above.

The resulting 13-cis retinal structures served as starting structures for the MD simulations of the different intermediate states (P200, P280, and P500-900) with protonated and deprotonated E90. For the starting structures for the P280-901 intermediate, we used the final structures of the P200 simulations after Schiff base deprotonation and D253 protonation.

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