Transient protonation changes in channelrhodopsin-2 and their relevance to channel gating

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The discovery of the light-gated ion channel channelrhodopsin (ChR) set the stage for the novel field of optogenetics, where cellular processes are controlled by light. However, the underlying molecular mechanism of light-induced cation permeation in ChR2 remains unknown. Here, we have traced the structural changes of ChR2 by time-resolved FTIR spectroscopy, complemented by functional electrophysiological measurements. We have resolved the vibrational changes associated with the open states of the channel (P_2^{390} and P_3^{520}) and characterized several proton transfer events. Analysis of the amide I vibrations suggests a transient increase in hydration of transmembrane α -helices with a $t_{1/2} = 60 \mu\text{s}$, which tallies with the onset of cation permeation. Aspartate 253 accepts the proton released by the Schiff base ($t_{1/2} = 10 \mu\text{s}$), with the latter being reprotonated by aspartic acid 156 ($t_{1/2} = 2 \text{ ms}$). The internal proton acceptor and donor groups, corresponding to D212 and D115 in bacteriorhodopsin, are clearly different from other microbial rhodopsins, indicating that their spatial position in the protein was relocated during evolution. Previous conclusions on the involvement of glutamic acid 90 in channel opening are ruled out by demonstrating that E90 deprotonates exclusively in the nonconductive P_4^{480} state. Our results merge into a mechanistic proposal that relates the observed proton transfer reactions and the protein conformational changes to the gating of the cation channel.

time-resolved IR | step scan | membrane protein | retinal | photosensory protein

Optogenetics provides new tools to neurophysiologists to steer cellular responses with unprecedented temporal and spatial resolution. The former takes advantage of light as an ultrashort trigger, whereas the latter is achieved by genetically encoding and directing photosensitive proteins to specific cell types. The most prominent among the optogenetics tools is channelrhodopsin (ChR), which was found to be the first light-gated ion channel of its kind (1, 2). This discovery paved the way for an exponentially growing number of neurophysiological applications, ranging from single cells to living animals (3). Light-gated ion permeation by ChR expands the various modes of action of the large family of microbial rhodopsins already comprising light-driven ion pumps and sensors (4). Among the various ChRs, which differ mostly in cation selectivity (3), ChR2 is used in the majority of optogenetic applications because of the higher expression yield in mammalian cells.

A projection structure of the heptahelical ChR2 showed a dimer with the contact interface between helices C and D suggested to form the cation channel (5). More recently, a chimeric ChR (C1C2) was constructed by linking the last two helices (F and G) of ChR2 to the first five (A to E) of ChR1 and resolved by X-ray crystallography to 2.3 Å (6). The high-resolution structure confirmed the dimeric arrangement and identified an electronegative extracellular pore in each monomer framed by helices A, B, C, and G. Accompanying electrophysiological experiments on point mutants indicated residues in the glutamate-rich helix B that delineate the cation channel (6). It is surprising that mutations of either C128 or D156 in ChR2 lead to far more drastic

functional alterations with intensified photocurrents and strongly retarded ground-state recovery kinetics (7) because these residues are located in helices C and D, respectively (i.e., remote from the proposed cation channel). The interaction of C128 and D156 was shown by FTIR difference spectroscopy to involve an H-bond between the termini of both side chains, the so-called “DC gate” (8). The structural basis of the DC gate could not be corroborated by the crystallographic model of C1C2, where the terminal S–H of C128 points away from D156 (6).

As in all other microbial rhodopsins, ChR is energized by the photo-induced isomerization of retinal. This feature renders ChR unique among all ion channels because the functional mechanism can be studied over the time scale from femtoseconds to seconds via triggering by a short light pulse. The chromophore retinal, linked to channelopsin via a protonated Schiff base (SB) with K257, exhibits an absorption maximum (λ_{max}) at $\sim 470 \text{ nm}$ in the dark state (9). The electronic properties of the retinal are modulated by the electrostatic structure in its immediate vicinity as ChR2 passes through a series of intermediate states (10). The photoreaction of ChR2 under continuous illumination is complex due to multiple photonic excitation (10), but single turnover conditions simplify the analysis when short laser pulses are applied (Fig. 1). On all-*trans* to 13-*cis* isomerization of the retinal cofactor and vibrational relaxation, the early red-shifted P_1^{500} intermediate ($\lambda_{\text{max}} \approx 500 \text{ nm}$) is formed in the picosecond range (11). Subsequently, the retinal SB deprotonates and the blue-shifted P_2^{390} state ($\lambda_{\text{max}} \approx 390 \text{ nm}$) is formed with $t_{1/2} \approx 10 \mu\text{s}$. Reprotonation of the SB proceeds with $t_{1/2} \approx 2 \text{ ms}$, leading to the late red-shifted P_3^{520} state ($\lambda_{\text{max}} \approx 520 \text{ nm}$), decaying with $t_{1/2} \approx 10 \text{ ms}$ to the late P_4^{480} state ($\lambda_{\text{max}} \approx 480 \text{ nm}$). Relaxation of

Significance

It was always a dream to control cells and living animals by light. Discovery of channelrhodopsin turned the dream into reality because this light-activated cation channel is able to elicit action potentials with unprecedented spatial and temporal resolution. To unravel the underlying molecular mechanism, we have applied time-resolved IR spectroscopy, and we suggest how the observed proton transfer and the protein conformational changes lead to opening of the cation channel. Our results will not only contribute to the rational design of channelrhodopsin variants with improved properties, but also help to decipher the temporal sequence in the gating of ion channels.

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Fig. 1. Scheme of the unidirectional photocycle of ChR2. Superscripts to each reaction intermediate (P_1^{500} , P_2^{390} , P_3^{520} , and P_4^{480}) indicate the wavelength of maximum absorption. The photocycle is initiated by the absorption of blue light, and each subsequent transition is characterized by the half life ($t_{1/2}$). Times given correspond to experiments on ChR2 when solubilized in dodecyl maltoside (25 °C, neutral pH).

P_4^{480} to the original ground state is unusually slow for a retinylidene protein and proceeds with $t_{1/2} \approx 20$ s.

The groups accepting and donating the proton from and to the SB are as yet unknown, as well as the proton transfer reactions responsible for the observed proton pump activity of ChR2 (12). Correlation of time-resolved UV/Vis spectroscopy and electrical experiments showed that the P_3^{520} state represents the conductive state (9). Whereas closing the channel is clearly associated with P_3^{520} decay, opening appeared to occur before the rise of P_3^{520} but after the rise of the preceding P_2^{390} state (9, 10). FTIR difference spectroscopy suggested that large conformational changes in the protein backbone take place in the early P_1^{500} and the late P_4^{480} states, which were trapped under continuous illumination (13, 14). Structural information on the P_2^{390} and P_3^{520} states, where opening and closing of the channel occurs, is scarce, however.

Inasmuch as the X-ray crystallographic structure sets the stage, elucidation of the functional mechanism of ChR2 requires the contribution of time-resolved methodologies with appropriate structural sensitivity. IR difference spectroscopy is a powerful technique, being sensitive not only to the transient conformational changes of the protein but particularly to protonation and H-bonding changes of amino acid side chains. Here, we have used time-resolved step-scan FTIR spectroscopy to study the photocycle of ChR2 with a time resolution of 6 μ s, sufficient to probe the rise and decay of the conductive P_2^{390} and P_3^{520} intermediates and to trace the associated internal proton transfers. We conclude that the primary acceptor of the proton of the retinal SB is D253

[D212 in bacteriorhodopsin (bR)] and the internal donor is D156 (D115 in bR), with the latter accounting for the functional relevance of the DC gate. These results are drastically different from bR, where D85 (E123 in ChR2) and D96 (H134 in ChR2) are the primary proton acceptors and donors (15), and where D115 (D156 in ChR2) plays only an indirect role in the proton pump mechanism (16). Analysis of the time evolution of the conformationally sensitive amide I vibrations of the protein backbone suggests an increase in the hydration of transmembrane α -helices that correlates to the onset of cation permeation. The hydration may be the result of the formation of a continuous water-filled channel inside the protein from previously disconnected internal cavities. We infer that the conformational rearrangements are triggered by the changes in electrostatics and H-bonding networks that are a consequence of the observed protonation changes.

Results

The conformational changes of ChR2 were studied by time-resolved FTIR spectroscopy following photonic excitation by a pulsed laser emitting at 450 nm. The IR difference spectra displayed a rich and complex pattern of positive and negative peaks (Fig. 2A), reflecting a temporal sequence of conformational changes of the holoprotein as a consequence of the photo-induced isomerization of the chromophore retinal. The IR data were complemented by time-resolved UV/Vis experiments (Fig. 2B), which were used to define the time of maximum transient concentration of the respective photointermediates: 6 μ s after the laser flash for the P_1^{500} state, 300 μ s after the laser flash for the P_2^{390} state, 6 ms after the laser flash for the P_3^{520} state, and 300 ms after the laser flash for the P_4^{480} state (Fig. 3).

The negative bands in Fig. 3A represent vibrations of the dark state of ChR2, which are missing in the respective intermediate states. The appearance of negative bands at 1,240, 1,200, and 1,154 cm^{-1} , typical for C–C stretching vibrations of all-*trans* retinal (17), are in accordance with the general view that the photocycle of ChR2 originates mostly from the photoisomerization of all-*trans* retinal (10). The negative band at 1,554 cm^{-1} is due to the ethylenic (C=C) vibration of ground-state retinal (18). The frequency correlates well with the electronic absorption of retinal at 470 nm of ground-state ChR2 (19).

The time-resolved IR difference spectrum of the P_1^{500} state (uppermost spectrum in Fig. 3A) exhibits similar spectral changes in the amide I region (negative band at 1,663 cm^{-1}) as the state trapped at 80 K (13, 14), albeit with discrepancies at other frequencies (SI Appendix, Fig. S1A). Thus, the large amide I changes previously found in static difference spectra were not due to a cryogenic artifact but are related to unusually fast conformational changes of the protein backbone. The time-resolved difference spectrum of the P_1^{500} intermediate shows a positive band at 986 cm^{-1} assigned to the hydrogen-out-of-plane vibration of the retinal, indicating a nonplanar retinal conformation in the

Fig. 2. Time-resolved IR and UV/Vis absorbance changes in ChR2 following retinal photoisomerization after 10 ns of laser excitation. Three-dimensional plot of the recorded time-resolved IR (A) and UV/Vis (B) difference spectra.

Fig. 3. Time-resolved IR and UV/Vis difference spectra of ChR2 at four selected times after pulsed excitation. (A) Extracted IR difference spectra. (B) Extracted UV/Vis difference spectra show from the positive bands that the extracted spectra correspond mainly to the P_1^{500} (6 μ s), P_2^{390} (300 μ s), P_3^{520} (6 ms), and P_4^{480} (300 ms) intermediates, respectively. The positive contribution from P_1^{500} appears upshifted to 515 nm as a result of the spectral overlap by the absorption of the initial dark state (14). Here and in Fig. 8, mOD refers to the change in absorbance of 10^{-3} .

early red-shifted intermediate like in other microbial rhodopsins (20–22). The positive band at $1,173\text{ cm}^{-1}$ appears asymmetrical and strongly red-shifted with respect to the C_{14} – C_{15} stretching of 13-*cis* retinal in other microbial rhodopsins [$1,190$ – $1,187\text{ cm}^{-1}$ (20–22)]. Its asymmetrical shape with a shoulder at $1,188\text{ cm}^{-1}$ (Fig. 3A and *SI Appendix*, Fig. S2) reflects heterogeneity in the conformation of the isomerized retinal, suggesting contributions from an intermediate preceding P_1^{500} . Two positive bands assignable to the retinal C_{14} – C_{15} stretching vibration were also observed in proteorhodopsin (23), with a dark state composed of a 70:30 mixture of all-*trans* and 13-*cis* retinal, as in ChR2 (18). Thus, it is inferred that the spectral heterogeneity of the C_{14} – C_{15} stretching might well indicate the concurrence of photo-intermediates from both the all-*trans* and 13-*cis* retinal photocycles, as was found in *Anabaena* sensory rhodopsin (24).

At 300 μ s after the laser pulse, the P_2^{390} state is the predominant intermediate, as indicated by positive absorption at 375 nm in the UV/Vis difference spectrum (Fig. 3B). However, residual absorption at around 520 nm is also observed. Thus, the IR difference spectrum recorded at 300 μ s includes minor contributions from a red-shifted intermediate state (Fig. 3A). A pure difference spectrum of the subsequent P_3^{520} intermediate was isolated at 6 ms after the laser flash (Fig. 3B). The positive band at $1,542\text{ cm}^{-1}$ is assigned to the ethylenic vibration of retinal (Fig. 3A). The frequency correlated well with the absorption maximum at 520 nm (19). The positive band from the C_{14} – C_{15} stretching of the isomerized retinal shifted to $1,177\text{ cm}^{-1}$ in P_3^{520} , which reflects different retinal conformations in the P_1^{500} and P_3^{520} intermediate states. The difference spectrum of the P_4^{480} state was resolved at 300 ms after the laser pulse. The absorption band of the ethylenic vibration of ground-state retinal is largely canceled due to the similar frequency of this mode in the P_4^{480} state (Fig. 3A). The IR difference spectrum of P_4^{480} is largely identical to the spectrum obtained under continuous illumination (*SI Appendix*, Fig. S1B) and very similar to the time-resolved difference spectrum of P_4^{480} when ChR2 is reconstituted in liposomes (25).

Proton Transfer and Hydrogen-Bonding Changes Involving Aspartate and Glutamate Side Chains.

The retinal SB of ChR2 deprotonates in the P_1^{500} -to- P_2^{390} transition and is reprotonated in the P_2^{390} -to- P_3^{520} transition (9). The groups accepting and donating the proton from and to the SB are yet unknown, but prime candidates are the terminal carboxylic groups of aspartate or glutamate residues and their corresponding acids as in other well-characterized rhodopsins. IR difference spectroscopy is particularly suited for the identification of protonation changes (26–28) because the C=O stretching vibration ($\nu\text{C=O}$) of protonated carboxylic groups appears in the $1,780$ to $1,690\text{ cm}^{-1}$ frequency range (29, 30). The vibrational bands in the carboxylic region of the four intermediate states exhibit significant spectral overlap (Fig. 3A and *SI Appendix*, Fig. S2), which is reduced by applying a well-established band-narrowing method [Fourier self-deconvolution (FSD)] (31) to the IR difference spectra (Fig. 4A). By these means, up to four positive ($1,760$, $1,745$, $1,728$, and $1,695\text{ cm}^{-1}$) and two negative ($1,737$ and $1,717\text{ cm}^{-1}$) bands were resolved (Fig. 4A). The assignment of these vibrational bands to molecular changes and the implications on the molecular mechanism of ChR2 will be discussed next.

Primary Proton Donor to the Retinal SB.

The rationale for the identification of the internal proton donor is that deprotonation of an aspartic or glutamic acid will generate a negative band whose kinetics match those of the formation of the P_3^{520} state. Two negative bands were resolved in the carboxylic region (Fig. 4A), but only the kinetics of the band at $1,737\text{ cm}^{-1}$ matched the rise and decay of the P_3^{520} state (Fig. 4B). In the IR difference spectrum of the P_3^{520} intermediate of D156E, the negative peak at $1,737\text{ cm}^{-1}$ is upshifted to $1,763\text{ cm}^{-1}$, whereas the negative band at $1,717\text{ cm}^{-1}$ is not affected (Fig. 4D, frequency shift indicated by an arrow). We generated the wildtype (WT) minus D156E double-difference spectrum, firmly establishing that the negative peak at $1,737\text{ cm}^{-1}$ in P_3^{520} originates from deprotonation of D156 and not from an environmental change (*SI Appendix*, Fig. S5A). As expected, the negative band at $1,737\text{ cm}^{-1}$ vanished in the IR difference spectrum of the P_2^{390} intermediate of the D156A

Fig. 4. Identification of the SB proton donor group. (A) IR difference spectra in the carboxylic region of WT ChR2 after FSD. Bands assigned to the $\nu\text{C=O}$ of either protonated D156 or E90 are indicated by the hatched areas. (B) Kinetics of the negative band at $1,737\text{ cm}^{-1}$ (red trace) compared with the kinetics at 540 nm (gray trace) measured on the same sample. The kinetics at 540 nm reflect the rise and decay of the P_3^{520} state. (C) Kinetics of the negative band at $1,717\text{ cm}^{-1}$ (blue trace) and the positive band at $1,728\text{ cm}^{-1}$ (green). The addition of both traces (magenta) resolves protonation changes of E90. (D, Upper) Difference spectra of the P_2^{390} state of WT ChR2 (gray trace) and the D156A mutant (orange) extracted at $300\text{ }\mu\text{s}$ and at 6 ms after the flash, respectively. (D, Lower) Difference spectra of the P_3^{520} state of WT ChR2 (gray trace) and the D156E mutant (blue) extracted at 6 ms after the flash. Missing and shifted bands are indicated by asterisks and arrows, respectively.

variant, as also did the bands at $1,760$ and $1,745\text{ cm}^{-1}$ (Fig. 4D, bands marked by asterisks). Thus, these two positive bands reflect H-bonding changes of the D156 carboxylic group in the P_1^{500} and P_2^{390} states, respectively. The remaining bands at $1,717$ and $1,728\text{ cm}^{-1}$ disappeared in the E90A variant (SI Appendix, Fig. S4), which supports their assignment to the $\nu\text{C=O}$ of the terminal carboxylic acid of E90 in the dark and photolyzed states, respectively (14).

Because D156 was identified as the internal proton donor to the retinal SB, we addressed the consequences of replacement of this residue by recording photocycle kinetics and channel functionality of D156 variants. The kinetics of SB reprotonation were accelerated in the D156E variant, leading to low P_2^{390} accumulation (Fig. 5A). The subsequent reprotonation of E156 was slowed down, resulting in a 10-fold delayed P_3^{520} decay (Fig. 5B). Both effects suggest a reduced proton affinity of the proton donor as a result of the D156E replacement. The D156E variant retains activity, as demonstrated by electrophysiology (Fig. 6 and SI Appendix, Fig. S3 B and C). In agreement with the slower P_3^{520} decay, closing of the channel was also 10-fold delayed with respect to the WT type (Fig. 6). It was previously shown that although the D156A variant is functional in cation permeation, it exhibits $\sim 10^4$ -fold delayed reprotonation of the retinal SB and strongly retarded channel closure (7), an observation that can now be rationalized by the absence of a suitable internal proton

donor. In conclusion, we infer that the reprotonation of D156 is a molecular determinant for efficient channel closure.

Primary Acceptor of the SB Proton. The crystallographic structure of the C1C2 chimera (6) locates E123 and D253 3.4 and $3.0\text{ }\text{\AA}$ away from the retinal SB (shortest distance), respectively. The close proximity renders these residues prime candidates as acceptors of the SB proton. However, a positive band for the $\nu\text{C=O}$ frequency of the SB proton acceptor, clearly observed in other rhodopsins in the range of $1,765$ – $1,710\text{ cm}^{-1}$ (SI Appendix, Table S1), is evidently missing in the P_2^{390} state IR difference spectrum of WT ChR2 (Fig. 3A). The three observed positive bands above $1,710\text{ cm}^{-1}$ can be exclusively assigned to H-bonding changes of D156 and E90. Because the latter two residues are both protonated in the dark state of ChR2, they cannot serve as proton acceptors of the SB proton after light excitation.

The only positive band left in the difference spectrum of P_2^{390} (Fig. 4A) is the band at $1,695\text{ cm}^{-1}$. The rise in the kinetics of this band tallies the rise of the P_2^{390} state (Fig. 7B) (i.e., compatible with the expected kinetics of the SB proton acceptor). However, the frequency is unusually low for a carboxylic $\nu\text{C=O}$ (32), and changes in the $\nu\text{C=O}$ of glutamine and asparagine side chains and in peptide bond vibrations may appear as well in this frequency range (32). Thus, we recorded time-resolved FTIR difference spectra of E123 and D253 variants to identify the internal proton acceptor group.

The residue equivalent to E123 of ChR2 is D85 of bR, which was shown to be the primary proton acceptor of the SB. Therefore, we replaced E123 by T and expected a strong impact on the functionality similar to that of the equivalent D85T mutation in bR (33). However, the replacement barely affected P_2^{390} and P_3^{520} formation and decay kinetics (Fig. 5), which agrees well with the preserved channel activity of the E123T variant, as was reported by Gunaydin et al. (34). Consistent with these observations, the IR difference spectrum of the P_2^{390} state of ChR2 is only moderately affected by the E123T mutation (SI Appendix, Fig. S4). A distinct difference, however, is the appearance of the positive band at $1,712\text{ cm}^{-1}$ (Fig. 7A). The kinetics of the rise of this band tallied with the rise of the P_2^{390} intermediate (Fig. 7C). The double-difference spectrum between WT and the E123T variant (SI Appendix, Fig. S5B) shows difference bands at $1,715(-)/1,697(+)\text{ cm}^{-1}$. The positive band at $1,715\text{ cm}^{-1}$ is assigned to the $\nu\text{C=O}$ stretch of D253 (*vide infra*), which is upshifted in the E123T variant. The positive band is apparently preserved in the succeeding P_3^{520} state of the E123T mutant, albeit slightly downshifted to $1,708\text{ cm}^{-1}$ by a change in hydrogen bonding (SI Appendix, Fig. S4). Thus, the proton acceptor remains protonated in P_3^{520} .

The results of the E123T variant with its modestly affected kinetics and spectra point to D253 as the SB proton acceptor. We scrutinized this possibility by studying the functionally active

Fig. 5. Transient absorbance changes in the UV/Vis range after pulsed excitation of WT ChR2 (gray trace) and variants D156E (blue), E123T (green), and D253E (red). The blue-shifted intermediate P_2^{390} was probed at 380 nm (A), and the red-shifted early P_1^{500} and late P_3^{520} intermediates were probed at 540 nm (B). Some of the transients have been scaled in amplitude to facilitate the comparison.

Fig. 6. Time-resolved photocurrents of ChR2 after pulsed laser excitation. WT ChR2 (gray trace), and variants D156E (blue) and D253E (red) have been expressed in HEK293 cells, and the whole-cell patch-clamp technique has been applied. The currents were recorded at room temperature ($\sim 22^\circ\text{C}$) at a holding potential of -60 mV . The traces were normalized to the maximum current amplitude of WT ChR2 to facilitate kinetic comparison. The accelerated kinetics of channel opening in the D253E mutant could not be resolved due to the response time of the electrical recordings in this experiment (red dashed trace additional details are provided in *SI Appendix*.)

D253E variant (Fig. 6 and *SI Appendix*, Fig. S3A and C), which exhibited a notable 50-fold acceleration of the rise of the P_2^{390} state (Fig. 5A). The decay of the P_2^{390} state was threefold slower on D253E replacement, leading to a low and delayed P_3^{520} accumulation (Fig. 5B) and, correspondingly, to retarded channel closure (Fig. 6). Despite the conservative exchange of D vs. E, the D253E mutation led to alterations in the IR difference spectrum of the P_2^{390} state exceeding, by far, those of E123T (*SI Appendix*, Fig. S4). A new positive band at $1,709\text{ cm}^{-1}$ (Fig. 7A) is observed in the IR difference spectrum of D253E in

Fig. 7. Identification of the acceptor of the SB proton. (A) IR difference spectra in the carboxylic region of the P_2^{390} state of E123T (green), D253E (red), and D253N (magenta) variants. Spectra have been normalized to the WT (gray) using the retinal fingerprint region. Band shifts are indicated by arrows, and the vibrational assignment is indicated by hatched areas. (B) Kinetic trace at $1,696\text{ cm}^{-1}$ (light blue trace) in comparison to the rise and decay of the P_2^{390} state (measured at 360 nm) for WT ChR2. (C) Kinetics at $1,713\text{ cm}^{-1}$ (green trace) and of the P_2^{390} state (gray trace) of the E123T variant. (D) Kinetics at $1,710\text{ cm}^{-1}$ (red trace) and of the P_2^{390} state (gray trace) of the D253E variant. The intensity of the IR traces was scaled to match the intensity of the respective traces at 360 nm .

the P_2^{390} state, whose kinetics match the rise of the P_2^{390} state (Fig. 7D). In analogy to the E123T replacement, the newly appearing positive band in the D253E variant is accompanied by a decrease of the area of the band at $1,695\text{ cm}^{-1}$ (Fig. 7A). The pair of bands in the double-difference spectrum between WT and D253E, at $1,712(-)/1,696(+)\text{ cm}^{-1}$ (*SI Appendix*, Fig. S5C), is consistent with the $\nu\text{C=O}$ frequency of protonated E253 and D253, respectively. The IR difference spectrum of the D253N variant showed significant alterations (*SI Appendix*, Fig. S4), most notably in the carboxylic region. First, the $1,717/1,728\text{ cm}^{-1}$ band feature vanished completely (Fig. 7A), suggesting that the D253N replacement also destabilized the protonated form of E90 in dark-state ChR2. Second, a positive band from the proton acceptor did not appear above $1,700\text{ cm}^{-1}$, unlike the case in the functionally active E123T and D253E variants (Fig. 7A). The D253N mutation did not exhibit channel behavior (*SI Appendix*, Fig. S3C) even though the amino acid exchange hardly affected the kinetics of the P_2^{390} and P_3^{520} states (*SI Appendix*, Fig. S7).

Protonation Changes of E90. The negative band at $1,717\text{ cm}^{-1}$ in the difference spectrum of P_4^{480} (Fig. 4A) has previously been assigned to the $\nu\text{C=O}$ of E90 (25). However, the latter work could not resolve the onset of deprotonation of E90 due to insufficient time resolution. The blue trace in Fig. 4C shows that the negative band at $1,717\text{ cm}^{-1}$ reached maximal intensity before $6\text{ }\mu\text{s}$ and decayed in two well-separated phases. The first phase mirrors the kinetics of the positive band at $1,728\text{ cm}^{-1}$ (Fig. 4C, green trace), suggesting a shift in the $\nu\text{C=O}$ of protonated E90 from $1,717$ to $1,728\text{ cm}^{-1}$ early in the photocycle. The deprotonation kinetics have been separated from H-bonding changes of E90 by adding the kinetic traces at $1,728$ and $1,717\text{ cm}^{-1}$ (Fig. 4C, magenta trace), clearly revealing that the deprotonated state of E90 is present only during the lifetime of the P_4^{480} intermediate.

Structural Changes of the Protein Backbone. Conformational and environmental changes in the protein backbone are reflected in frequency changes of the amide I vibrations comprising predominantly the $\nu\text{C=O}$ mode of the peptide backbone (35). Steady-state IR difference spectra recorded under continuous illumination have shown intense bands in the range of α -helices, namely, a negative peak at $1,663\text{ cm}^{-1}$ and a positive peak at $1,648\text{ cm}^{-1}$ (13, 14). Interestingly, the intense negative difference band also appeared when illuminating ChR2 at 80 K , arguing for large structural changes even at cryogenic temperatures. Our time-resolved data confirm the presence of the negative band at $1,663\text{ cm}^{-1}$ that persisted from $6\text{ }\mu\text{s}$ until the end of the photocycle, albeit with varying intensity (Fig. 8A). The intense positive band at $1,650\text{ cm}^{-1}$ is weak at $6\text{ }\mu\text{s}$ but rises in intensity later on. Despite their different initial intensity, the transient intensity changes of the bands at $1,663(-)$ and $1,650(+)\text{ cm}^{-1}$ followed a similar temporal profile, suggesting a frequency downshift of the amide I band (Fig. 8B). The time traces of both bands reached maxima at 1 ms [i.e., between the maximal concentrations of the P_2^{390} ($350\text{ }\mu\text{s}$) and P_3^{520} (4 ms) states]. The maximal photocurrent amplitude in ChR2 is also detected at 1 ms after photoexcitation (9) (Fig. 6). However, the rise of the changes at $1,650\text{ cm}^{-1}$ (Fig. 8B, green trace) lags behind the deprotonation of the SB (Fig. 8B, gray trace), with a $t_{1/2}$ of $60\text{ }\mu\text{s}$ vs. $10\text{ }\mu\text{s}$, respectively. This delay suggests the presence of two P_2^{390} substrates, with the transition between them linked to the onset of protein backbone alterations.

Structural changes are also reflected in the amide II vibration of the protein backbone, a coupled mode of the C–N stretching and N–H in-plane bending vibrations (35). Fig. 8E shows the IR difference spectrum at 300 ms after the laser pulse (i.e., when P_4^{480} dominates). This time was chosen because the ethylenic vibrations of the retinal, overlapping in frequency with the amide II, are reduced in intensity in P_4^{480} here. Indeed, bands are observed at $1,561(+)/1,541(-)\text{ cm}^{-1}$, in the range of α -helical structures

Fig. 8. Changes in amide I and II vibrations of the protein backbone during the ChR2 photocycle. (A) IR differences in the raw spectra (black lines) and after applying FSD (gray lines). Relevant bands are indicated, as well as their integrated areas. (B) Kinetics of the intensity changes at 1,663 cm^{-1} (red trace) and 1,650 cm^{-1} (green trace). Scaled kinetics of P_2^{390} measured at 360 nm on the same sample used for the IR experiments (gray trace). (C) Kinetics of the negative band at 1,663 cm^{-1} probed by integrating the area from 1,671 to 1,641 cm^{-1} (light blue trace). This procedure canceled the overlapping contribution from the hydration of helices by assuming similar amide I extinction coefficients for nonhydrated and hydrated amide groups. The kinetics of the positive band at 1,631 cm^{-1} were determined by tracing the integrated area between 1,636 and 1,625 cm^{-1} (orange trace). (D) Kinetics of the band area integrated between 1,249 and 1,227 cm^{-1} , which reflect ground-state recovery (black trace), and an exponential fit (dashed red trace). (E) IR difference spectrum of P_4^{480} , including the amide I and II region (black line). The FSD-treated spectrum is also shown (gray line). Bands assigned to the hydration of α -helices are labeled.

(35). It was deduced from experiments and simulations that the hydration of α -helices shifts the amide I vibration frequency down by 8–20 cm^{-1} due to H-bonding of water to the amide C=O (36, 37). Correspondingly, a frequency upshift is expected for the amide II frequency, given that the H-bonding of water to the amide C=O will weaken the H-bond between amide C=O and N–H pairs, leading to a stronger N–H bond. Thus, the bands at 1,663(–)/1,650(+) cm^{-1} and at 1,561(+)/1,540(–) cm^{-1} may report the hydration of α -helices during the ChR2 photocycle (i.e., transient access of bulk water to parts of the transmembrane region).

The appearance of the two intense bands at 1,663(–)/1,631(+) cm^{-1} as soon as 6 μs after pulsed laser excitation (Fig. 8A) indicates that protein backbone changes precede the hydration of helices. The two bands exhibit the same kinetics after accounting for overlapping contributions (Fig. 8C). Based on their frequencies, they can be tentatively assigned to the submicrosecond elongation of β -hairpins [1,640–1,625 cm^{-1} (35)] from extramembrane loops [$\sim$ 1,662 cm^{-1} (38)]. However, the negative absorption at 1,663 cm^{-1} may also comprise changes in the $\nu\text{C}=\text{N}$ of the protonated SB, the frequency of which has been assigned to 1,657 cm^{-1} by resonance Raman spectroscopy (18).

There is consensus that the channel of ChR2 closes with P_3^{520} decay ($t_{1/2} \approx 10$ ms) (9, 10). We probed the recovery of the initial dark state by recording the integrated area of the retinal band at 1,240 cm^{-1} [mostly $\text{C}_{12}\text{--C}_{13}$ stretching of ground-state all-*trans* retinal (17)], as done before for bR (39). The kinetics are clearly biphasic, where the dominant phase (75%) proceeded concomitant to P_3^{520} decay, with $t_{1/2} \approx 10$ ms (Fig. 8D). The remaining 25% of the molecules decay $\sim$ 1,000 times slower ($t_{1/2} \approx 20$ s), concomitant to P_4^{480} decay (Fig. 8D). This result indicates that the majority of ChR2 molecules decay directly to the initial dark state without passing the P_4^{480} state, presumably by means of a branch at the level of the P_3^{520} state. These molecules close the channel in the

P_3^{520} -to-ChR2 transition by restoring the initial retinal and protein conformation. The fraction of ChR2 molecules that transit the P_4^{480} state close the channel without reverting most of the conformational changes in the protein backbone, as indicated by the very similar IR differences in the amide I region of the P_3^{520} and P_4^{480} states (Fig. 8A). Notably, the bands assigned to the hydration of transmembrane helices are quantitatively preserved in P_4^{480} , with the 1,663/1,650 cm^{-1} bands showing 25% of their maximal intensity at 300 ms (Fig. 8B).

Discussion

We have traced the molecular changes of photoactivated ChR2 by time-resolved IR difference spectroscopy. By spectral and kinetic analysis, the timing of the protonation and deprotonation of three crucial internal carboxylic groups was determined during opening and closing of the channel. The vibrational assignment was achieved with the help of the mutants E90A, E123T, D156E, D156A, D253E, and D253N. We found that the terminal carboxylate of D253 gets protonated with the rise of the P_2^{390} intermediate (Fig. 7) (i.e., when the retinal SB deprotonates). D156 deprotonates in the P_2^{390} -to- P_3^{520} transition, concomitant to SB reprotonation (Fig. 4A, B, and D). Thus, we assign D253 and D156 to act as the internal proton acceptor and donor of the retinal SB, respectively. Finally, E90 deprotonates only in the P_4^{480} intermediate (Fig. 4A and C).

An acidic residue at position 253 but not at position 123 is conserved in all ChRs sequenced so far (40). Whereas the replacement of D253 by nonprotonatable residues leads to the loss of channel function [D253A in C1C2 (6) and D253N in ChR2; *SI Appendix, Fig. S3*], the functionality of E123 variants is preserved even on mutation to a nonpolar residue like alanine (34). The role of D253 as the primary proton acceptor is further corroborated by the 50-fold accelerated deprotonation kinetics of the

retinal SB in D253E (Fig. 5). The longer side chain of the glutamate residue might place the accepting carboxylate group closer to the positively charged SB, leading to acceleration of proton transfer by electrostatic coupling (41). Such behavior is reminiscent of the D85E mutant in bR (42).

The frequency of the $\nu\text{C=O}$ vibration of protonated D253 is $1,696\text{ cm}^{-1}$, which is low enough to challenge its identification. For such low vibrational energy to occur, the C=O group must interact with an H-bonding donor and the O–H group must be H-bonded to a strong acceptor (29, 30). $\nu\text{C=O}$ frequencies lower than $1,700\text{ cm}^{-1}$ have been reported for carboxylic groups only when the H-bonding donor is the NH_3^+ group from a lysine (29, 30) or when the H-bond acceptor is a COO^- group (43). Thus, we infer that the terminal carboxylate of E123 is the H-bond acceptor of protonated D253 in the P_2^{390} state of ChR2 (Fig. 9A). The strong interaction of the carboxylic O–H group of D253 with the E123 carboxylate is weakened by the E123T replacement, which leads to the observed frequency upshift of the $\nu\text{C=O}$ of protonated D253 from $1,696$ to $1,715\text{ cm}^{-1}$ (Fig. 7 and *SI Appendix, Fig. S5B*). A similar frequency upshift occurs in D253E (Fig. 7 and *SI Appendix, Fig. S5C*), where the aforementioned H-bond with E123 would be geometrically less favorable because of the longer side chain of E253.

The identification of D156 as an internal proton donor to the SB explains the extremely long lifetime of the P_2^{390} intermediate in the D156A variant (7). In the absence of the internal proton donor, SB reprotonation is rate-limiting for the photocycle. We may infer strongly pH-dependent photocycle kinetics in D156A, akin the D96 variants of bR (44). Moreover, we are now in a position to rationalize the kinetic alterations found in variants of C128 (7), a residue H-bonded to D156 to form the DC gate (8). One of the roles of the DC gate could be to raise the pK_a of D156. On C128A and C128T mutations, the deprotonated state of D156 is stabilized, which provides a clue to the long lifetime of the P_3^{520} state in these mutants (7). We suggest that the pK_a of D156 is lowered in C128 variants, even to be deprotonated in ground-state ChR2 at neutral pH, as was observed for D115 in bR by the homologous T90A mutation (45). A partially deprotonated D156 in the dark state, and thus a pH-dependent inactive fraction of the primary proton donor, elegantly explains why a

long-lived P_2^{390} state is also present in C128 mutants and why the P_2^{390} accumulates with increasing pH (7).

The observed proton transfer reactions are put into structural context (Fig. 9B) using the structural model of ChR2 based on recent X-ray crystallographic data of the C1C2 chimera (6). In this scheme, D253 accepts the proton from the SB with a $t_{1/2}$ of $\sim 10\text{ }\mu\text{s}$. The SB is reprotonated from D156 with a $t_{1/2}$ of $\sim 2\text{ ms}$. The latter two groups are $9.7\text{ }\text{\AA}$ apart, which is comparable to the $12.0\text{-}\text{\AA}$ distance between the SB and D96 in bR (46). We recently monitored transient pH changes in the aqueous medium with the help of a pH-indicating dye (47). Proton release to the bulk aqueous medium was detected with the rise of the P_3^{520} state. Because D253 appears to remain protonated in P_3^{520} , we postulate the presence of a proton release group in ChR2.

The recovery kinetics of the initial dark state proceed in two well-separated phases (Fig. 8D), which is explained by branching out of the ChR2 photocycle at the level of the P_3^{520} intermediate. About 75% of the ChR2 molecules relax from P_3^{520} directly to the ground state, taking up a proton from the bulk to reprotonate D156. In the remaining 25% of the population, E90 releases its proton and P_3^{520} relaxes to P_4^{480} . Finally, protons will be taken up from the bulk to reprotonate E90 in the P_4^{480} -to-ChR2 transition. The latter interpretation is corroborated by pH indicator experiments on the E90A variant, where the slow phase of proton uptake was absent (*SI Appendix, Fig. S8*). In the pH indicator experiments on WT ChR2, the slow phase exhibits a relative amplitude of 20% (47), which is close to the 25% derived to transit the P_4^{480} state.

Large changes in amide I bands have been observed during the course of the photocycle, which were previously suggested to reflect conformational changes in the backbone of the transmembrane α -helices (13, 14). Moreover, we found opposite frequency shifts for the amide I and amide II vibrations, which were interpreted to arise from the transient hydration of some transmembrane α -helices. The putative backbone hydration takes place after SB deprotonation at a time that coincides with the onset of channeling activity as reported by time-resolved electrical measurements (9). P_3^{520} is generally accepted to be the conductive state of ChR2 (10); thus, the structural changes reporting the hydration of transmembrane helices appear to take place too early to be related to the opening of the channel. However, compelling evidence for P_2^{390} also being conductive comes from the D156A variant. Although it shows a long-lived P_2^{390} intermediate and barely any P_3^{520} formation, the ion conductance is as high as in C128 mutants that exhibit long-lived P_3^{520} states (7). Thus, we may infer the existence of two consecutive P_2^{390} states with different peptide backbone structures that relate to the opening of the ion gate.

From the X-ray structural model, a pore was suggested to intrude from the extracellular medium, which is framed by transmembrane helices A, B, C, and G (6). E90 (on helix B), H-bonded to N258 (on helix G), is located at the tip of the intruding pore funnel (6). After light excitation, isomerization of all-*trans* retinal leads to weakening of the H-bond between E90 and N258, explaining the 11 cm^{-1} upshift of the $\nu\text{C=O}$ vibration frequency of E90 in the early P_1^{500} state (Fig. 5A and C). The structural consequences of retinal isomerization might reach the extramembrane loops on a submicrosecond scale, leading to the elongation of β -hairpins (Fig. 8C), which are present in both the intracellular and extracellular domains of the C1C2 crystallographic structure (6). The subsequent transfer of the proton from the SB to D253 may lead to tilting of some of the transmembrane helices surrounding the pore as in models proposed for voltage-gated ion channels (48). Mechanistically, this structural change will be driven by the reorganization of intramolecular H-bonding networks, which follow retinal conformation and proton transfer from the SB to D253. In particular, changes in H-bonds involved in interhelical interactions (e.g., those of the DC gate) might be critical to transduce electrostatic changes into pK_a and

Fig. 9. Proton transfers in the ChR2 photocycle. (A) Schematic representation of the proposed direct H-bonding of D253 with E123 in the P_2^{390} state. Other H-bonds are purely tentative. (B) Cartoon of ChR2 based on the C1C2 structure (6), showing relevant carboxylic groups and the proposed proton transfers reactions along with their $t_{1/2}$ times. Proton release and uptake from E90 are arbitrarily set to the intracellular side. The location of the proton release group (PRG) is unknown but tentatively placed in the extracellular domain of ChR2.

conformational changes (16). Indeed, the frequency change of the $\nu\text{C=O}$ of D156 attests to the adaptive response of the DC gate during the different steps of the photocycle (Fig. 4A). The tilt of helices will be sufficient to extend the length of the pore to the cytoplasmic medium, and will also lead to the hydration of transmembrane helices. Once formed, the conductivity and selectivity of the channel will be mostly regulated by residues facing the channel interior.

What remains elusive is the role of the deprotonation of E90, which takes place during the rise of the P_4^{480} state. Closure of the channel during the P_3^{520} -to- P_4^{480} transition does not require the reversal of the large protein structural changes (Fig. 8A). We infer that deprotonation of E90 leads to the transient formation of a hydrogen bond with N258. The hydrogen bond may act as a lock that constrains the dynamics of the adjacent helices framing the channel and, by this means, may help to reduce the ion conductance. Saving the obvious differences, this scenario is reminiscent of the ionic lock between E134, R135, and E247 in bovine visual rhodopsin (49). E134 is ionic in the ground state, and its transient protonation leads to the disruption of the salt bridge, identified as a thermodynamic contributor to the formation of the active state of rhodopsin. Similarly, deprotonation of E90 could contribute to the inhibition of channel conductance on continued illumination (desensitization), which was observed to be reduced (50) or even abolished (25) in E90 variants.

In conclusion, we have traced the sequence of proton transfer reactions in ChR2 by time-resolved IR spectroscopy. D253 was identified as the primary proton acceptor of the retinal SB and D156 as the internal proton donor. Intriguingly, the proton donor and acceptor groups to the retinal SB of ChR2 are different in location from all other microbial rhodopsins known so far.

The observed elementary reactions were related to molecular changes that eventually lead to on- and off-gating of the cation channel. Although neither D253 nor D156 is part of the putative ion channel, mutation of either of these two residues led to the most drastic alterations in the photocycle and channel behavior of ChR2 so far described. Specifically, mutation of D253 to a nonprotonable group prevents channel opening, whereas mutations of D156 strongly delay channel closure. Thus, the proton transfer reactions identified in the present work are clearly rate-limiting to channel gating. Evidently, the change in electrostatic pattern created by internal proton relocation leads to channel opening. Still, the mechanistic link remains elusive. However, ChR2 is unique because it offers the opportunity to study details of gating at high time resolution via triggering and synchronizing channel activity by a short laser pulse. Thus, it is possible to access the structure of intermediate states experimentally during ion permeation across the membrane that are usually hidden.

Materials and Methods

WT ChR2 and the point mutants E90A, E123T, D156E, D156A, D253E, and D253N were heterologously expressed in *Pichia pastoris*. The His-tagged

proteins were solubilized in 0.2% dodecyl maltoside, 100 mM NaCl, and 20 mM Hepes (pH 7.4) as described elsewhere (8, 13). The resulting protein-detergent micelle sufficiently mimics the properties of the lipid bilayer, as was concluded in previous spectroscopic studies (18, 51). For experiments measuring the transient pH changes of E90A, the buffer was substituted with 15 μM bromoxylene blue, a pH indicator (47). Samples used for FTIR spectroscopy were concentrated to ~ 4 mg/mL ChR2 in an aqueous solution of 5 mM NaCl and 5 mM Hepes at pH 7.4, and ~ 8 μL was dried on top of a BaF₂ window. The protein film was rehydrated with the saturated vapor phase of a glycerol/water mixture (3:7 wt/wt) (52). Sample hydration was sufficient, as deduced from the absorbance IR spectrum (SI Appendix, Fig. S9) and from the similar kinetics compared with UV/Vis experiments performed on ChR2 in solution (SI Appendix, Table S2). The hydrated films of ChR2 were placed into the FTIR spectrometer (Vertex 80v; Bruker). Sample holder was kept at a constant temperature by a circulating water bath set to 25 °C (F25; JULABO). Time-resolved UV/Vis experiments were performed on these samples at specific wavelengths and on solubilized ChR2 in solution from 320 to 600 nm in 20-nm steps, using a commercial flash photolysis spectrometer (LKS80; Applied Photophysics). The photoreaction in the IR and UV/Vis experiments was induced by a short laser pulse emitted by a neodymium yttrium aluminum garnet laser (Nd:YAG from Quanta-Ray; Spectra-Physics), which drives an optical parametric oscillator (OPTA). The resulting emission was set to a wavelength of 450 nm with a 10-ns pulse width and a 3-mJ/cm² energy density at the sample, leading to a photoconversion of about 10% of the molecules (47). Time-resolved step-scan FTIR difference spectra were recorded in the time range of 6.25 μs to 125 ms. Rapid-scan FTIR spectroscopy was used to trace the slower kinetic range (90 ms to 95 s). All data were recorded at a spectral resolution of 8 cm⁻¹. The repetition rate of the pulsed laser was set to 0.25 Hz for the step-scan experiments and 0.01 Hz for the rapid-scan experiments, with the former being sufficiently fast to perform the experiments within a reasonable time frame (5 d of data accumulation for WT ChR2) but slow enough to prevent artifacts from excitation of slow photocycle intermediates (SI Appendix, Fig. S11). After data acquisition, the time-resolved IR spectra were quasilogarithmically averaged to 20 spectra per decade and offset to zero in the range of 1,820–1,780 cm⁻¹. The step-scan and rapid-scan data were merged and noise-filtered by singular value decomposition (SVD), retaining the first five major components (SI Appendix, Fig. S12). FSD and global fitting were applied, as described in SI Appendix, SI Materials and Methods. The flash photolysis experiments in the UV/Vis range were performed on two time scales, with the faster time range (<300 μs) recorded with the light source (Xe arc lamp) in pulsed mode and the slower time scale (>30 μs) recorded with the lamp in continuous operation. Repetition frequency of the photolysis laser was 0.01 Hz. The kinetic data were quasilogarithmic-averaged (100 points per decade) and noise-reduced by means of SVD by retaining the first five major components. Difference spectra were generated by spline interpolation between the measured absorption changes at each wavelength (a detailed description of the acquisition, processing, and analysis of the time-resolved FTIR data and of the electrophysiological recordings is provided in SI Appendix, SI Materials and Methods).

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- Nagel G, et al. (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci USA* 100(24):13940–13945.
- Nagel G, et al. (2002) Channelrhodopsin-1: A light-gated proton channel in green algae. *Science* 296(5577):2395–2398.
- Zhang F, et al. (2011) The microbial opsin family of optogenetic tools. *Cell* 147(7):1446–1457.
- Spudis JL (2006) The multitasking microbial sensory rhodopsins. *Trends Microbiol* 14(11):480–487.
- Müller M, Bamann C, Bamberg E, Kühlbrandt W (2011) Projection structure of channelrhodopsin-2 at 6 Å resolution by electron crystallography. *J Mol Biol* 414(1):86–95.
- Kato HE, et al. (2012) Crystal structure of the channelrhodopsin light-gated cation channel. *Nature* 482(7385):369–374.
- Bamann C, Gueta R, Kleinlogel S, Nagel G, Bamberg E (2010) Structural guidance of the photocycle of channelrhodopsin-2 by an interhelical hydrogen bond. *Biochemistry* 49(2):267–278.
- Nack M, et al. (2010) The DC gate in Channelrhodopsin-2: Crucial hydrogen bonding interaction between C128 and D156. *Photochem Photobiol Sci* 9(2):194–198.
- Bamann C, Kirsch T, Nagel G, Bamberg E (2008) Spectral characteristics of the photocycle of channelrhodopsin-2 and its implication for channel function. *J Mol Biol* 375(3):686–694.
- Stehfest K, Hegemann P (2010) Evolution of the channelrhodopsin photocycle model. *Chemphyschem* 11(6):1120–1126.
- Verhoeven MK, et al. (2010) The photocycle of channelrhodopsin-2: Ultrafast reaction dynamics and subsequent reaction steps. *Chemphyschem* 11(14):3113–3122.
- Feldbauer K, et al. (2009) Channelrhodopsin-2 is a leaky proton pump. *Proc Natl Acad Sci USA* 106(30):12317–12322.
- Radu I, et al. (2009) Conformational changes of channelrhodopsin-2. *J Am Chem Soc* 131(21):7313–7319.
- Ritter E, Stehfest K, Berndt A, Hegemann P, Bartl FJ (2008) Monitoring light-induced structural changes of Channelrhodopsin-2 by UV-visible and Fourier transform infrared spectroscopy. *J Biol Chem* 283(50):35033–35041.
- Heberle J (2000) Proton transfer reactions across bacteriorhodopsin and along the membrane. *Biochim Biophys Acta* 1458(1):135–147.

16. Perálvarez-Marín A, et al. (2007) Inter-helical hydrogen bonds are essential elements for intra-protein signal transduction: The role of Asp115 in bacteriorhodopsin transport function. *J Mol Biol* 368(3):666–676.
17. Smith SO, Lugtenburg J, Mathies RA (1985) Determination of retinal chromophore structure in bacteriorhodopsin with resonance Raman spectroscopy. *J Membr Biol* 85(2):95–109.
18. Nack M, Radu I, Bamann C, Bamberg E, Heberle J (2009) The retinal structure of channelrhodopsin-2 assessed by resonance Raman spectroscopy. *FEBS Lett* 583(22):3676–3680.
19. Aton B, Doukas AG, Callender RH, Becher B, Ebrey TG (1977) Resonance Raman studies of the purple membrane. *Biochemistry* 16(13):2995–2999.
20. Dioumaev AK, Braiman MS (1997) Two bathointermediates of the bacteriorhodopsin photocycle, distinguished by nanosecond time-resolved FTIR spectroscopy at room temperature. *J Phys Chem B* 101(9):1655–1662.
21. Hackmann C, et al. (2001) Static and time-resolved step-scan Fourier transform infrared investigations of the photoisomerization of halorhodopsin from *Natronobacterium pharaonis*: Consequences for models of the anion translocation mechanism. *Biophys J* 81(1):394–406.
22. Hein M, Wegener AA, Engelhard M, Siebert F (2003) Time-resolved FTIR studies of sensory rhodopsin II (NpSRII) from *Natronobacterium pharaonis*: Implications for proton transport and receptor activation. *Biophys J* 84(2):1208–1217.
23. Friedrich T, et al. (2002) Proteorhodopsin is a light-driven proton pump with variable vectoriality. *J Mol Biol* 321(5):821–838.
24. Kawanabe A, Furutani Y, Jung KH, Kandori H (2006) FTIR study of the photoisomerization processes in the 13-*cis* and all-*trans* forms of *Anabaena* sensory rhodopsin at 77 K. *Biochemistry* 45(14):4362–4370.
25. Eisenhauer K, et al. (2012) In channelrhodopsin-2 Glu-90 is crucial for ion selectivity and is deprotonated during the photocycle. *J Biol Chem* 287(9):6904–6911.
26. Rothschild KJ, Zagaeski M, Cantore WA (1981) Conformational changes of bacteriorhodopsin detected by Fourier transform infrared difference spectroscopy. *Biochem Biophys Res Commun* 103(2):483–489.
27. Engelhard M, Gerwert K, Hess B, Kreutz W, Siebert F (1985) Light-driven protonation changes of internal aspartic acids of bacteriorhodopsin: An investigation by static and time-resolved infrared difference spectroscopy using [4-¹³C]aspartic acid labeled purple membrane. *Biochemistry* 24(2):400–407.
28. Braiman MS, et al. (1988) Vibrational spectroscopy of bacteriorhodopsin mutants: Light-driven proton transport involves protonation changes of aspartic acid residues 85, 96, and 212. *Biochemistry* 27(23):8516–8520.
29. Nie B, Stutzman J, Xie A (2005) A vibrational spectral maker for probing the hydrogen-bonding status of protonated Asp and Glu residues. *Biophys J* 88(4):2833–2847.
30. Takei K, Takahashi R, Noguchi T (2008) Correlation between the hydrogen-bond structures and the C=O stretching frequencies of carboxylic acids as studied by density functional theory calculations: Theoretical basis for interpretation of infrared bands of carboxylic groups in proteins. *J Phys Chem B* 112(21):6725–6731.
31. Kauppinen JK, Moffatt DJ, Mantsch HH, Cameron DG (1981) Fourier self-deconvolution: A method for resolving intrinsically overlapped bands. *Appl Spectrosc* 35(3):271–276.
32. Barth A (2000) The infrared absorption of amino acid side chains. *Prog Biophys Mol Biol* 74(3-5):141–173.
33. Sasaki J, et al. (1995) Conversion of bacteriorhodopsin into a chloride ion pump. *Science* 269(5220):73–75.
34. Gunaydin LA, et al. (2010) Ultrafast optogenetic control. *Nat Neurosci* 13(3):387–392.
35. Goormaghtigh E, Cabiliaux V, Ruysschaert JM (1994) Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. I. Assignments and model compounds. *Subcell Biochem* 23:329–362.
36. Walsh ST, et al. (2003) The hydration of amides in helices; a comprehensive picture from molecular dynamics, IR, and NMR. *Protein Sci* 12(3):520–531.
37. Paschek D, et al. (2008) The solvent-dependent shift of the amide I band of a fully solvated peptide as a local probe for the solvent composition in the peptide/solvent interface. *Chemphyschem* 9(18):2742–2750.
38. Lórenz-Fonfría VA, et al. (2003) Structural and functional implications of the instability of the ADP/ATP transporter purified from mitochondria as revealed by FTIR spectroscopy. *Biophys J* 85(1):255–266.
39. Lórenz-Fonfría VA, Kandori H, Padrós E (2011) Probing specific molecular processes and intermediates by time-resolved Fourier transform infrared spectroscopy: Application to the bacteriorhodopsin photocycle. *J Phys Chem B* 115(24):7972–7985.
40. Watanabe HC, et al. (2012) Structural model of channelrhodopsin. *J Biol Chem* 287(10):7456–7466.
41. Li H, Robertson AD, Jensen JH (2005) Very fast empirical prediction and rationalization of protein pK_a values. *Proteins* 61(4):704–721.
42. Heberle J, Oesterheld D, Dencher NA (1993) Decoupling of photo- and proton cycle in the Asp85→Glu mutant of bacteriorhodopsin. *EMBO J* 12(10):3721–3727.
43. Iliadis G, Zundel G, Brzezinski B (1994) Aspartic proteinases—Fourier transform IR studies of the aspartic carboxylic groups in the active site of pepsin. *FEBS Lett* 352(3):315–317.
44. Otto H, et al. (1989) Aspartic acid-96 is the internal proton donor in the reprotonation of the Schiff base of bacteriorhodopsin. *Proc Natl Acad Sci USA* 86(23):9228–9232.
45. Perálvarez-Marín A, Márquez M, Bourdelande JL, Querol E, Padrós E (2004) Thr-90 plays a vital role in the structure and function of bacteriorhodopsin. *J Biol Chem* 279(16):16403–16409.
46. Luecke H, Schobert B, Richter HT, Cartailler JP, Lanyi JK (1999) Structure of bacteriorhodopsin at 1.55 Å resolution. *J Mol Biol* 291(4):899–911.
47. Nack M, et al. (2012) Kinetics of proton release and uptake by channelrhodopsin-2. *FEBS Lett* 586(9):1344–1348.
48. Tombola F, Pathak MM, Isacoff EY (2006) How does voltage open an ion channel? *Annu Rev Cell Dev Biol* 22:23–52.
49. Vogel R, et al. (2008) Functional role of the “ionic lock”—An interhelical hydrogen-bond network in family A heptahelical receptors. *J Mol Biol* 380(4):648–655.
50. Sugiyama Y, et al. (2009) Photocurrent attenuation by a single polar-to-nonpolar point mutation of channelrhodopsin-2. *Photochem Photobiol Sci* 8(3):328–336.
51. Stehfest K, Ritter E, Berndt A, Bartl F, Hegemann P (2010) The branched photocycle of the slow-cycling channelrhodopsin-2 mutant C128T. *J Mol Biol* 398(5):690–702.
52. Noguchi T, Sugiura M (2002) Flash-induced FTIR difference spectra of the water oxidizing complex in moderately hydrated photosystem II core films: Effect of hydration extent on S-state transitions. *Biochemistry* 41(7):2322–2330.