Monitoring Light-induced Structural Changes of Channelrhodopsin-2 by UV-visible and Fourier Transform Infrared Spectroscopy

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Channelrhodopsin-2 (ChR2) is a microbial type rhodopsin and a light-gated cation channel that controls phototaxis in Chlamydomonas. We expressed ChR2 in COS-cells, purified it, and subsequently investigated this unusual photoreceptor by flash photolysis and UV-visible and Fourier transform infrared difference spectroscopy. Several transient photoproducts of the wild type ChR2 were identified, and their kinetics and molecular properties were compared with those of the ChR2 mutant E90Q. Based on the spectroscopic data we developed a model of the photocycle comprising six distinguishable intermediates. This photocycle shows similarities to the photocycle of the ChR2-related Channelrhodopsin of Volvox but also displays significant differences. We show that molecular changes include retinal isomerization, changes in hydrogen bonding of carboxylic acids, and large alterations of the protein backbone structure. These alterations are stronger than those observed in the photocycle of other microbial rhodopsins like bacteriorhodopsin and are related to those occurring in animal rhodopsin. UV-visible and Fourier transform infrared difference spectroscopy revealed two late intermediates with different time constants of \( \tau = 6 \) and 40 s that exist during the recovery of the dark state. The carboxylic side chain of Glu40 is involved in the slow transition. The molecular changes during the ChR2 photocycle are discussed with respect to other members of the rhodopsin family.

Channelrhodopsins (ChRs)3 are light-gated cation channels (1, 2) that share homology with other microbial rhodopsins such as bacteriorhodopsin (BR), halorhodopsin (HR), and sensory rhodopsin (SR). In nature they serve as sensory photoreceptors for photophobic responses and phototaxis in green algae (3–5). The light-induced ion conductance leads to depolarization of the cell membrane within milliseconds. Because of this property, in recent years ChRs have been widely used in the neuroscience field as a tool for depolarization of selected cell types or cell ensembles (6, 7). Furthermore, channelrhodopsins were used to control neuronal activity in C. elegans, Drosophila, zebras, chicken embryos, and mice (8–13).

As is typical for rhodopsins, light absorption induces isomerization of the ChR-chromophore with subsequent conformational changes in the protein (photocycle). Based on UV-visible spectroscopic and electrophysiological measurements, several schemes for this photocycle have been presented. A recent model for recombinant Volvox channelrhodopsin (VChR), purified from green monkey COS cells, comprises the two dark states D470 and D480, characterized by a fine structured UV-visible absorption spectrum with maxima at 470 and 480 nm, respectively (14). These two states, which exist in a pH-dependent equilibrium, are both converted by light via retinal isomerization and transient Schiff base deprotonation to the conducting state P510 or, under acidic conditions, to P530. These intermediates thermally relax back to the dark state equilibrium in a biphasic reaction on a millisecond time scale. A similar model was deduced for ChR2 from Chlamydomonas reinhardtii (15), in which the conducting state, termed P520, was also observed to decay biexponentially with time constants of 10 ms and 5 s. No absolute spectra and no early photocycle intermediates were analyzed in this study.

Here we present more detailed spectroscopic studies on wild type Chlamydomonas ChR2 and the mutant E90Q, which is 10 times more selective for Na+ than for H+ as compared with the wild type (4). Because UV-visible studies solely provide information about the protonation state of the Schiff base and constraints of the retinal chromophore, we additionally applied Fourier transform infrared (FTIR) difference spectroscopy, which allowed us to analyze structural changes beyond the direct protein-chromophore interaction. The FTIR difference spectra of formation and decay of three different intermediates were used to control neuronal activity in Caenorhabditis elegans, Drosophila, zebras, chicken embryos, and mice (8–13).

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change, changes in hydrogen bonding of the protonated carboxylic acid Glu\(^{90}\), and structural alterations of the protein backbone. As indicated by the spectra, these changes of the secondary structure are large compared with other microbial rhodopsins but are comparable with visual rhodopsin, which is a G-protein-coupled receptor. Such large changes are already observed in an intermediate cryotrapped at 80 K. FTIR difference spectroscopy indicated that the glutamic acid side chain of Glu\(^{90}\) changes its hydrogen bonding pattern during the photocycle. The late transitions, finally leading to the recovery of the dark state, comprise two intermediates with decay time constants of 6 and 40 s. In contrast to VChR, ChR2 does not show any pH-dependent equilibrium in the dark.

**EXPERIMENTAL PROCEDURES**

ChR2 Expression in COS-1 Cells—For expression in COS-1 cells (ATCC, CRL-1650), a human codon-optimized synthetic ChopDNA fragment (corresponding to amino acids 1–311 of the native protein; accession number AF461397), plus the C-terminal ETSQVAPA sequence (1D4 epitope (16)) was designed and purchased from GeneArt (Regensburg, Germany). The synthetic Chop DNA was inserted between the EcoRI-NotI sites of the expression vector pMT4. Tissue culture, transient transfection with the resulting ChR2-pMT4 vector, reconstitution with chromophore, and subsequent purification of ChR2 was performed basically as described for bovine rhodopsin (17). Three days after transfection, the cells were harvested and reconstituted with all-trans-retinal (final concentration, 30 \(\mu\)M). ChR2 was solubilized with dodecyl maltoside and purified by immunoaffinity adsorption using rho 1D4 antibody coupled to CNBr-activated Sepharose 4 Fast Flow (GE Healthcare). ChR2 was eluted with 100 \(\mu\)M of an 18-mer peptide corresponding to the C-terminal rhodopsin sequence in 0.03% (w/v) dodecyl maltoside, 10 mM 1,3-bis-[tris(hydroxymethyl)methylamino]-propane, pH 6.0 (17). The eluates were concentrated using Centricon YM-10 (Millipore) concentrators and stored at −80 °C. The mutant ChR2-E90Q was generated by site-directed mutagenesis (QuickChange kit; Stratagene).

UV-visible Spectroscopy—For absorption spectroscopy and slow kinetics at 20 °C, a Cary 50 Bio spectrophotometer (Varian Inc.) was used at a spectral resolution of 2 nm. The samples were illuminated for 10 s with a blue Luxeon LED (Philips Lumileds, San Jose, CA) with a wavelength of 456 nm, 90 milliwatt cm\(^{-2}\), 2 \(\times\) 1021 photons m\(^{-2}\) s\(^{-1}\). Transient spectroscopy was performed on a LKS.60 flash photolysis system (Applied Photophysics Ltd., Leatherhead, UK) at 22 °C. Excitation pulses (10 ns, 456 nm) were provided by a tunable Rainbow OPO/Nd: YAG laser system. Laser energy was adjusted to 5 mJ/shot. The instrument used a xenon lamp (150 W) as monitoring light source, which was pulsed during short time experiments. Monochromators placed before and after the sample were set to spectral resolutions of 2 nm. For detection, a 466 photomultiplier (Hamamatsu Photonics) was used, and the signal was recorded with an Infinium Oscilloscope (Agilent Technologies). 32,000 data points were recorded in each measurement and compressed by LKS.60 software to files of 500 data points. 50 of these points were recorded before laser excitation, and 450 points were recorded after laser excitation. To avoid artifacts and scatter, only data points 60–500 were used for analysis. Data analysis was performed with Matlab 7.01 (The MathWorks, Natick, MA). Singular value decomposition of representative data sets was performed to identify significant components that were used for reconstruction of the three-dimensional spectra. Time constants were obtained by fitting exponential functions to the data. For measurements at pH 6, freshly prepared ChR2 was used. For experiments at pH 8 and 4.5, freshly prepared ChR2 was diluted with buffer containing 0.03% dodecyl maltoside. For measurements at 80 K, an Oxford Instruments Optistat DN LN\(_2\) cooled cryostat was used. In this case, the same sample preparation as described below for FTIR measurements was used.

**FTIR Difference Spectroscopy**—The pH of solubilized channelrhodopsin was adjusted by diluted NaOH or HCl (100 mM). Subsequently, 20 \(\mu\)l of the sample were dried on a 15-mm-diameter BaF\(_2\) window, rehydrated by 1 \(\mu\)l of buffer (1,3-bis-[tris(hydroxymethyl)methylamino]-propane) of the appropriate pH value, sealed by a second BaF\(_2\) window, and placed into a temperature-controlled cuvette as described (18). A Bruker ifss66v/S FTIR spectrometer with a LN\(_2\)-cooled mercury cadmium telluride detector (Kolmar Technologies Inc.) and a 1950 cm\(^{-1}\) optical cut-off filter was used to record the infrared spectra with a scanning velocity of 200 kHz and a spectral resolution of 2 cm\(^{-1}\). Before the measurement the sample was equilibrated 45 min in the spectrometer. Illumination was performed by blue LEDs (475 nm, 7200 mcd; led1.de). FTIR spectra were recorded in the rapid scan mode with a time resolution of 5 s. In parallel, UV-visible spectra were recorded every millisecond. For measurements at room temperature, each experiment was repeated at least 30 times to increase the S/N ratio. To allow the sample to quantitatively recover the dark state, the delay between two single experiments was 10 min at 25 °C. Illumination time was 60 s, and FTIR spectra were measured before, during, and after illumination. Spectra before and during illumination were setwise averaged, and the difference spectrum for the transition from the dark state to the photostationary state (termed photostationary minus dark state difference spectrum) was calculated. For the measurements at 80 K, each experiment was repeated three times. Between the experiments, the sample was equilibrated at 25 °C for 5 min. A set of dark spectra was recorded over a period of 2 min. After illumination, a second set of data were collected, and the difference spectrum was calculated as described. For further data analysis, a mathematical procedure was developed using the numerical computation language GNU Octave (19). It combines singular value decomposition with a rotation procedure and global fitting to the spectral data as described (20). Time constants were obtained from the global fitting procedure.

**RESULTS**

**UV-visible Spectroscopic Characterization of ChR2**—The UV-visible spectrum of dark-adapted wild type (WT) ChR2 is shown in Fig. 1A (gray). It is identical under acidic and alkaline conditions. Interestingly, this spectrum exhibits a fine structure comprising two maxima at 450 and 470 nm and a shoulder at
413 nm. These absorption maxima indicate that the Schiff base linkage between chromophore and protein is protonated. Upon continuous illumination with blue light (10 s, 456 nm), the fine structure is diminished, and a red-shifted maximum around 480 nm is observed (Fig. 1A, blue, green, and red spectra), because of accumulation of a photostationary state that mainly consists of late photocycle intermediates and the dark form.

Illumination under acidic conditions (pH 4.5) results in strong depletion of the 450-nm band and higher accumulation of the late 480-nm intermediates (Fig. 1A, red line) compared with more alkaline conditions (Fig. 1A, green and blue lines). Accordingly, the recovery kinetics is slower under acidic conditions (time constants $\tau$ in Fig. 1B). Because of the strong 470-nm absorption in the spectrum of the dark adapted form, we term this state D470, whereas the illuminated form is termed P480. We also purified recombinant ChR2-E90Q, a mutant that shows reduced electrical conductance for $H^+$ and consequently a comparatively higher selectivity for Na$^+$. The spectrum of dark-adapted E90Q is virtually identical to the WT spectrum and shows a similar red shift when the sample is continuously illuminated, as illustrated in the inset of Fig. 1A. Although the recovery kinetics of the dark-adapted state of E90Q is faster than that of WT under all pH conditions applied, low pH also resulted in the slowest recovery rate of the dark state (Fig. 1B and $\tau$ values).

For further characterization of early photocycle intermediates, purified ChR2 was excited with 10-ns laser flashes (pH 6, 456 nm), and time-resolved UV-visible spectra were recorded. The three-dimensional plots in Fig. 2 (A–F) show flash-induced absorbance changes of WT (A–C) and E90Q mutant (D–F) at pH 6 in the spectral range between 300 and 650 nm, which visualize formation and decay of photocycle intermediates. The spectra were recorded with a resolution of 10 nm over a period of 15 $\mu$s (A and D), 8 ns (B and E), or 30 ms (C and F) after the actinic flash. The plots were reconstructed using the two most significant components obtained by singular value decomposition (see “Experimental Procedures”) as shown in Fig. 2 (G (WT) and H (E90Q)). An immediate absorbance increase was found at 520 nm at a time scale of nanoseconds (Fig. 2, A and G (WT) and D and H (E90Q), early spectrum, blue lines). Generally, maxima of difference spectra are shifted when the bands of the initial and the final state overlap. Therefore, the 520-nm absorption observed in the early difference spectra corresponds to a photointermediate that absorbs maximally at around 500 nm and was termed P500 accordingly. Within microseconds P500 converts into a species with a maximum around 390 nm (P390), indicating a deprotonated Schiff base (Fig. 2, G and H, medium spectrum, green line). The kinetics of formation and decay of P390 is shown in Fig. 2I for the WT (blue) and the E90Q mutant (green). In the mutant the chromophore deprotonates faster ($\tau = 4$ $\mu$s) than WT ChR2 ($\tau = 25$ $\mu$s), whereas the decay kinetics is similar for both ChR2 variants. The deprotonated P390 decays with time constant of 1 ms into the third photoproduct P520 (Fig. 2, G and H, late spectrum, red line). Subsequently, P520 converts into an intermediate termed P480 within 10 ms (Fig. 2, C and F). Because of its slow decay, this intermediate is accumulated in continuous light, hence dominating the light adapted ChR2 form shown in Fig. 1A.

**FIGURE 1.** UV-visible spectroscopy of the photostationary state and the late conversions of purified ChR2. A, absorbance spectra of purified WT ChR2 in dodecyl maltoside solution of the dark-adapted state at pH 6 (gray line) and after 10 s of illumination with blue light at pH 6 (456 nm; green line), pH 8 (blue line), and at pH 4.5 (red line), normalized to the band at 280 nm. Inset, difference spectra (light minus dark, pH 6) of WT and the E90Q mutant. B, recovery of dark-adapted ChR2 WT (blue line) and E90Q mutant (green line). Absorbance changes at 456 nm after illumination are shown as a function of time. The time constants and the respective standard deviations for the recovery process at the different pH values are given in the table. All of the fits showed a $R^2 > 0.994$. 

| $\tau$ (s) | WT / SD | E90Q / SD |
|------------|---------|-----------|
| pH 4.5     | 123 / 0.5 | 105 / 0.5  |
| pH 6.0     | 39 / 0.06 | 27 / 0.07  |
| pH 8.0     | 34 / 0.08 | 21 / 0.08  |

A Cryotrapped Intermediate of the Photocycle at 80 K—To obtain additional information about the photocycle, an intermediate of ChR2 was cryotrapped at 80 K. The inset in Fig. 3A shows the illuminated minus dark state UV-visible difference spectrum of ChR2 at 80 K (maroon). It comprises negatives bands at 413, 442, and 475 nm and a positive band at 504 nm. This spectrum is qualitatively similar to the photostationary minus dark state difference spectrum shown in Fig. 1A (inset). Fig. 3A (maroon) shows the respective illuminated minus dark state FTIR difference spectrum of ChR2 recorded at this temperature. Negative bands correspond to vibrations of the dark state, whereas positive bands occur because of vibrations of the photoprodut. In the fingerprint region between 1350 and 1150 cm$^{-1}$, indicative for the isomeric state of the chromophore, the spectrum exhibits negative bands at 1245, 1235, 1202, and 1182 cm$^{-1}$ and positive bands at 1190 and 1176 cm$^{-1}$. In the amide II region around 1550 cm$^{-1}$, indicative for changes of the secondary structure and for C=C stretching modes of the retinal, we observe a broad negative band at 1542 cm$^{-1}$. However, the largest spectral alterations occur in the structurally sensitive amide I region as a strong negative band at 1665 cm$^{-1}$, which does not downshift in D$_2$O. An additional negative band in this region is found at 1638 cm$^{-1}$ in H$_2$O and at 1635 cm$^{-1}$ in D$_2$O (data not shown). In the spectral region above 1700 cm$^{-1}$, indicative for C=O stretching vibrations of protonated carboxylic acids, a bilobe at 1735 (−)/1741 (+) cm$^{-1}$ is a significant feature of the spectrum. Green light (520 nm) reconverts the 80 K intermediate back to a dark state-like structure, as concluded from the photoprodut minus 80 K intermediate FTIR and UV-visible difference spectra (Fig. 3A, cyan lines). The
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FTIR difference spectrum is largely a mirror image of the difference spectrum of the forward pathway in the range of amide I and protonated carboxylic acids. However, as indicated by bands in the fingerprint region of the FTIR spectrum and by the UV-visible spectrum (Fig. 3B, inset, cyan line), the isomeric state of the chromophore is not exactly reversed.

The Photostationary State—Continuous illumination of the Chr2 dark state D470 with blue light leads to formation of a photostationary state that is expected to consist mainly of the intermediates with the slowest decay kinetics and the dark state, depending on the illumination conditions. The photostationary state minus dark state FTIR difference spectrum (Fig. 3A, blue line) was calculated by subtracting the spectra of the dark state from the spectra measured during continuous illumination at 25 °C. Recording time of each set (dark and illuminated state spectra) was 50 s, and monitoring of the illuminated state spectra was started 2 s after the onset of the illumination to ensure that the photostationary state is observed exclusively.

The chromophore fingerprint region between 1150 and 1350 cm⁻¹ of this spectrum exhibits positive bands around 1174 cm⁻¹ and negative bands at 1184, 1200, 1235, and 1245 cm⁻¹. Similar bands in this spectral region occur in the 80 K intermediate, albeit more pronounced. In the amide II region the strong negative band that was observed at 1542 cm⁻¹ in the 80 K spectrum is slightly upshifted to 1548 cm⁻¹. The most significant alterations appear in the amide I region and comprise a negative band at 1663 cm⁻¹ and positive bands at 1648 and 1632 cm⁻¹. In the range of the C=O stretching vibrations above 1700 cm⁻¹, the bilobe at 1735 (-)/1741 (+) cm⁻¹ in the difference spectrum of the 80 K intermediate has vanished. Instead, we now observe a positive band at 1730 cm⁻¹ accompanied by a negative band at 1718 cm⁻¹ and a weak negative band at 1738 cm⁻¹.

FTIR Difference Spectroscopy of Deuterated Samples and of the E90Q Mutant—Measurements in D₂O were performed to facilitate a first assignment of bands in the spectral region between 1700 and 1800 cm⁻¹. The photostationary minus dark state difference spectrum obtained in D₂O is shown in Fig. 3B (red) with extended scale in comparison with the spectrum recorded in H₂O (blue). The bands at 1730 and 1718 cm⁻¹ in H₂O are downshifted to 1722 and 1713 cm⁻¹ in D₂O. The negative band at 1738 cm⁻¹ is not shifted and gains intensity in D₂O. The most likely reason for this behavior is a partial overlap of the negative band at 1738 cm⁻¹ with a positive band at 1730 cm⁻¹ in H₂O. Because of deuteration, the band at 1730 cm⁻¹ is downshifted, which reduces the overlap and thus leads to an increased intensity of the 1738 cm⁻¹ band. Such deuteration-induced spectral shifts are typical for protonated carboxylic acids, which change their chemical environment. Similar shifts were observed for comparable spectra of other photoreceptors recorded in D₂O (21, 22). Therefore, we tentatively assign the 1730/1718 cm⁻¹ band to the C=O stretch of a protonated carboxylic acid.

In Fig. 3C we show the photostationary minus dark state difference spectrum of ChR2-E90Q (green line), which was recorded as described above. The difference spectrum of the wild type is given for comparison (blue line). Interestingly, the most significant vibration at 1663 cm⁻¹ (amide I band) remains unchanged in the spectrum of the mutant. However, the difference band at 1730/1718 cm⁻¹, which we assigned to a C=O vibration of a protonated carboxylic acid, largely lost intensity, indicating that it is probably caused by the glutamate side chain of protonated Glu."
Amide I region, most prominent at 1665 cm⁻¹ isomerization. Structural alterations are reflected by difference bands in the FTIR difference at 298 K in the spectral region between 1600 and 1780 cm⁻¹. The decay of the photostationary state. In Fig. 4, the dark state, we recorded the FTIR difference spectra during the thermal back reaction from the photostationary state to the dark state. The difference spectrum of the E90Q mutant at 298 K. The 1730 cm⁻¹ band and 1718 cm⁻¹ band are indicative for chromophore isomerization. Structural alterations are reflected by difference bands in the amide I region, most prominent at 1665 cm⁻¹. A unique feature of the spectrum is the bilobe at 1735/1741 cm⁻¹ in the range of the C=O stretching vibration of protonated carboxylic acids. Inset, maroon, UV-visible difference spectrum (photoproduct minus dark state) of the same sample at 80 K. Negative bands at 413, 442, and 475 nm and a positive band at 504 nm indicate depletion of the dark state and formation of a photoproduct. The further red-shifted absorption maximum shows that the photoproduct bears a protonated Schiff base. Cyan lines, UV-visible and FTIR difference spectra of illumination of the 80 K intermediate with 520-nm light. Blue, photostationary minus dark state FTIR difference recorded at 298 K. The sample was illuminated with 475-nm light. Difference bands in the chromophore fingerprint region occur with decreased intensities, whereas the strong difference band in the amide I region at 1663 cm⁻¹ shows a comparable intensity as in the spectrum obtained at 80 K. B, blue, photostationary state minus dark state FTIR difference at 298 K in the spectral region between 1600 and 1780 cm⁻¹. Red, the spectrum, recorded under the same conditions but in D₂O. Bands at 1730 and 1718 cm⁻¹ undergo deuteration-induced downshifts. C, blue, photostationary minus dark state FTIR difference spectrum at 298 K (same spectrum as in B, blue), C, dark green, photostationary state minus dark state difference spectrum of the E90Q mutant at 298 K. The 1730 (+) and 1718 (−) cm⁻¹ difference band is not observed in this spectrum.

Decay of the Photostationary State—To further characterize the thermal back reaction from the photostationary state to the dark state, we recorded the FTIR difference spectra during the decay of the photostationary state. In Fig. 4A we compare the FTIR difference spectra for the formation (photostationary state minus dark state, blue line) and decay (final decay product (10 min) minus photostationary state, red line) of the photostationary state. Because the two spectra are almost perfect mirror images of each other as seen by the difference (black line), we can state that all of the structural changes that led to the photostationary state and that can be monitored in the infrared spectral region are reverted during the thermal decay.

As mentioned above, the most intense band at 1663 cm⁻¹ is a marker band for changes of the secondary structure of the protein during the photocycle. To analyze the kinetics of this transition, the intensity change of this band was tracked after the light was switched off. In agreement with UV-visible spectroscopic data, the decay of the band is in the range of minutes. As shown by the residuals (upper black line), the data cannot exactly be described by an one-phase exponential function (red), as indicated by the residuals (upper light green line). For further characterization of the data, singular value decomposition in combination with a rotation procedure and global fitting was performed. We could identify a slow and a fast component, with time constants of 40 s (red) and 6 s (orange), respectively. A biexponential function comprising these two components fits the data as shown by the residuals (upper light green line), C, FTIR difference spectra (b-spectra) of the pure components obtained by singular value decomposition and global analysis of the transition from the photostationary state to the dark state (orange; fast component $\tau = 6$ s, red; slow component $\tau = 40$ s).
two components with time constants of $\tau_1 = 6\ s$ and $\tau_2 = 40\ s$ at a ratio of 1:9. As indicated by the residuals (light green line), a biexponential function comprising these two components fits the data. The corresponding FTIR difference spectra of the pure first (Fig. 4C, orange spectrum) and second component (Fig. 4C, red spectrum) have also been obtained by application of this procedure.

The spectrum of the first component shows only few characteristic difference bands. Most significant are the positive band at 1663 cm$^{-1}$ and two smaller, negative bands at 1648 and 1633 cm$^{-1}$. In the amide II region, only a small band occurs at 1555 cm$^{-1}$. The intensity of the pattern observed in the chromophore region is relatively small and comprises bands at 1200, 1234, and 1244 cm$^{-1}$. However, their intensities are hardly above the noise level. Interestingly, in the region above 1700 cm$^{-1}$, no significant difference bands are observed in this component.

The spectrum of the slow component (red) is more similar to the spectrum of the photostationary state (Fig. 4A). It exhibits intense positive bands at 1663 and 1546 cm$^{-1}$ in the amide I and amide II regions, and the difference band at 1718 (+)/1730 (−) cm$^{-1}$. The pattern in the chromophore region is also more pronounced and comprises bands at 1186, 1201, 1234, and 1244 cm$^{-1}$.

**DISCUSSION**

The Photocycle of ChR2—The combination of UV-visible and FTIR spectroscopy as applied in this study is a powerful tool to investigate reaction mechanisms of retinal proteins, because the two techniques allow extensive and even complementary structural and mechanistic conclusions (23).

Absorption maxima of the UV-visible spectra mainly provide information about the protonation state of the retinal Schiff base, charge distributions within the polyene chain of the chromophore, and protein/chromophore interactions. Generally, blue-shifted absorption maxima in the range below 400 nm indicate intermediates in which the retinal Schiff base is deprotonated, whereas more red-shifted absorption maxima usually reveal the Schiff base in its protonated form (24, 25). FTIR difference spectra allow analysis of structural changes in both the chromophore and the protein. Difference bands in the spectral region between 1300 and 900 cm$^{-1}$ reflect changes of the retinal geometry and direct protein chromophore interactions (21). Bands in the amide I region near 1650 cm$^{-1}$ are indicative for changes of the secondary structure (26). Changes of the protonation states of amino acid residues and alterations of hydrogen bonding in the protein are reflected by bands in the spectral range between 1700 and 1800 cm$^{-1}$. Therefore, our experiments allow us to propose a photocycle model for ChR2, which contains both information about the chromophore including its direct interaction with the environment as well as information about changes of the protein part during the photocycle (Fig. 5).

The UV-visible spectrum of the ChR2 dark state comprises three absorption maxima at 413, 450, and 470 nm indicative for a protonated Schiff base (Fig. 1A). These different maxima most likely indicate the existence of substates of the ground state that differ in the chromophore rigidity or planarity.

After light excitation the dark state is converted via two photocycle intermediates P500 and P390 into P520. This reaction sequence is similar to the one observed for *Volvox* VChR (14) with respect to the kinetics and absorption maxima. P500 is formed within nanoseconds after the actinic flash and still bears a protonated Schiff base. Subsequent deprotonation of the Schiff base leads with a time constant of 25 μs to formation of P390, a step significantly slower than deprotonation of ChR2-E90Q. VChR2, or ChR1.5 P390 is in a pH-dependent equilibrium with the Schiff base reprotonated form P520. The latter has been hypothesized to be the conducting state, because formation and decay parallels the rise and decay of the photocurrent after a flash (14, 15). However, this correlation needs further consideration. Whereas Bamann et al. (15) reported P520 to be formed with $\tau = 0.15$ ms, our studies reveal a time constant of 1.5 ms (Fig. 5). Photocurrents in ChR2 expressing *Xenopus* oocytes or HEK cells rise after a laser flash with $\tau = 0.2$ ms at pH 7.5 and −100 mV (2), which is closer to the value of Bamann et al. (15). However, the rise is slowed down at higher (less negative) voltage and approaches 1.5 ms at 0 volts, which is close to the value found for the recombinant protein in detergent.

P520 converts within 10 ms into the P480 intermediates, which relax within only minutes (WT) back to the dark state. The kinetics is thought to reveal the dark adaptation process as

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5 S. P. Tsunoda and P. Hegemann, unpublished observations.
6 A. Bernd and P. Hegemann, unpublished observations.
observed in electrical measurements (2, 27). There is no pH-dependent equilibrium between D470 and other dark states, as observed in VChR (14). Thus, we conclude that the last step of the photocycle, i.e. the formation of D470, is irreversible. Because the recovery kinetics of D470 starting from P480 is a pH-dependent process, an additional pH-dependent equilibrium and therefore an additional photoproduction must be involved that could, however, not be distinguished from the previously described P480 species by UV-visible spectroscopy. By combining FTIR difference spectroscopy and singular value decomposition (20), we could show that the recovery of the dark state P480, as suggested by the UV-visible data (28, 29), can be explained by excitation of P520 with green light (Fig. 5) as seen from the light-induced reversibility of the ChR2 80 K FTIR difference spectrum (Fig. 3A, cyan lines).

Finally, P480 must be also photoreactive. Otherwise ChR2 would be nearly completely trapped as P480 in continuous light, because the decay of P480 is the rate-limiting step of the photocycle and roughly 4000 times slower than the decay of the conducting state P520. Because only one-third of the molecules are accumulated as P480 at saturating light (pH 7.5, –100 mV), light should photo-convert P480 either to the dark state P470 (2, 28) or directly to P500 photocycle intermediate as outlined in Fig. 5 (14, 27).

The Cryotrapped Intermediate at 80 K—Information on the early steps of the photocycle has been achieved from the 80 K FTIR and UV-visible difference spectra. The absorption maximum of the UV-visible difference spectrum at 504 nm (Fig. 3, inset) indicates that this intermediate contains the Schiff base in its protonated form. A contribution of P390 to this spectrum can be excluded, but an exact assignment to P500 or P520 was not possible.

In the infrared, the bands in the fingerprint region between 1150 and 1350 cm⁻¹ indicate an isomerized state of the chromophore most likely caused by all-trans to 13-cis isomerization. This is based on the observation that the earliest 13-cis BR-K state minus BR dark state FTIR difference spectrum of bacteriorhodopsin with positive bands at 1194 and 1187 cm⁻¹ and negative bands at 1216, 1202, and 1169 cm⁻¹ (29, 30) is in the fingerprint region similar to the ChR2 difference spectra recorded at 80 K. Thus, we conclude that the photoconversion of dark-adapted channelrhodopsin to the 80 K intermediate is triggered by an all-trans to 13-cis isomerization of the retinal. An exact assignment of these FTIR bands is beyond the scope of this study and would require techniques such as Raman spectroscopy or FTIR spectroscopic studies on channelrhodopsin with ¹³C- or ¹⁵N-labeled retinoids. Nevertheless, the conclusion is consistent with behavioral studies on white Chlamydomonas cells that were reconstituted with different retinal analogues (31, 32).

Further structural changes of the protein appear in the spectral region between 1700 and 1600 cm⁻¹. These bands can either be caused by the amide I vibration, indicative of secondary structure changes, or by the C=N stretching vibration of the protonated Schiff base (21). The invariability of the large negative band at 1665 cm⁻¹ in D₂O indicates that it originates from an amide I mode than from the C=N stretching vibration (33, 34). Consequently, this band suggests that already the formation of the 80 K intermediate is accompanied by significant alterations of the protein secondary structure.

Another significant feature of the 80 K difference spectrum of ChR2 is the bilobe at 1741 cm⁻¹ (+)/1735 (–) cm⁻¹. A similar pattern was observed in the BR-K state minus BR dark state difference spectrum at 1741 (–)/1732 (+) cm⁻¹. This difference band was assigned to changes of the Asp¹¹³ hydrogen-bonding pattern (35–38). This amino acid is conserved in all microbial rhodopsins and is D156 in ChR2. Hydrogen bonding changes around this amino acid indicate alterations of the interactions between helices 3 and 4 (39).

The Photostationary State—Information about the late photocycle states P480, and P480, can be collected from the photostationary minus dark state FTIR difference spectrum at 298 K, where in particular these two states are accumulated with an enrichment of P480, at low pH. The small bands in the retinal fingerprint region suggest that the retinal geometry is more similar to the all-trans dark state compared with the 80 K intermediate. In contrast, the intense difference bands in the amide I region at 1648 (+)/1663 (–) cm⁻¹ indicate strong structural alterations. This is surprising because in the difference spectra of the related system BR, comparable bands are less intense, suggesting smaller structural changes during the photocycle of BR. The FTIR measurements in D₂O of the E90Q mutant revealed that the 1718 (–)/1730 (+) cm⁻¹ difference band is most likely caused by Glu⁹⁰ (Fig. 3, B and C). Its position at relatively low wavenumbers suggests that Glu⁹⁰ is strongly hydrogen-bonded (high pK), which means that it is located within the transmembrane helices consistent with the assignment of Nagel et al. (1) and Suzuki et al. (40) but inconsistent with that of Sineshchekov et al. (3). The upshift from 1718 cm⁻¹ in the dark state to 1730 cm⁻¹ in the photostationary state would then indicate that this residue moves into a less hydrogen-bonded environment.

The Reformation of the Dark State from the Photostationary State Involves Two Steps with Different Kinetics—Singlular value decomposition of the FTIR data revealed that the recovery of the dark state from P480 involves two components that decay with time constants of 6 and 40 s, respectively, whereas the UV-visible data suggest a mono-exponential process. The FTIR difference spectra of these components were calculated from the original time-resolved data set by singular value decomposition and global analysis. The spectra in Fig. 4C suggest that the first process involves smaller changes of the secondary structure as indicated by the lower intensity of the amide I band at 1663 cm⁻¹, compared with the spectrum of the second transiton. Therefore, we assign the first component to the formation of the P480, intermediate starting from earlier P480, (Fig. 5). In the FTIR difference spectrum of the second process, the band at 1663 cm⁻¹ in the amide I region is most significant, indicating that the transition from P480, to the dark state D470 involves major structural changes. Interestingly, the difference band at 1730/1718 cm⁻¹, connected to changes of residue Glu⁹⁰ during
the photocycle, is more pronounced in the difference spectrum of the second step. Thus, we conclude that the major changes of the chemical environment of Glu<sup>90</sup> are linked to structural changes occurring during the reformation of the dark state on the way from P480<sub>b</sub> to D470.

Large Structural Changes Occur in ChR2 in Contrast to BR—Despite the sequence similarities ChR shares with other microbial rhodopsins like BR, HR, and SR (41), there are significant differences between the spectra. The microbial proteins BR, HR, and SR show smaller difference bands in the amide I region, which is consistent with limited conformational alterations as they occur during the photocycle (42). It is interesting that this applies to SR, whose function is to activate a transducer protein rather than ion pumping. But the SR mechanism is based on interaction between SR and its transducer in the membrane, involving similar structural changes as observed for BR (43–45).

In contrast, visual rhodopsins, whose function is to activate a G-protein, undergo comparatively large structural alterations upon activation, including backbone perturbation such as helix movement (46) and helix formation or elongation (47). Consequently, the FTIR difference spectra of these rhodopsins feature large amide I modes, which are the most intense bands in the spectra of their active states (21, 48).

In summary, FTIR difference spectra of ChR2 show similarities with both the bacteriorhodopsin family and visual rhodopsins. On the one hand, the chromophore fingerprint region of ChR2 is similar to the corresponding region of BR, HR, and SR, indicating that the predominating initial event in the photocycle of ChR2 is most likely an all-trans- to 13-cis-isomerization of the retinal. On the other hand the prominent ChR2 amide I mode at 1663 cm<sup>-1</sup> reflects large structural changes of the protein backbone (Fig. 3), as in visual rhodopsin. Thus, we conclude that the photocycle of ChR2 is linked to significant structural alterations of the protein backbone. These changes are possibly necessary for the formation of the ion conducting pore, whereas in BR and HR one ion is pumped per absorbed photon through already preformed hydrogen-bonded networks (49–52). The uphill pump mechanism is based on deprotonation and dipole rearrangement of the Schiff base, which requires only small rearrangements of the secondary protein structure (42, 53), whereas down hill channeling of many charges in a short time unit requires large water-filled pores that in case of ChR have to be rapidly opened and closed after light excitation.

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