



**Title:** Photoexcitation of the P4480 State Induces a Secondary Photocycle That Potentially Desensitizes Channelrhodopsin-2

**Author(s):** Saita, M., Pranga-Sellnau, F., Resler, T., Schlesinger, R., Heberle, J., & Lorenz-Fonfria, V. A.

**Document type:** Postprint

provided by institutional repository of the Freie Universität Berlin

View metadata, citation and similar papers at CORE.ac.uk

powered by COBE

right to use is granted. This document is intended solely for personal, non-commercial use.

**Citation:** This document is the Accepted Manuscript version of a Published Work that appeared in final form in The Journal of Physical Chemistry Letters, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see <https://doi.org/10.1021/jacs.8b03931>.  
Saita, M., Pranga-Sellnau, F., Resler, T., Schlesinger, R., Heberle, J., & Lorenz-Fonfria, V. A. (2018). Photoexcitation of the P4480 State Induces a Secondary Photocycle That Potentially Desensitizes Channelrhodopsin-2. *Journal of the American Chemical Society*, 140(31), 9899–9903. <https://doi.org/10.1021/jacs.8b03931>

# Photoexcitation of the $P_4^{480}$ state induces a secondary photocycle that potentially desensitizes channelrhodopsin-2.

Mattia Saita<sup>†</sup>, Franziska Pranga-Sellnau<sup>†</sup>, Tom Resler<sup>†</sup>, Ramona Schlesinger<sup>‡</sup>, Joachim Heberle<sup>†</sup>, and Victor A. Lorenz-Fonfria<sup>§,\*</sup>

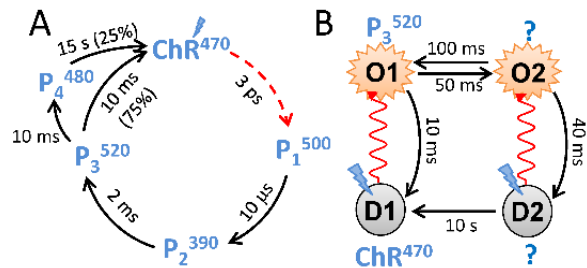
<sup>†</sup>Experimental Molecular Biophysics, Department of Physics, Freie Universität Berlin, 14195 Berlin, Germany; <sup>‡</sup>Genetic Biophysics, Department of Physics, Freie Universität Berlin, 14195 Berlin, Germany; <sup>§</sup>Institute of Molecular Science, Universitat de València, 46980 Paterna, Spain; and Department of Biochemistry and Molecular Biology, Universitat de València, 46100 Burjassot, Spain.

**ABSTRACT:** Channelrhodopsins (ChRs) are light-gated cation channels. In spite their wide use to activate neurons with light, the photocurrents of ChRs rapidly decay in intensity under both continuous illumination and fast trains of light pulses, broadly referred to as desensitization. This undesirable phenomenon has been explained by two interconnected photocycles, each of them containing a non-conductive dark state (D1 and D2) and a conductive state (O1 and O2). While the D1 and O1 states correspond to the dark-state and  $P_3^{520}$  intermediate of the primary all-*trans* photocycle of ChR2, the molecular identity of D2 and O2 remains unclear. We show that  $P_4^{480}$ , the last intermediate of the all-*trans* photocycle, is photoactive. Its photocycle, characterized by time-resolved UV/Vis spectroscopy, contains a red-shifted intermediate,  $I_3^{530}$ . Our results indicate that the D2 and O2 states correspond to the  $P_4^{480}$  and  $I_3^{530}$  intermediates, connecting desensitization of ChR2 with the photochemical properties of the  $P_4^{480}$  intermediate.

## INTRODUCTION

Channelrhodopsins (ChRs) are membrane proteins belonging to the family of microbial rhodopsins, with a covalently linked retinal chromophore.<sup>1</sup> ChR2 from *Chlamydomonas reinhardtii*, CrChR2, a light-gated cation-conductive channel,<sup>2</sup> is widely used in optogenetics<sup>3</sup> and has been studied in detail by electrophysiology and molecular spectroscopies.<sup>4,5</sup> The non-conductive dark state of CrChR2, whose structure was recently solved by X-ray crystallography,<sup>6</sup> bears retinal in all-*trans*,15-*anti* configuration,<sup>7,8</sup> hereafter ChR<sup>470</sup> state. Depending on the illumination history we also find in the dark minor proportions (up to 25-30%) of CrChR2 with 13-*cis*,15-*syn* retinal,<sup>8,9</sup> hereafter ChR<sup>460</sup> state. [Superscripts refer to the approximate  $\lambda^{\max}$  of the retinal in nm.]

The most detailed spectroscopic investigations on CrChR2 and related ChRs have used illumination protocols where short (< 10 ns) light pulses were applied interleaved by long (seconds) periods in the dark.<sup>10-13</sup> Photoexcitation of the ChR<sup>470</sup> state leads to a photocycle with four intermediate states:  $P_1^{500}$ ,  $P_2^{390}$ ,  $P_3^{520}$  and  $P_4^{480}$  (see Fig. 1A for a kinetic scheme), the three formers containing 13-*cis* retinal and the latter retinal in all-*trans* configuration.<sup>5</sup> The  $P_3^{520}$  intermediate decays monoexponentially with  $\tau = 6-10$  ms in detergent-solubilized CrChR2 (20-25 °C and pH ~ 7)<sup>10,14,15</sup> much as the photocurrents do under equivalent conditions in cells.<sup>15,16</sup> The same good kinetic correlation is preserved in various mutants,<sup>10,16</sup> supporting the conductivity of the  $P_3^{520}$  intermediate and the relevance of time-resolved spectroscopic studies of CrChR2 in detergent.<sup>5</sup>  $P_4^{480}$  is a non-conductive intermediate with an unusual slow decay,  $\tau = 5-20$  s,<sup>10,17</sup> bypassed by the ~75% of the molecules relaxing back to the ChR<sup>470</sup> state (Fig. 1A).<sup>10,13</sup>



**Figure 1.** Photocycle models of CrChR2 with approximate time constants derived from experiments performed under (A) single turnover conditions, with intermediates characterized spectroscopically<sup>5</sup> and (B) multiple turnover conditions, with intermediates conductivities characterized by electrophysiology.<sup>18,19</sup>

In electrophysiological experiments, CrChR2 is typically excited by trains of light pulses of tens-to-hundreds of milliseconds in length.<sup>2,18</sup> In contrast to laser flash experiments, the photocurrents under these conditions decay bi-exponentially ( $\tau_1 \approx 4-8$  ms and  $\tau_2 \approx 30-50$  ms),<sup>18</sup> and display new phenomena such as inactivation (i.e., stationary photocurrents lower than peak photocurrents) and light adaptation (i.e., decrease of the peak currents with repetitive illumination).<sup>2</sup> A simple model consisting of two intercrossing photocycles, with two non-conductive dark-states (D1 to D2) and two conductive states (O1 and O2), has been employed to quantitatively relate the photocurrents to the intensity, duration, and frequency of the light pulses (Fig. 1B).<sup>18,19</sup> Inactivation emerges from the ~10-to-20-fold lower conductance of O2 with respect to O1 and the increased O2/O1 ratio under sustained illumination,<sup>18,20</sup> while light adaptation originates from the dependence of the D2/D1 ratio on the illumination history.<sup>19</sup>

Desensitization and light adaptation are among the main drawbacks of *CrChR2* as an optogenetic tool.<sup>3</sup> Thus, characterization of the secondary photocycle in *CrChR2* is of immense practical relevance. There is consensus that D1 and O1 correspond to the well-characterized  $\text{ChR}^{470}$  and  $\text{P}_3^{520}$  states (Fig. 1B). Instead, the molecular identity of D2 and O2 remains disputed. It has been suggested that D2 corresponds to the  $\text{ChR}^{460}$  state, the dark state with 13-*cis*,15-*syn* retinal.<sup>8</sup> However, in opposition to this assignment, the decay of  $\text{ChR}^{460}$  to  $\text{ChR}^{470}$  takes several hours,<sup>8</sup> while the decay of D2 to D1 proceeds with  $\tau \approx 10$  s.<sup>18</sup>

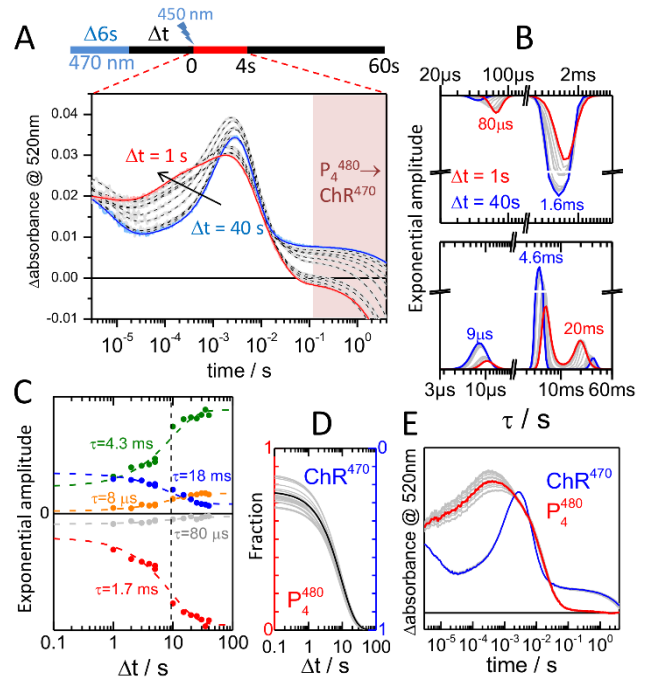
Here, we propose that the D2 state corresponds to the  $\text{P}_4^{480}$  intermediate, and O2 to one of its photoproducts. To validate this hypothesis, we characterized the photoactivity of  $\text{P}_4^{480}$  by transient UV/Vis absorption spectroscopy.

## RESULTS

The slow decay of the  $\text{P}_4^{480}$  intermediate, three orders slower than the previous  $\text{P}_3^{520}$  intermediate,<sup>5</sup> was the basis for our strategy to address the photoreaction of the  $\text{P}_4^{480}$  state. In contrast to previous time-resolved studies,<sup>10,11</sup> we pre-illuminated the sample using a light emitting diode (LED) to generate a photo-stationary mixture enriched in  $\text{P}_4^{480}$ .<sup>21</sup> After the LED was switched off, we introduced a variable time delay,  $\Delta t$ , long enough ( $\geq 1$  s) to ensure that all intermediates other than  $\text{P}_4^{480}$  fully relax to  $\text{ChR}^{470}$ , an expectation confirmed by time-resolved FTIR experiments (Fig. S1). Subsequently, the sample was excited with a 10 ns laser flash and transient absorption UV/Vis changes were recorded (Fig. 2A top for a scheme).

We measured transient absorption changes at 520 nm with time delays between 1 s to 40 s (Fig. 2A). Additional transients have been recorded at 380 nm and analyzed (Fig. S2). The variable time delays generate different  $\text{P}_4^{480} / \text{ChR}^{470}$  ratios before the sample is interrogated by the laser excitation. For long time delays (e.g.,  $\Delta t = 40$  s), the time trace matches that measured before for the  $\text{ChR}^{470}$  photocycle, showing contributions from the well-known red-shifted intermediates  $\text{P}_1^{500}$ ,  $\text{P}_3^{520}$  and  $\text{P}_4^{480}$  (Fig. 2A).<sup>5</sup> As the time delay is reduced, the time traces at 520 nm gradually change in shape, indicating that: a)  $\text{P}_4^{480}$  is photoactive and; b) its photocycle contains at least one red-shifted intermediate. A rank analysis by singular value decomposition supports the conclusion that only two photocycles, from  $\text{ChR}^{470}$  and  $\text{P}_4^{480}$ , contribute to the time traces in Fig. 2A (see Fig. S3).

The laser flash typically excites less than a 10% of the *ChR2* molecules in the  $\text{ChR}^{470}$  state.<sup>15</sup> A similar incomplete photoexcitation is anticipated for the  $\text{P}_4^{480}$  state. As a consequence, the recorded kinetic traces in Fig. 2A also reflect the thermal decay of the unexcited  $\text{P}_4^{480}$  intermediate back to  $\text{ChR}^{470}$  (see Fig. S4 for a control experiment without laser flash). This contribution interferes with the recorded time traces above 100 ms (shaded area in Fig. 2A). Being well-described by a single exponential decay its contribution can be numerically corrected when needed (Fig. S5).



**Figure 2.** Generation of variable mixtures of  $\text{ChR}^{470}$  and  $\text{P}_4^{480}$  states by pre-illumination, and characterization of their transient absorption changes at 520 nm. (A) Kinetic traces at 520 nm (continuous lines), recorded after a variable delay ( $\Delta t$  from 1 s to 40 s) between the end of pre-illumination (470 nm, 6 s, 15 mW/cm<sup>2</sup>) and a laser flash (450 nm, 10 ns, 2 mJ/cm<sup>2</sup>). Global exponential fitting (dashed lines). The shaded area marks contributions from the  $\text{P}_4^{480}$ -to- $\text{ChR}^{470}$  decay. (B) Lifetime distribution analysis of the time-traces in (A), split in rising (top) and decaying (bottom) components. (C) Exponential amplitudes as a function of  $\Delta t$  (solid circles) globally fitted to a single exponential decay (colored dashed lines) with  $\tau = 9.3$  s (vertical dashed line). (D) Estimated population of  $\text{ChR}^{470}$  and  $\text{P}_4^{480}$  as a function of  $\Delta t$  (black trace) with uncertainties (grey traces). (E) Estimated pure kinetic traces at 520 nm for the  $\text{ChR}^{470}$  (blue trace) and  $\text{P}_4^{480}$  (red trace) photocycles, with uncertainties (grey traces).

We conducted a maximum entropy lifetime distribution analysis of the time traces at 520 nm to discriminate exponential components arising from the  $\text{ChR}^{470}$  and the  $\text{P}_4^{480}$  photocycles (Fig. S6). Exponential components are resolved here as bands, which for displaying purposes we split into exponential rising components, giving negative bands (Fig. 2B top), and exponential decaying components, giving positive bands (Fig. 2B bottom). With  $\tau \approx 1.6$  ms and  $\tau \approx 4.6$  ms we resolve the rise and decay of the  $\text{P}_3^{520}$  intermediate.<sup>14</sup> As expected, the amplitude of these two components decreases as  $\Delta t$  decreases, (Fig. 2B, compare blue and red curves). On the contrary, two components at  $\tau \approx 80$   $\mu$ s and  $\tau \approx 20$  ms, marginally present in the dark-adapted sample, increase their amplitude with shorter values of  $\Delta t$  (Fig. 2B, compare blue and red curves). We assign them to the rise ( $\tau \approx 80$   $\mu$ s) and decay ( $\tau \approx 20$  ms) of a red-shifted intermediate in the  $\text{P}_4^{480}$  photocycle.

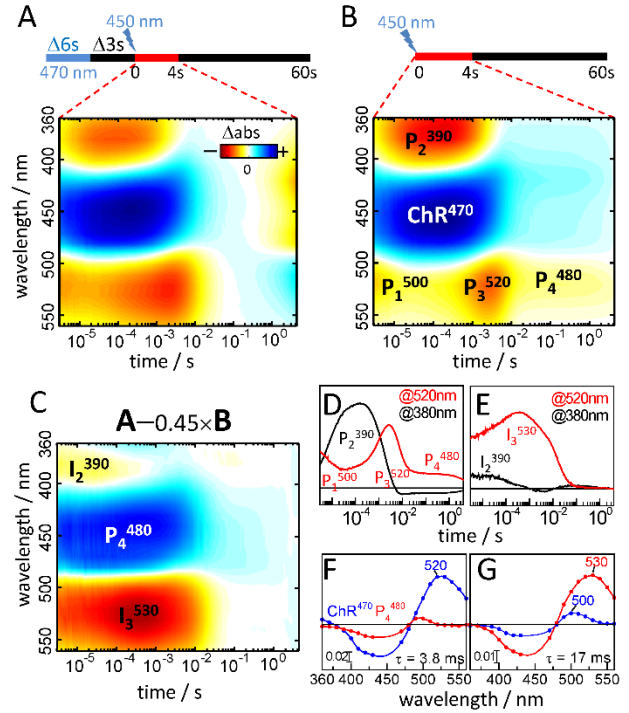
Figure 2C shows the exponential amplitudes as a function of  $\Delta t$ , obtained by global exponential fitting of the experimental data (Fig. 2A, dashed traces). All amplitudes exhibit a dependence on  $\Delta t$  well-modeled by a single exponential with  $\tau = 9.3 \pm 1.5$  s (Fig. 2C). A similar dependence is observed for the exponential amplitudes of the transients at 380 nm (Fig. S2B).

This recovery time constant agrees with the time constant for the decay of  $P_4^{480}$  to  $ChR^{470}$  reported in the literature,  $\tau = 6 - 20$  s,<sup>10,14,15</sup> confirming that the second photocycle of  $CrChR2$  observed here originates from photo-excitation of  $P_4^{480}$ . Note also the similarity of the data in Fig. 2C with the “peak current vs time delay” plots used in electrophysiology experiments to assess dark adaptation (reversal of light adaptation),<sup>15,22</sup> suggesting a fundamental link between the decay of  $P_4^{480}$  to  $ChR^{470}$  and the decay of D2 to D1.

The exponential amplitudes in Fig. 2C for the  $\tau \approx 1.7$  ms and  $\tau \approx 4.3$  ms components take a  $24 \pm 8\%$  of their maximum value when extrapolated to zero time delay, indicating a mixture of  $24 \pm 8\%$   $ChR^{470}$  and  $76 \pm 8\%$   $P_4^{480}$  during continuous illumination. After combining these percentages with the time constant of  $P_4^{480}$  decay ( $9.3 \pm 1.5$  s, Fig. 2C), we calculated the fractions of  $P_4^{480}$  and  $ChR^{470}$  as a function of the delay time in the dark (Fig. 2D). And from these fractions we obtained the kinetics at 520 nm for the pure  $ChR^{470}$  and  $P_4^{480}$  photocycles by least-squares (Fig. 2E).

In order to get further insights into the  $P_4^{480}$  photocycle, we recorded a spectral map from 360 nm to 560 nm in 10 nm steps: the sample was pre-illuminated for 6 s with a blue LED, followed by a 3 s delay time in the dark prior to laser flash excitation and data acquisition (Fig. 3A). As a control, we measured a spectral map without pre-illumination (Fig. 3B), corresponding to the  $ChR^{470}$  photocycle. We subtracted the data in Fig. 3A and Fig. 3B with a factor of 0.45 (derived from Fig. 2D) to obtain the spectro-temporal changes associated with the  $P_4^{480}$  photocycle (Fig. 3C). From the result, we conclude that the photocycle of  $P_4^{480}$  contains at least two intermediates: a  $P_2$ -like intermediate with  $\lambda^{max} \approx 390$  nm ( $I_2^{390}$ ), and a  $P_3$ -like intermediate with  $\lambda^{max} \approx 530$  nm ( $I_3^{530}$ ). Time slices at 520 nm and 380 nm for the  $ChR^{470}$  and  $P_4^{480}$  photocycles are presented in Figs. 3D and 3E, respectively. Those at 520 nm might contain minor contributions from the 13-*cis* photocycle, but restricted to the microsecond range.<sup>12</sup> The  $I_2^{390}$  intermediate rises and decays much earlier than its  $P_2^{390}$  homologue, with a very low accumulation (Fig. 3E and Fig. S2C). The  $I_3^{530}$  intermediate rises earlier and decays slower than its  $P_3^{520}$  homologue, showing a similar accumulation level (Fig. 3D-E and Fig. 2E).

We performed a global exponential analysis of the spectral maps in Figs. 3A-C, to obtain eight time constants and their decay associated spectra (DAS) (see Fig. S7). The  $P_3^{520}$  intermediate decays with  $\tau = 3.8$  ms, as indicated by the positive peak at 520 nm in the corresponding DAS-5 from the  $ChR^{470}$  photocycle (Fig. 3F, blue curve).  $I_3^{530}$  decays with  $\tau = 17$  ms, as supported by the positive peak at 530 nm in DAS-6 from the  $P_4^{480}$  photocycle (Fig. 3G, red curve). In addition, the  $ChR^{470}$  photocycle shows in DAS-6 a minor but significant positive amplitude at 500 nm (Fig. 3F, blue curve), explaining why the amplitude of the  $\tau = 18$  ms component in Fig. 2B,C does not fully decay to zero at long  $\Delta t$ .



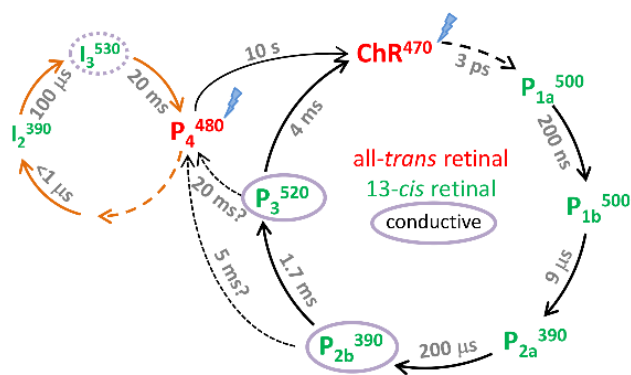
**Figure 3.** Spectral analysis of the  $P_4^{480}$  photocycle. (A) Transient absorption changes of  $CrChR2$  with pre-illumination and (B) without pre-illumination. (C) Spectral map of the  $P_4^{480}$  photocycle, obtained by subtracting data in (A) and (B), and correcting for the  $P_4^{480}$ -to- $ChR^{470}$  decay. (D, E) Extracted kinetics at 520 nm and 380 nm for the (D)  $ChR^{470}$  and (E)  $P_4^{480}$  photocycles. (F, G) Decay associated spectra for (F)  $\tau = 3.8$  ms and (G)  $\tau = 17$  ms for the  $ChR^{470}$  (blue) and  $P_4^{480}$  (red) photocycles.

Based on the above results we propose a modified photocycle scheme (Fig. 4), which includes the photoreactions after the sequential absorption of two photons by  $ChR^{470}$  and  $P_4^{480}$ . The hereby characterized  $P_4^{480}$  photocycle shows a  $P_2$ -like intermediate,  $I_2^{390}$ , which rises with  $\tau < 1$   $\mu$ s.  $I_2^{390}$  decays with  $\tau \approx 100$   $\mu$ s to  $I_3^{530}$ , a  $P_3$ -like intermediate. The  $I_3^{530}$  intermediate rises in two phases (see Fig. S8), with  $\tau < 1$   $\mu$ s (65%) and with  $\tau \approx 100$   $\mu$ s (35%), decaying to the parent  $P_4^{480}$  state with  $\tau \approx 20$  ms.

## DISCUSSION

The photocycle of  $P_4^{480}$  (Fig. 4) shares two key features with the secondary photocycle of  $CrChR2$  introduced to model the photocurrents under continuous illumination (Fig. 1B). First, the  $I_3^{530}$ -to- $P_4^{480}$  transition is 4-5 times slower than the  $P_3^{520}$ -to- $ChR^{470}$  transition ( $\tau \approx 4$  ms vs 20 ms), much like the decay of O2-to-D2 is 4-5 times slower than decay of O1-to-D1 ( $\tau \approx 4-8$  ms vs 30-50 ms).<sup>18</sup> Second,  $P_4^{480}$  thermally decays to  $ChR^{470}$  with  $\tau \approx 9.3$  s in wild-type  $CrChR2$  (Fig. 2C) and with  $\tau \approx 3$  s in E123T,<sup>12</sup> much like D2 thermally decays to D1 with  $\tau \approx 10$  s in the wild type (Fig. 1B)<sup>18</sup> and with  $\tau \approx 1$  s in the E123T variant.<sup>22</sup> Having both  $ChR^{470}$  and  $P_4^{480}$  an all-*trans* retinal<sup>9</sup> it is conceivable that both photocycles display intermediates with similar properties and, thus, that  $I_3^{530}$  is, like  $P_3^{520}$ , conductive. Future electrophysiology experiments will be required to confirm the conductivity of the  $I_3^{530}$  intermediate, which we expect to be 10-20 times smaller than for the  $P_3^{520}$  intermediate

based on the O1/O2 conductivity ratio.<sup>18,20</sup> Overall, the present results are fully consistent with the notion that the P<sub>4</sub><sup>480</sup> and I<sub>3</sub><sup>530</sup> intermediates correspond to the non-conductive D2 and conductive O2 states, respectively.



**Figure 4.** Photocycle model of *CrChR2*, including the ChR<sup>470</sup> photocycle<sup>12,16</sup> and the P<sub>4</sub><sup>480</sup> photocycle, ignoring possible back-reactions.<sup>23</sup> We assume that I<sub>2</sub><sup>390</sup> and I<sub>3</sub><sup>530</sup> hold a 13-*cis* retinal. We hypothesize that the I<sub>3</sub><sup>530</sup> intermediate is conductive. Time constants for dotted transitions are chosen to allow for 75% of the photo-excited ChR<sup>470</sup> molecules to bypass the P<sub>4</sub><sup>480</sup> intermediate.

Our results indicate that the recovery of the photocurrents from light adaptation is controlled by the slow thermal decay of P<sub>4</sub><sup>480</sup> to ChR<sup>470</sup>. Why is this decay so slow? Although the P<sub>4</sub><sup>480</sup> intermediate appears to be structurally similar to P<sub>3</sub><sup>520</sup>,<sup>10,24,25</sup> the decay of P<sub>3</sub><sup>520</sup> to ChR<sup>470</sup> is 3 orders of magnitude faster, suggesting that the rate-limiting factor in the decay of P<sub>4</sub><sup>480</sup> to ChR<sup>470</sup> is not the reversal of protein conformational changes. The decay of P<sub>4</sub><sup>480</sup>, but not of P<sub>3</sub><sup>520</sup>, involves reprotonation of E90.<sup>10</sup> In the E90A variant the decay of P<sub>4</sub><sup>480</sup> is equally slow though (Fig. S9), indicating that its reprotonation is not rate-limiting either. Both P<sub>4</sub><sup>480</sup> and ChR<sup>470</sup> contain all-*trans* retinal, as concluded from retinal C=C vibrations probed by resonance Raman spectroscopy and retinal extraction followed by chromatography.<sup>9</sup> P<sub>4</sub><sup>480</sup> and ChR<sup>470</sup> differ in the C–C stretching vibrations of the retinal,<sup>9</sup> indicating that the configuration of some C–C bonds might differ between them. Although highly speculative, it is possible that thermal isomerization around C–C bonds of the retinal, for instance from all-*trans*,15-*syn* to all-*trans*,15-*anti*, might be the rate-limiting step in the decay of P<sub>4</sub><sup>480</sup>.

Given the identity between P<sub>4</sub><sup>480</sup> and D2, decreasing the formation of the P<sub>4</sub><sup>480</sup> intermediate should reduce both light adaptation and inactivation of the photocurrents. Thus, understanding the formation of the P<sub>4</sub><sup>480</sup> intermediate is of utmost relevance to design strategies for this goal. We have proposed that P<sub>4</sub><sup>480</sup> is formed as a result of a branch in the ChR<sup>470</sup> photocycle.<sup>5</sup> Because of the structural similarity of P<sub>4</sub><sup>480</sup> with the P<sub>2b</sub><sup>390</sup> and P<sub>3</sub><sup>520</sup> intermediates,<sup>10,16,24,25</sup> it is reasonable to assume that the branching takes place at any of these two intermediates, with the competing time constants defining the branching factor and, thus, the accumulation of P<sub>4</sub><sup>480</sup> (Fig. 4). Another hypothesis are parallel photocycles originated from heterogeneities in ChR<sup>470</sup><sup>13</sup> with only a subpopulation of ChR<sup>470</sup> giving rise to the P<sub>4</sub><sup>480</sup> intermediate. The recent dimeric X-ray structure of the dark state of *CrChR2* reveals conformational differences between the two protomers for some side chains and internal water molecules,<sup>6</sup> although the relevance of these subtle heterogeneities remains unclear.

The reported photoreactivity of the P<sub>4</sub><sup>480</sup> intermediate should not come as a complete surprise. It was previously shown that the photoexcitation of the P<sub>2</sub><sup>390</sup> and P<sub>3</sub><sup>520</sup> intermediates leads to a fast shutdown of the photocurrents by recovery of the ChR<sup>470</sup> state.<sup>15,23</sup> In bacteriorhodopsin, photoexcitation of the K, L and M intermediates (13-*cis* retinal) leads also to the recovery of the dark state,<sup>26</sup> while the N intermediate (13-*cis* retinal) displays a more complex photoreaction.<sup>26</sup> Photoexcitation of the O intermediate (all-*trans* retinal) has resulted in contradictory reports,<sup>26</sup> but might involve a photocycle returning back to O through the formation of a N-like intermediate,<sup>27</sup> a process somehow reminiscent to the photocycle of P<sub>4</sub><sup>480</sup> that we report here.

## OUTLOOK

We have established and characterized the photoactivity of the P<sub>4</sub><sup>480</sup> state of *CrChR2*. We have provided evidence for a link between its photocycle and the desensitization of the photocurrents. Further characterization of the P<sub>4</sub><sup>480</sup> state and its photocycle will provide new perspectives in improving the properties of *CrChR2* as an optogenetic tool.

## ASSOCIATED CONTENT

### Supporting Information.

Experimental Section and Figures S1-S10. The Experimental Section provides details regarding the preparation of the *CrChR2* sample, the acquisition of time-resolved UV/Vis and FTIR experiments, and their analysis (global exponential fit, lifetime distribution analysis, singular value decomposition, and estimation of pure time-traces for the P<sub>4</sub><sup>480</sup> photocycle). Figure S1 – Relaxation kinetics of *CrChR2* after pre-illumination monitored by time-resolved rapid-scan FTIR spectroscopy. Figure S2 – Transient absorption changes at 380 nm of pre-illuminated *CrChR2*. Figure S3 – Singular value decomposition analysis of the transient absorption changes in Fig. 2A. Figure S4 – Transient absorption changes of pre-illuminated *CrChR2* at 520 nm, without laser excitation. Figure S5 – Correction for the decay of unexcited P<sub>4</sub><sup>480</sup> to ChR<sup>470</sup> in pre-illuminated time-resolved UV/Vis experiments. Figure S6 – Full lifetime distributions from Fig. 2B. Figure S7 – Decay associated spectra from the global fitting of the data in Fig. 3B and 3C. Figure S8 – Lifetime distribution analysis of the kinetics of the P<sub>4</sub><sup>480</sup> photocycle at 520 nm. Figure S9 – Comparison of the decay kinetics of P<sub>4</sub><sup>480</sup> in *CrChR2* WT and E90A. Figure S10 – UV/Vis absorption spectrum of the *CrChR2* sample before and after UV/Vis time-resolved experiments.

## AUTHOR INFORMATION

### Corresponding Author

[victor.lorenz@uv.es](mailto:victor.lorenz@uv.es)

### ORCID

Victor A. Lorenz-Fonfria: 0000-0002-8859-8347  
 Ramona Schlesinger: 0000-0002-7716-4439  
 Joachim Heberle: 0000-0001-6321-2615  
 Mattia Saita: 0000-0003-0638-1644

### Notes

The authors declare no competing financial interests.

## ACKNOWLEDGMENT

Funding was provided by the Spanish Ministry of Economy, Industry and Competitiveness through the project BFU2016-768050-P and a Ramon y Cajal fellowship RYC-2013-13114 to V.A.L.-F., and by the German Research Foundation through the SFB-1078, projects B3 to J.H and B4 to R.S.

## REFERENCES

- (1) Govorunova, E. G.; Sineshchekov, O. A.; Li, H.; Spudich, J. L. *Annu. Rev. Biochem.* **2017**, *86*, 845.
- (2) Nagel, G.; Szellas, T.; Huhn, W.; Kateriya, S.; Adeishvili, N.; Berthold, P.; Ollig, D.; Hegemann, P.; Bamberg, E. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 13940.
- (3) Fenno, L.; Yizhar, O.; Deisseroth, K. *Annu. Rev. Neurosci.* **2011**, *34*, 389.
- (4) Stehfest, K.; Hegemann, P. *Chemphyschem* **2010**, *11*, 1120.
- (5) Lórenz-Fonfría, V. A.; Heberle, J. *Biochim. Biophys. Acta* **2014**, *1837*, 626.
- (6) Volkov, O.; Kovalev, K.; Polovinkin, V.; Borshchevskiy, V.; Bamann, C.; Astashkin, R.; Marin, E.; Popov, A.; Balandin, T.; Willbold, D.; Buldt, G.; Bamberg, E.; Gordeliy, V. *Science* **2017**, *358*.
- (7) Becker-Baldus, J.; Bamann, C.; Saxena, K.; Gustmann, H.; Brown, L. J.; Brown, R. C.; Reiter, C.; Bamberg, E.; Wachtveitl, J.; Schwalbe, H.; Glaubitz, C. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 9896.
- (8) Bruun, S.; Stoeppler, D.; Keidel, A.; Kuhlmann, U.; Luck, M.; Diehl, A.; Geiger, M. A.; Woodmansee, D.; Trauner, D.; Hegemann, P.; Oschkinat, H.; Hildebrandt, P.; Stehfest, K. *Biochemistry* **2015**, *54*, 5389.
- (9) Nack, M.; Radu, I.; Bamann, C.; Bamberg, E.; Heberle, J. *FEBS Lett.* **2009**, *583*, 3676.
- (10) Lórenz-Fonfría, V. A.; Resler, T.; Krause, N.; Nack, M.; Gossing, M.; Fischer von Mollard, G.; Bamann, C.; Bamberg, E.; Schlesinger, R.; Heberle, J. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, E1273.
- (11) Kuhne, J.; Eisenhauer, K.; Ritter, E.; Hegemann, P.; Gerwert, K.; Bartl, F. *Angew. Chem. Int. Ed. Engl.* **2014**, *54*, 4953.
- (12) Lórenz-Fonfría, V. A.; Schultz, B. J.; Resler, T.; Schlesinger, R.; Bamann, C.; Bamberg, E.; Heberle, J. *J. Am. Chem. Soc.* **2015**, *137*, 1850.
- (13) Szundi, I.; Li, H.; Chen, E.; Bogomolni, R.; Spudich, J. L.; Kliger, D. S. *J. Biol. Chem.* **2015**, *290*, 16573.
- (14) Resler, T.; Schultz, B. J.; Lórenz-Fonfría, V. A.; Schlesinger, R.; Heberle, J. *Biophys. J.* **2015**, *109*, 287.
- (15) Bamann, C.; Kirsch, T.; Nagel, G.; Bamberg, E. *J. Mol. Biol.* **2008**, *375*, 686.
- (16) Lórenz-Fonfría, V. A.; Bamann, C.; Resler, T.; Schlesinger, R.; Bamberg, E.; Heberle, J. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E5796.
- (17) Verhoeven, M. K.; Bamann, C.; Blocher, R.; Förster, U.; Bamberg, E.; Wachtveitl, J. *Chemphyschem* **2010**, *11*, 3113.
- (18) Nikolic, K.; Grossman, N.; Grubb, M. S.; Burrone, J.; Toumazou, C.; Degenaar, P. *Photochem. Photobiol.* **2009**, *85*, 400.
- (19) Hegemann, P.; Ehlenbeck, S.; Gradmann, D. *Biophys. J.* **2005**, *89*, 3911.
- (20) Richards, R.; Dempski, R. E. *J. Biol. Chem.* **2017**, *292*, 7314.
- (21) Ritter, E.; Stehfest, K.; Berndt, A.; Hegemann, P.; Bartl, F. J. *J. Biol. Chem.* **2008**, *283*, 35033.
- (22) Gunaydin, L. A.; Yizhar, O.; Berndt, A.; Sohal, V. S.; Deisseroth, K.; Hegemann, P. *Nat. Neurosci.* **2010**, *13*, 387.
- (23) Bamann, C.; Gueta, R.; Kleinlogel, S.; Nagel, G.; Bamberg, E. *Biochemistry* **2010**, *49*, 267.
- (24) Krause, N.; Engelhard, C.; Heberle, J.; Schlesinger, R.; Bittl, R. *FEBS Lett.* **2013**, *587*, 3309.
- (25) Sattig, T.; Rickert, C.; Bamberg, E.; Steinhoff, H. J.; Bamann, C. *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 9705.
- (26) Balashov, S. P. *Isr. J. Chem.* **1995**, *35*, 415.
- (27) Tóth-Boconádi, R.; Keszthelyi, L.; Stoeckenius, W. *Biophys. J.* **2003**, *84*, 3857.

SYNOPSIS TOC (Word Style "SN\_Synopsis\_TOC"). If you are submitting your paper to a journal that requires a synopsis graphic and/or synopsis paragraph, see the Instructions for Authors on the journal's homepage for a description of what needs to be provided and for the size requirements of the artwork.

