Proton transfer reactions in the red light-activatable channelrhodopsin variant ReaChR and their relevance for its function

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Channelrhodopsins (ChRs) are light-gated ion channels widely used for activating selected cells in large cellular networks. ChR variants with a red-shifted absorption maximum, such as the modified Volvox carteri ChR1 red-activatable channelrhodopsin (“ReaChR,” \( \lambda_{\text{max}} \) = 527 nm), are of particular interest because longer wavelengths allow optical excitation of cells in deeper layers of organic tissue. In all ChRs investigated so far, proton transfer reactions and hydrogen bond changes are crucial for the formation of the ion-conducting pore and the selectivity for protons versus cations, such as Na\(^+\), K\(^+\), and Ca\(^{2+}\) (1). By using a combination of electrophysiological measurements and UV-visible and FTIR spectroscopy, we characterized the proton transfer events in the photocycle of ReaChR and describe their relevance for its function. 1) The central gate residue Glu\(^{130}\) (Glu\(^{256}\) in Chlamydomonas reinhardtii (Cr) ChR2) undergoes a hydrogen bond change in \( \text{D} \to \text{K} \) transition and (ii) deprotonates in \( \text{K} \to \text{M} \) transition. Its negative charge in the open state is decisive for proton selectivity. 2) The counter-ion Asp\(^{229}\) (Asp\(^{254}\) in CrChR2) receives the retinal Schiff base proton during M-state formation. Starting from \( \text{M} \), a photocycle branching occurs involving (i) a direct \( \text{M} \to \text{D} \) transition and (ii) formation of late photointermediates \( \text{N} \) and \( \text{O} \). 3) The DC pair residue Asp\(^{196}\) (Asp\(^{216}\) in CrChR2) deprotonates in \( \text{N} \to \text{O} \) transition. Interestingly, the D196N mutation increases 15-syn-retinal at the expense of 15-anti, which is the predominant isomer in the wild type, and abolishes the peak current in electrophysiological measurements. This suggests that the peak current is formed by 15-anti species, whereas 15-syn species contribute only to the stationary current.

The concept of parallel 15-anti and 15-syn photocycles also delivers an explanation for the observed isomeric light-dark adaptation of ChRs by assuming transitions between the different subspecies by C13=C14 and C15=N double isomerization: the initial dark state (IDA) of ChRs, which is formed after hours of dark adaptation, adopts a pure all-trans-retinal isomer according to recent NMR studies (16, 17). The apparent dark state (DA\(_{\text{app}}\)) instead is formed within minutes after recovery of the illuminated state and represents a mixture of \( 13\text{-trans},15\text{-anti} \) (D) and 13-cis,15-syn-retinal (D’) as shown by retinal extraction and subsequent HPLC analysis (15, 18, 19) and resonance Raman experiments (16, 18, 19). Although the parallel

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6 The abbreviations used are: ChR, channelrhodopsin; C1C2, chimera of CrChR1 and CrChR2 from C. reinhardtii; CaChR1, Chlamydomonasaugustae channelrhodopsin-1; C1, counter-ion 1 (Glu\(^{130}\) in ReaChR); C2, counter-ion 2 (Asp\(^{229}\) in ReaChR); O1, C. reinhardtii channelrhodopsin-1; CrChR2, C. reinhardtii channelrhodopsin-2; D, 13-trans,15-anti-retinal; D’, 13-cis,15-syn-retinal; DA\(_{\text{app}}\), apparent dark state; IDA, initial dark state; NMG, N-methyl-D-glucamine; P4ChR2, P. subcordiformis channelrhodopsin-2; ReaChR, red-activatable channelrhodopsin; RSB, retinal Schiff base; RSBH\(^{+}\), protonated retinal Schiff base; SVD, singular value decomposition; \( I_p \), transient photocurrent; \( I_{s} \), stationary photocurrent.
Proton transfer reactions in ReaChR

The light-induced photocycles of ChRs involve a number of changes in hydrogen bonding and proton transfers that affect a variety of channel properties, such as ion selectivity, conductivity, and photocycle kinetics. The photocycle of the best studied ChR, ChR2 from Chlamydomonas reinhardtii (CrChR2), involves proton dynamics of the central gate residue Glu\textsuperscript{130} (Glu\textsuperscript{130} in ReaChR; Fig. 1), the counter-ion residue Asp\textsuperscript{253} (Asp\textsuperscript{293} in ReaChR), and the DC pair residue Asp\textsuperscript{196} (Asp\textsuperscript{196} in ReaChR). Glu\textsuperscript{90} plays a major role for both formation of the conducting pore and ion selectivity (20, 21). Asp\textsuperscript{253}, in the following referred to as Ci2 (counter-ion 2), receives the RSBH\textsuperscript{+} proton that is released prior to channel opening (22). Alternatively, it was proposed that both Ci1 (Glu\textsuperscript{123}) and Ci2 (Asp\textsuperscript{253}) serve as proton acceptors (21). Furthermore, it was suggested that the DC pair residue Asp\textsuperscript{196} reprotonates the RSB in the P390 → P520 transition (M → N in ReaChR) (22) as mutations of this residue highly decelerate the photocycle kinetics (23). However, it is unclear whether the proton dynamics of CrChR2 also apply to red-shifted ChRs, such as ReaChR, as the bathochromically shifted absorption maximum points to molecular alterations near the retinal-binding pocket and the active site as compared with CrChR2; e.g. threonine 159, in the direct environment of Asp\textsuperscript{196}, is exchanged for a cysteine in ReaChR (Cys\textsuperscript{199}).

The present study, based on a combination of FTIR difference spectroscopy, time-resolved UV-visible spectroscopy, electrophysiological measurements, and site-directed mutagenesis, showed that Glu\textsuperscript{130}, Ci2, and the DC pair residue Asp\textsuperscript{196} (Fig. 1) also experience proton dynamics in ReaChR. Our data show that the Glu\textsuperscript{130}-helix 2-tilt-model for CrChR2 (21) applies to ReaChR as well. Additionally, we now present a more detailed mechanism for ReaChR and show that deprotonation of Glu\textsuperscript{130} occurs in two distinct mechanistic steps. It undergoes a change in hydrogen bonding in the D → K transition and deprotonates before formation of the M-state. Consistent with CrChR2, the M-state (P390 in CrChR2) is formed by a proton transfer from the RSBH\textsuperscript{+} to Ci2, but after M formation a photocycle branching occurs that was not observed in CrChR2 WT. The main branch involves M → N → O transitions, and water is likely to serve as the proton donor for the reprotonation of the RSB during M → N transition because no amino acid residue could be identified as proton donor. In parallel, a direct M → D transition, involving reprotonation of the RSB by Ci2, takes place.

In either case, Asp\textsuperscript{196} does not serve as the proton donor for reprotonation of the RSB in ReaChR as its homologue Asp\textsuperscript{156} in CrChR2 does (22). Instead, Asp\textsuperscript{196} deprotonates in the N → O transition. Neutralization of Asp\textsuperscript{156} as in the D196N mutant increases D\textsuperscript{-} at the expense of D under conditions of continuous illumination and abolishes the peak current in electrophysiological measurements. This interesting correlation provides experimental evidence for the concept that the peak current is mainly formed by a conducting state with 13-cis,15-anti-retinal conformation, whereas the stationary current contains contribu-
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copy and compared with the wild type (11). The IDA, i.e. the state before any light exposure of the protein, is slightly red-shifted for the counter-ion mutants, E163T (λ_{max} = 530 nm) and D293N (λ_{max} = 528 nm), with respect to the wild type (λ_{max} = 527 nm; Fig. 3A). However, the bathochromic shift is minor compared with CrChR2, C1C2, and ChR2 from *Platy- monas subcordiformis* (PsChR2), which show 10–25-nm shifts upon counter-ion neutralization (29, 30). In contrast, the IDA of E130Q (λ_{max} = 513 nm) and D196N (λ_{max} = 523 nm) are blue-shifted, which was not reported for analogous mutations in CrChR2 (23, 31). Upon extended illumination (60 s, 530 nm) and subsequent recovery in the dark (10 min), the DA app is formed. Corresponding UV-visible spectra of all samples recorded upon 500-ms illumination.

To further evaluate this interpretation, FTIR difference spectra of the wild type (gray filled curves), E130Q, E163T, D293N, and D196N were recorded (Fig. 3B). In the retinal fingerprint region, two negative bands at 1234 and 1199 cm^{-1} in the WT spectrum reflect depletion of the 13-cis,15-anti dark state (D) (18, 36), and the positive band at 1176 cm^{-1} is assigned to 13-trans-retinal (37–39). This band pattern is only slightly altered by mutations of Glu130, Ci1, and Ci2. However, in the D196N spectrum, an additional negative band at 1183 cm^{-1} in the D196N mutant. Additionally, a more pronounced positive band at 1173 cm^{-1} points to the 13-trans photoproduction of a photoconverted D' state. This finding implies that 1) DA app is composed of a mixture of 13-trans,15-anti-retinal (D) and 13-cis,15-syn-retinal (D') isomers and 2) the contribution of the syn conformation (D') to DA app is relatively increased in the D196N mutant. D and D', which together form DA app, undergo light-induced photocycles comprising the intermediates K, L, M, N, and O as well as K', L', M', N', and O', respectively (Fig. 3C, left) (11). Due to similar absorption maxima, which are not more
than 10 nm shifted (39), it is not a simple task to separately investigate both photocycles. Additionally, the contribution of the syn photocycle is small as compared with the anti photocycle because 1) ReaChR WT populates only around 20% of D/H11032 in DAapp (11) and 2) a lower quantum efficiency of the photoactivation of the 13-cis,15-syn D/H11032 is assumed (43). Therefore, the observed photocycle dynamics and half-life times are primarily assigned to the anti photocycle branch. The influence of key residues on the photocycle was studied by flash photolysis experiments on the mutants E130Q, E163T, D196N, and D293N (Fig. 3, D–G). Purified proteins were excited with green laser flashes (10 ns, 530 nm, 5 mJ), and UV-visible absorption changes were recorded. Half-life times (t_{1/2}) were derived from global analysis (table in Fig. 3C; wild type values are taken from Figure 3. UV-visible spectroscopy of ReaChR WT and the mutants E130Q, E163T, D293N, and D196N. IDA and DA app spectra of recombinant wild type and mutants at pH 7.4 are shown in A. The wild type IDA spectrum is adapted from Ref. 11. DA app is formed upon extended illumination (60 s, 530 nm) and subsequent recovery in the dark (10 min). The observed spectral shifts from IDA to DA app indicate that the dark-state composition is altered upon first illumination. The significant blue shift of DA app of D196N as compared with WT can be explained by the retinal fingerprint region in FTIR spectra of ReaChR wild type (gray filled curves) and the mutants E130Q, D196N, E163T, and D293N (B). D196N shows an additional negative band at 1183 cm\(^{-1}\) and a strong positive band at 1173 cm\(^{-1}\), hinting to a relative gain in 15-syn-retinal isomer at the expense of 15-anti-retinal in comparison with the wild type. C, the proposed ReaChR photocycle comprises two branches, a main branch starting from 13-trans,15-anti-retinal (D) and a side branch starting from 13-cis,15-syn-retinal (D'), which together form DA app (11). In either branch, light induces a photoreaction involving photointermediates K, L, M, N, and O as well as K', L', M', N', and O', respectively. Half-life times (t_{1/2}) of the evolution-associated difference spectra are derived by global analysis of flash photolysis data (D–G) using a sequential model comprising four or five components. The values of t_{1/2} are summarized in the table (C, right); life-times of the wild type are taken from Ref. 11. Transient absorption changes (in mOD) induced by green laser flashes (10 ns, 530 nm, 5 mJ) were measured for E130Q (D), E163T (E), D196N (F), and D293N (G) for at least 15 cycles from 40 ns to 10 s. Single-wavelength kinetics of E163T and D293N are illustrated in H and I, respectively. Raw data (dots) and global fits (lines) are shown. Wild type data are taken from Ref. 11 (J), abs., absorbance; norm., normalized.
Monitoring proton transfer processes and changes in hydrogen bonding by FTIR difference spectroscopy

The formation of the photocycle intermediates is accompanied by changes of hydrogen bonding and/or proton transfer processes involving acidic and alkaline amino acid side chains. To address the question in which stage of the photocycle these events occur and to elucidate the role of specific amino acids, FTIR difference spectra of WT and selected mutants were recorded at cryogenic temperatures to stabilize photocycle intermediates. The steady-state spectrum obtained at 80 K mainly represents the transition from the dark state to the early K intermediate (11). Fig. 4A shows the FTIR difference spectra of the D → K transition in H₂O (gray filled curves) and in D₂O buffer (black) of ReaChR WT and the mutants E130Q, E163T, D293N, and D196N.

Early proton dynamics in Glu₁³₀

Bands in the spectral region between 1800 and 1700 cm⁻¹ are predominantly caused by carbonyl C=O stretching modes of protonated acidic amino acid side chains, and therefore corresponding difference bands reflect changes in hydrogen bonding strength and/or proton transfer processes. In the WT spectrum (Fig. 4A), a prominent difference band at 1729(+/−)1721(−) cm⁻¹ and a positive band at 1713 cm⁻¹ are observed. In D₂O, the negative band at 1721 cm⁻¹ is downshifted to 1713 cm⁻¹, whereas its positive counterpart at 1729 cm⁻¹ is shifted to 1721 cm⁻¹. However, this band maximum is superimposed by the negative band at 1721 cm⁻¹ due to incomplete deuteration so that two apparent maxima at 1725 and 1719 cm⁻¹ are seen. The band intensities and positions of the 1729(+/−)/1721(−) cm⁻¹ difference band in the wild type are only slightly affected by mutations of the counter-ions, E163T and D293N, or by the DC pair mutation D196N. As this difference band is completely absent in E130Q, it is assigned to a vibration of Glu₁³₀. The characteristic pattern with one positive and one negative band suggests that Glu₁³₀ undergoes a hydrogen bond change in the D → K transition rather than a deprotonation, which would be indicated by only one single negative band.

The impact of the D196N mutation on this difference band might be explained by its influence on the ratio of 15-anti and 15-syn retinal (see Fig. 3B). The slight effect of E163T and D293N on the Glu₁³₀ difference band is explained by an altered interaction of these residues with Glu₁³₀. In the E163T spectrum, a negative band at 1712 cm⁻¹ instead of the positive WT band at 1713 cm⁻¹ is observed. The origin of the positive wild type band is unclear; however, protonation of Ci1 is unlikely because in that case the positive band would be absent in the spectrum of the E163T mutant rather than being replaced by a negative band.
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Proton transfer reactions in later photocycle intermediates

In the FTIR difference spectrum of the wild type at 293 K representing the O-state with minor contributions of M, N, and L (Fig. 4B, gray filled curve), we observe a band pattern at 1753(−)/1738(+)/1723(−) cm\(^{-1}\). The spectra of the mutants (Fig. 4B) support the assignment of the 1723 cm\(^{-1}\) band to Glu\(^{130}\) because it is absent only in E130Q. In D\(_2\)O, this negative band is downshifted by 11 cm\(^{-1}\) to 1712 cm\(^{-1}\). The residual band at 1723 cm\(^{-1}\) indicates only partial sample deuteration. The existence of only one band assigned to Glu\(^{130}\) instead of a difference band as observed in the spectrum at 80 K (Fig. 4A) now shows deprotonation of Glu\(^{130}\). Deprotonation of the homologous residue Glu\(^{90}\) was observed by various authors for the photocycle of CrChR2, although it is still under debate in which intermediate this deprotonation occurs (20–22).

The positive band at 1738 cm\(^{-1}\) is affected by all mutants analyzed. Its intensity is significantly reduced in both the E163T and D293N mutants and thus reflects protonation of Ci2 during M-state formation. The influence of both mutations on the 1738 cm\(^{-1}\) band can be explained by an accelerated proton release from the unmutated counter-ion residue due to a lowered pK\(_a\) value if the other respective residue is mutated. In E130Q, instead of one positive band at 1738 cm\(^{-1}\), two positive bands arise at 1739 and 1731 cm\(^{-1}\). This observation is explained by an overlap of the positive counter-ion band with a negative band that arises in E130Q only, possibly due to deprotonation of a so far unidentified residue.

In the spectrum of the D196N mutant, the band at 1738 cm\(^{-1}\) is upshifted to 1748 cm\(^{-1}\). The high upshift of this band can be explained by the influence of this mutation on the ratio of the 15-anti and 15-syn photocycle branches (see Fig. 3B), leading to a weaker chromophore–counter-ion interaction in line with the accumulation of the red-shifted N-intermediate in the steady state (Fig. 3F).

At 293 K, a negative band arises at 1753 cm\(^{-1}\) in the WT spectrum that is not observed in the spectrum of any mutant. An assignment of this band is nevertheless possible based on the spectra at 260 K. In these spectra, this vibration of the WT is slightly altered in E130Q, E163T, and D293N, but D196N is the only mutant in which it is not observed (Fig. 4B, inset column). Thus, we assign this band to a vibration of Asp\(^{196}\), reflecting deprotonation of this residue. Interestingly, this band is not D\(_2\)O-sensitive, indicating that Asp\(^{196}\) is buried within the receptor and not accessible to bulk water, similar to observations made for C1C2 (36). The absence of this band in any mutant at 293 K indicates that Asp\(^{196}\) is already reprotonated in the steady state of the mutants at this temperature, which is in agreement with faster photocycle progression of the mutants compared with WT (Fig. 3C).

M-state-related proton transfer

Although proton transfer reactions involving Glu\(^{130}\), the counter-ion complex, and Asp\(^{196}\) were shown to occur in the photocycle of ReaChR, it is still unclear in which photocycle intermediate(s) these transfers actually occur. Accordingly, we investigated proton transfers related to M-state formation and decay. The slow-cycling DC pair mutant C168S (C128S in CrChR2) is suitable to investigate proton transfer reactions in the M-state because it highly accumulates M upon illumination (60 s, 530 nm) in the steady state at 293 K (Fig. 5A, inset). In the FTIR difference spectra of C168S in H\(_2\)O (orange) and D\(_2\)O (magenta) reflecting D → M transition (Fig. 5A), the positive band at 1738 cm\(^{-1}\), which was assigned to the protonation of Ci2 (Fig. 4B), is split into two positive bands arising at 1742 and 1729 cm\(^{-1}\), similar to the E130Q mutant (Fig. 4B). This observation can again be explained by an overlap of a single positive counter-ion band with an additional negative band arising in the M-state. The negative band at 1721 cm\(^{-1}\) assigned to deprotonation of Glu\(^{130}\) is downshifted to 1711 cm\(^{-1}\) in D\(_2\)O, similar to WT. This indicates that Glu\(^{130}\) deprotonates at the latest during M-formation. An early deprotonation of the homologous residue Glu\(^{90}\) was observed during the photocycle of CrChR2 (21). Asp\(^{196}\) is not deprotonated in M because the spectra lack the negative band at 1753 cm\(^{-1}\).

Ion conductance of channelrhodopsins can be rapidly switched on and off with alternating illumination of suitable wavelengths as shown in electrical studies (44, 45). We applied a similar illumination protocol with alternating green (520 nm) and UV (390 nm) light to the wild type. To estimate the independent spectral components contributing to the signal change, we evaluated the results using a combination of singular value decomposition (SVD) and a rotation procedure (46, 47) as performed for CrChR2-C128T (15) (Fig. 5B and see “Experimental procedures” for details). The first component represents formation of the late intermediates, mainly O, which can be derived from similarity of this spectral component to the steady-state spectrum at 293 K (Fig. 4B, gray filled curves). This component displays a negative band at 1753 cm\(^{-1}\), indicating deprotonation of Asp\(^{196}\). It arises with green illumination, remains throughout the whole illumination period, and slowly decays after the end of the illumination. A further component strictly follows the illumination protocol. Green illumination induces a steady state including M, which can be depopulated by UV light, so that formation and decay of M, which contains contributions of both M\(_1\) and M\(_2\) (see Fig. 3, H–J), can be directly observed. This component involves a difference band at 1766(+)/1756(−) cm\(^{-1}\), which demonstrates a change of hydrogen bonding of still protonated Asp\(^{196}\) coincident with formation and decay of M, whereas the negative band in the first component confirms later deprotonation of Asp\(^{196}\), i.e. after formation of the N-state. Thus, it can be excluded that Asp\(^{196}\) serves as proton donor for the RSB during M decay in contrast to the homologue Asp\(^{156}\) in CrChR2 (22). Another likely candidate for reprotonation of the RSB is Ci2, which receives the RSBH\(^{−}\) proton during M formation. Indeed, the component following the illumination protocol comprises a band pattern with two maxima at 1744 and 1731 cm\(^{-1}\) that was already assigned to Ci2 protonation (see Fig. 5A). This shows that Ci2 receives a proton during M formation and releases it during light-induced decay. In contrast, the band at 1737 cm\(^{-1}\) in the first component shows that Ci2 remains partially protonated in the O state. This finding shows that a photocycle branching takes place after M formation, involving a light-induced shortcut from M to D, as observed earlier in ReaChR (11).
To elucidate the role of the observed proton transfers during the thermal $M\rightarrow D$ and $O\rightarrow D$ transitions, respectively, we investigated the decay process starting from the steady state at 293 K (Fig. 5C). As revealed by SVD and rotational analysis, two components, which were fitted by a global analysis procedure using a biexponential function (31), contributed to the decay process. The first decay component, which almost mirrors the component following alternating green and UV illumination (Fig. 5B) and thus reflects the direct thermal $M\rightarrow D$ transition involving $C_{i2}$ deprotonation, comprises a band at 1731 cm$^{-1}$ that implies late deprotonation of $C_{i2}$. These findings support the above mentioned concept that the photocycle branches after the $M$-state, involving 1) a direct $M\rightarrow D$ transition where the RSB is presumably reprotonated by $C_{i2}$ and 2) a late $O\rightarrow D$ transition. In the latter case, both $A_{s}p^{196}$ and $C_{i2}$ are excluded as proton donors to the RSB.

Discussion

In this study, the electrical properties and the photocycle dynamics of ReaChR WT were compared with properties of selected mutants by a combined spectroscopic and electrophysiological approach. In the following, the key proton transfers are characterized with respect to their functional relevance (Fig. 6).

Glu$^{130}$ is a determinant for ion selectivity

Our observation that replacement of Glu$^{130}$ by Gln reduces H$^+$ conductance in favor of Na$^+$ (Fig. 2D) raised questions about the dynamics of this residue during the photocycle, which were then addressed by spectroscopic measurements. We show that Glu$^{130}$ undergoes a hydrogen bond change with formation and decay of $M$, $C$, $C=O$ stretching region of the steady state of the wild type at 293 K and its two decay components revealed by SVD and a rotational analysis and fitted by a global fit using a biexponential function ($R^2 > 0.99$). Time constants are indicated below the respective components. Dark-state recovery is accomplished by a fast and a slow transition that occur in parallel: the fast decay component mainly represents $M\rightarrow D$ transition, which is inferred from its similarity to the second component in B, whereas the slow decay component represents $O\rightarrow D$ transition.
Glu130 is linked to ion selectivity. We showed that, upon light activation of ReaChR, Glu130 deprotonates, and therefore a negative charge close to the chromophore is neutralized, and the negative charge close to the chromophore is neutralized, and the electrostatic interaction of deprotonated Asp196 with the retinal polyene chain facilitates the back-reaction from 15-anti to 15-syn-retinal during dark adaptation. Due to the D196N mutation, this negative charge at the chromophore is neutralized, and the syn → anti reaction is impaired. Moreover, the DC pair residues or other amino acid pairs (DT in bacteriorhodopsin) at this position in other microbial rhodopsins form a hydrogen bond between helix 3 and helix 4 that is essential for the protein stability and the retinal-binding pocket in particular. Destruction of this bond in many cases destabilizes the retinal-binding pocket and the protein stability in general (54), resulting in a slowdown of both M formation and decay, and might also alter the syn/anti ratio accordingly.

Asp196 is a determinant of dark adaptation

The two-photocycle model, comprising two closed (D and D’) and two conducting states, was proposed to explain the photocurrent kinetics and amplitudes of the transient and photostationary photocurrents (1, 12–14, 51). This model was supported by spectroscopic data (52) and chromophore isomer analysis and extended with the finding that during one photocycle the retinal remains in 15-anti conforma- tion and during the other the retinal remains in 15-syn conformation (11, 15, 16). Similarly, in bacteriorhodopsin, two different dark states exist, BR548 with 13-cis,15-syn-retinal and BR668 with 13-trans,15-anti-retinal (32, 53). Based on the observed differences between IDA and DAapp (Fig. 3A), we assumed that this applies to ReaChR as well. The photocycle of ReaChR is anticipated to start from IDA with 100% 13-trans,15-anti-retinal (D) by both trans/cis and, with lower efficiency, C13=C14 and C15=N double isomerization of the retinal (16). Incomplete thermal back-reaction leads to an altered syn/anti ratio in DAapp as compared with IDA (see Fig. 3A). This effect is most prominent in D196N (see Fig. 3, A and B). The observation that the D196N mutation abolishes the transient current (Fig. 2A) can thus be explained by the assumption that the 15-anti branch, which is reduced in D196N, causes the peak current, whereas both the 15-anti and 15-syn branches contribute to the stationary current. Accordingly, we provide further evidence that the two open states observed in electrophysiological experiments represent the conducting states of the respective photocycle branches as described earlier (15).

For the question how Asp196 affects the retinal syn/anti ratio, especially because it is located at more than 9-Å distance from the RSB (Fig. 6), the protonation state of Asp196 is decisive: Asp196 deprotonates during N → O transition, rendering a negative charge close to the chromophore. The electrostatic interaction of deprotonated Asp196 with the retinal polypeptide chain facilitates the back-reaction from 15-syn to 15-anti-retinal during dark adaptation. Due to the D196N mutation, this negative charge at the chromophore is neutralized, and the syn → anti reaction is impaired. Moreover, the DC pair residues or other amino acid pairs (DT in bacteriorhodopsin) at this position in other microbial rhodopsins form a hydrogen bond between helix 3 and helix 4 that is essential for the protein stability and the retinal-binding pocket in particular. Destruction of this bond in many cases destabilizes the retinal-binding pocket and the protein stability in general (54), resulting in a slowdown of both M formation and decay, and might also alter the syn/anti ratio accordingly.

environment in ReaChR as compared with the environment of Glu90 in CrChR2, we propose a similar scenario for ReaChR. Accordingly, the observed upshift of the Glu130 band by 8 cm⁻¹ in the K spectrum (Fig. 4A) reflects weakening of hydrogen bonding of Glu130 and is in agreement with a reduction of the number of hydrogen bonds between Glu130 and Asn298 from two (weak hydrogen bonds) to one (48). Subsequently, Glu130 deprotonates in the K → M transition, moves outward, and presumably forms a hydrogen bond to Lys133 (Lys93 in CrChR2) as it does in CrChR2 (21).

Now we focus on how the change of the protonation state of Glu130 is linked to ion selectivity. We showed that, upon light activation of ReaChR, Glu130 deprotonates, and therefore a negative charge in the central gate is created that persists in the conducting M₂- and N-states (11). The E130Q mutation has the other consequence. First, in the dark state, no hydrogen bond to Gln130 and thus a preactive conformation might be stabilized. Moreover, the DC pair residues or other amino acid pairs (DT in bacteriorhodopsin) at this position in other microbial rhodopsins form a hydrogen bond between helix 3 and helix 4 that is essential for the protein stability and the retinal-binding pocket in particular. Destruction of this bond in many cases destabilizes the retinal-binding pocket and the protein stability in general (54), resulting in a slowdown of both M formation and decay, and might also alter the syn/anti ratio accordingly.

Figure 6. Summary of proton transfer processes in ReaChR. 1) During D → K transition, Glu130 experiences a hydrogen bond change and then 2) deprotonates at the latest in the M state. 3) Asp196 receives a proton from the RSBH⁺ during M formation. 4) M decays via two pathways: (i) direct transition to D and (ii) transition to N. 5) Asp196 deprotonates after M decay, presumably in the N → O transition. The proton transfer reactions are reversed in the O → D and M → D transitions, respectively. The structure was obtained by homology modeling with SWISS-MODEL using the crystal structure of C12 (Protein Data Bank code 3u9g) (25) as a template. Residues discussed in this work are highlighted.
It remains unclear whether the influence of Asp196 on the syn/anti equilibrium is applicable to CrChR2 as well: although mutations of Asp196 abolish the peak current of CrChR2 wild type similarly to D196N in ReaChR (23, 27), the retinal fingerprint region in FTIR spectra does not imply a significant alteration of the retinal isomer composition (22). The environment of Asp196 is different from its homologues in CrChR2 or C1C2, which is reflected by the higher frequency of the C=O stretch vibration (1753 cm⁻¹) as compared with the respective vibrations at 1738–1737 cm⁻¹ (22, 55). This could, among other things, be due to the adjacent Cys1199, which is exchanged for a threonine in these blue-absorbing ChRs.

Proton transfers in the RSBH⁺ counter-ion complex coincide with photocycle branching

The counter-ion complex of the RSBH⁺ is formed by Ci1 (Glu163), Ci2 (Asp196), and presumably water molecules (56–58). Ci2 receives the RSB proton during M-state formation (Fig. 3G), which is in agreement with the findings for Ci2 in CrChR2 (22) but different to CaChR1 where Ci1 was proposed as proton acceptor (59). Although in both CrChR2 and ReaChR the counter-ion complex interacts with a lysine (Lys133 in ReaChR) that

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Proton transfer reactions in ReaChR

Neutralization of Glu130 enhances Na⁺ conductivity by altered electrostatics of the central gate.

After formation of the M-state, important mechanistic differences between ReaChR and CrChR2 become evident: (i) the photocycle branches with M-state decay, (ii) Asp196 is excluded as proton donor for RSB reprotonation, and (iii) deprotonation of Asp196 facilitates thermal syn → anti isomerization of the chromophore during dark-state recovery. These differences are explained by distinct structural differences between ReaChR and blue-absorbing ChRs at the active site and Asp196 environment, respectively. Asp196 represents an interesting target for elucidating the actual functional relevance of 15-syn-retinal species in ChRs that is poorly understood so far.

Experimental procedures

Molecular biology

For spectroscopic experiments, DNA encoding ReaChR (GenBank™ accession number KF448069.1; amino acids 1–345) with a C-terminal 1D4 epitope (TETSQVAPA) was inserted into the pMT4 expression vector (60) between EcoRI and NotI (Thermo Fischer Scientific, Waltham, MA), whereas for patch clamp recordings ReaChR was C-terminally fused to the mCerulean3 fluorophore (61) and cloned into the pEGFP-N1 vector between HindIII and XbaI (Thermo Fischer Scientific). The mutations E130Q, E163T, C168S, D196N, and D293N were created via site-directed mutagenesis (QuikChange, Agilent Technologies, Santa Clara, CA).

Electrical measurements

HEK293 cells were seeded (0.75 × 10⁵ cells/ml) in Petri dishes on poly-l-lysine-coated glass coverslips and supplemented with 1 μM all-trans-retinal. One day later, cells were transiently transfected with DNA encoding the respective construct using FuGENE HD (Promega, Madison, WI). Recordings took place 48 h after transfection. Signals were amplified and digitized using an AxoPatch 200B and a DigiData 1440 (Molecular Devices, Sunnyvale, CA). Light for activation was provided by a Polychrome V (TILL Photonics, Planegg, Germany) coupled to the optical path of an inverted IX-70 microscope (Olympus, Shinjuku, Japan) and controlled with a programmable shutter (Vincent Associates, Rochester, NY). Standard buffer conditions were as follows: internal 140 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM KCl, 1 mM CsCl, 10 mM HEPES, 10 mM EGTA, pH 7.2, and external 140 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM KCl, 1 mM CsCl, 10 mM HEPES. If not indicated otherwise, data were recorded under that conditions in the whole-cell configuration at a holding potential of −60 mV. Raw traces were baseline-corrected and filtered with a low-pass Gaussian filter (1000-Hz cutoff). Data are displayed as mean ± S.E.

For selectivity measurements, the patch was established at standard conditions, and then extracellular solution was exchanged (five times) for 140 mM NMGCl₂, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM KCl, 1 mM CsCl, 10 mM HEPES, pH 7.2 (NMG, pH 7.2); then 140 mM NMGCl₂, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM KCl, 1 mM CsCl, 10 mM Tris, pH 9.0 (NMG, pH 9.0); and finally 140 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 1 mM KCl, 1 mM CsCl, 10 mM Tris, pH 9.0 (NaCl, pH 9.0). Reversal potentials (E_rev)
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were calculated from the respective stationary currents via linear interpolation between the two nearest data points and corrected for the respective liquid junction potential (23 °C) except for NMG, pH 9.0, where the values were obtained through linear extrapolation from the currents recorded at −60 and −40 mV.

Expression and purification of ReaChR

Expression in HEK293T and purification via immunoaffinity chromatography (1D4) were performed as described before (11).

UV-visible spectroscopy

Absorption spectra were recorded by a Cary 50 Bio spectrophotometer (Varian Inc., Palo Alto, CA) at 22 °C. IDA spectra represent spectral properties of protein purified under safe light (>600 nm), but DA_{290} spectra were measured after preillumination (530 nm, 60 s, 1 × 10^{20} photons m^{-2} s^{-1}; Luxeon light-emitting diode, Philips, Amsterdam, Netherlands) and subsequent recovery phase in the dark (10 min). The difference spectrum of ReaChR-C168S was achieved upon prolonged illumination (60 s, 530 nm). Flash photolysis experiments and data processing and data were performed as described elsewhere (49). In short, a tunable optical parametric oscillator (Rainbow, MagicPrism™, Optek Inc., Carlsbad, CA) was pumped by the third harmonic (355 nm) of a neodymium-doped yttrium aluminium garnet laser (Rainbow, BrilliantB, Quantel, Les Ulis, France). Excitation wavelength was adjusted manually by a micrometer drive and calibrated with an Andor iStar intensified charge-coupled device camera (Andor Technology Ltd., Belfast, Ireland). Absorption changes were probed by a 150-watt xenon short-arc XBO lamp (Osram, München, Germany) and detected by the intensified charge-coupled device camera. Samples with an optical density of A_{290} = 1.0 were excited with green flashes (10 ns, 530 nm, 5 mJ/flash). Global analysis is based on a sequential model with four or five spectral components and was performed with Glotaran (62, 63).

FTIR measurements

FTIR samples in Dulbecco’s PBS, pH 7.4, and 0.03% (w/v) n-dodecyl β-D-maltopyranoside were prepared on a BaF_{2} window by repeated drying under a nitrogen stream and subsequent hydration. After preparation, the sample was sealed with a second BaF_{2} window. Until use, the samples were stored at −40 °C for cryostatic samples and at 4 °C for room temperature samples. For deuteration experiments, pD was adjusted to 7.8. Samples were deuterated by repeated buffer exchange using Centricon centrifugal filter units (GE Healthcare) and subsequent equilibration for 3 days minimum.

Samples were illuminated with light-emitting diodes (maximum emission wavelengths of ~530 nm and ~390 nm for alternating illumination experiments). For cryostatic measurements, the cryostat DN (Oxford Instruments, Abingdon, UK) was used. Samples were equilibrated at the respective temperature for at least 45 min. After measurement, the sample was heated up again to a minimum 20 °C to allow relaxation.

FTIR measurements were performed using an if5660/s FTIR spectrometer (Bruker Optics, Karlsruhe, Germany) with an LN_{2}-cooled mercury cadmium telluride detector (Kolmar Technologies, Newburyport, MA). A 1850 cm^{-1} optical cutoff filter was used. Spectra were recorded with a 200-kHz sampling rate and a spectral resolution of 2 cm^{-1}. For each data set, >2500 spectra of the samples were collected and averaged in the dark and after illumination. At cryotemperatures, this procedure was performed for every sample at least twice (n ≥ 2), and measurements at 293 K were conducted at least 18 times (n ≥ 18) to exclude instabilities of pH and temperature that might affect the reproducibility of the data set.

The difference spectra were corrected for baseline drifts using a spline algorithm and the baseline correction mode implemented in the OPUS 6.5 software package (Bruker Optics). The “steady state” was defined by the steady state of the kinetics of the strongest absorption band in the amide I region (~1660 cm^{-1}). Results obtained from alternating illumination were evaluated by a combination of SVD and a rotation procedure to allow for the estimation of independent spectral components (15). Briefly, the first set of independent spectral components (b-spectra; $S$) and corresponding kinetics components (V) were estimated by SVD. These data were then subjected to a rotation procedure based on the autocorrelation function of V to increase the signal content in a smaller number of vectors as described previously (47). Recovery kinetics were obtained by application of a method combining SVD with a rotation procedure and a global fitting approach using a multieponential function, the latter supplying the time constants of the decay of the steady state (46).

Author contributions—J. C. D. K. performed FTIR spectroscopic measurements. B. S. K. expressed and purified protein and performed UV-visible spectroscopic measurements. C. G. conducted electrophysiological experiments. E. R. provided evaluation software and experimental expertise. E. R., P. H., and F. J. B. designed and interpreted experiments. J. C. D. K. and F. J. B. wrote the manuscript with further contributions from all authors. All authors reviewed the results and approved the final version of the manuscript.

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