Proton uptake mechanism in bacteriorhodopsin captured by serial synchrotron crystallography

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One Sentence Summary:

Time-resolved serial crystallography at a synchrotron completes the dynamic view of proton
pumping in bacteriorhodopsin

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Abstract:

Conformational dynamics are essential for proteins to function. We adapted time-resolved serial crystallography developed at X-ray lasers to visualize protein motions using synchrotrons. We recorded the structural changes in the light-driven proton-pump bacteriorhodopsin over 200 ms in time. The snapshot from the first 5 ms after photoactivation shows structural changes associated with proton release at comparable quality to previous X-ray laser experiments. From 10-15 ms onwards we observe large additional structural rearrangements up to 9 Å on the cytoplasmic side. Rotation of Leu93 and Phe219 opens a hydrophobic barrier leading to the formation of a water chain connecting the intracellular Asp96 with the retinal Schiff base. The formation of this proton wire recharges the membrane pump with a proton for the next cycle.

Main Text:

Proteins are intrinsically dynamic molecules that follow defined conformational rearrangements to drive the fundamental biochemistry of life. Structural studies of these dynamic processes are challenging, because structural intermediates are often short-lived. Pump-probe crystallography offers the unique opportunity to capture successive temporal snapshots of light-activated processes to study protein dynamics within the crystallographic lattice at the level of individual atoms, at physiological temperatures and on wild-type proteins (1).

X-ray free electron lasers (XFELs) and the emerging method of time-resolved serial femtosecond crystallography (TR-SFX) have brought exciting opportunities for structural biologists (2, 3). Recent studies on photoactive yellow protein (4, 5) show that TR-SFX is a powerful new tool to study the structural dynamics of proteins within a crystalline environment. The ultrafast but bright X-ray pulses from these powerful linear accelerators outrun most radiation damage effects (6) and allow the use of small crystals, resulting in high protein activation levels (4). The serial injection of crystals further favors the study of non-reversible reactions and the collection of redundant datasets of high quality. The great potential of the TR-SFX method to study membrane transport can be realized when comparing results (7) from freeze trapping studies of the light-driven proton pump bacteriorhodopsin (bR) to a whole series of structural states ranging from the femtosecond (8) to the early millisecond regime (9).

Primary energy conversion processes in biology are variations around one central theme: proton transfer across membranes. Time-resolved crystallographic studies provided much insight into how proton pumping is achieved in bR. In particular they focused on the early events in the pumping cycle including the ultrafast process of energy capture through photoisomerization of retinal (8) and the proton release step from the retinal Schiff base (SB) towards the extracellular

side of the membrane (9). The fundamental piece missing in our dynamic view of proton transport is how the protein rearranges to reprotonate the SB from the cytoplasmic side to reload substrate for the next pumping cycle.

We answer this question by adapting injector-based serial crystallography for time-resolved measurements in the millisecond range at widely available synchrotron sources. Requirements for time-resolved crystallography using the Laue method were never met for bR (7). Therefore, pioneering works to determine the structures of spectroscopic intermediates were carried out using freeze-trapping and mutagenesis (10). While mutagenesis and freeze trapping provide valuable insights and allow to trap individual structural intermediates, major controversies remained because mutations modify critical activation switches and structures of mutants may not adequately reflect rearrangements in the native protein. Such structures may further suffer from radiation damage, are obtained at non-physiological temperatures and lack the temporal dimension critical to understanding protein dynamics. Serial millisecond crystallography (SMX) approaches work well with high viscosity injectors (11), as the injectors extrude crystals slowly and allow sufficient X-ray exposure to collect high-resolution data with the photon flux available at synchrotrons (12). Our recent work has shown that modern high frame-rate and low-noise detectors make SMX a viable method for routine room-temperature structure determination (13). By extending the setup with a simple class 3R laser diode we enable time-resolved studies with millisecond time resolution (TR-SMX) and bring dynamic serial crystallography from the few operational XFELs to the large community of synchrotron users worldwide. We demonstrate the technology by recording the temporal evolution of two structurally distinct intermediate states of bR and how they decay back to the dark-state. The closed-state obtained by TR-SMX at 0-5 ms shows the structural rearrangements necessary for proton release which are associated with the

spectroscopic M-state of bR with comparable quality and in agreement with previous XFEL experiments. The open-state we obtained from data collected 10-15 ms after activation is characterized by large conformational rearrangements up to 9 Å on the cytoplasmic side which have been associated with the spectroscopic N-state of bR and were not yet resolved by dynamic
5 crystallography. These open the protein for the formation of a Grotthuss proton wire, a transient water chain that allows the reprotonation of the Schiff base (SB) from Asp96 the primary proton donor at the cytoplasmic side of the membrane. The observations align with the classical alternate access model of membrane transport and further complete the dynamic view of vectorial proton transport in bR by resolving the critical proton uptake step at the end of the pumping cycle.

Time-resolved serial millisecond crystallography

The most straightforward experiment possible with our TR-SMX setup (**Fig. 1**) leaves the laser diode on while data from continuously light-activated protein molecules within the crystal are being collected. By comparing these data to serial data collected without illumination (**Fig. S1**) it
15 is possible to reveal dominant conformational changes in a photocycle analogous to steady-state spectroscopic techniques. From bR microcrystals with a size of about $35 \times 35 \times 3 \mu\text{m}^3$, we collected 114'052 light and 119'374 dark diffraction patterns in the steady-state mode with data extending to a resolution of 1.8 Å (**Table S1**). The quality of difference electron density maps ($F_o(\text{light}) - F_o(\text{dark})$) improved with the number of included images with characteristic difference density
20 peaks starting to plateau at about 10'000 images, indicating data convergence (**Fig. S2**). The estimated activation level was 38% (see **Materials and Methods**) and resulted in well-resolved difference density map features, with the strongest positive and negative peaks at 7.3 σ and 8.5 σ , respectively. Structure factor extrapolation allows to selectively refine the activated species in a

crystal by removing the contribution of non-activated states from structure factor amplitudes (14) (Fig S3, Materials and Methods). Refinement against extrapolated steady-state data revealed two activated species of bR (Table S2). While one subspecies had been observed in previous TR-SFX structures obtained at 1.7 ms and 8.3 ms after photoactivation which we termed the ‘closed’-state, the second subspecies featured large rearrangements on the cytoplasmatic side indicating that the dynamic view of proton pumping in bR had not yet been completed in the previous studies, hence we termed this intermediate the ‘open’-state (Table S2).

In order to follow the rise and decay of these intermediates and to obtain separate structures, we developed a data collection scheme, which triggers the laser diode using a transistor-transistor-logic signal issued from a delay generator to achieve laser exposure synchronized with data collection at the detector (Fig. 1). In this setup bR crystals were exposed for 5 ms with laser light at the X-ray interaction region, while tracking the photoreaction by collecting 5 ms long timepoints over 40 consecutive frames. This data collection sequence was continuously repeated while new crystals were resupplied by the high viscosity injector (11). After about 5 hours of data collection, each of the timepoints contained ~13’000 indexable diffraction patterns with the resolution extending to 2.3 Å (Table S1).

Analysis of the data provides a ‘continuous’ molecular movie of structural changes over 200 ms in time during which bR molecules complete their photocycle within the crystal. Within this time frame we observe a long-term decay of difference map signal and complete restoration of the dark-state within about 100 ms (Movie S1). The rate of this long-term decay (Fig. S4) and the evolution of structural states as determined by occupancy refinements (Fig. 1) occur within the temporal range established by time-resolved spectroscopy on bR crystals (9, 15, 16). Successive structural intermediates typically overlap which presents a general problem for time-resolved experiments.

Following the rise and decay of intermediates in small steps as demonstrated here allows to choose time delays at which occurring intermediates can best be separated. These results demonstrate that time-resolved serial crystallography is not limited to XFEL sources but can also be done efficiently at synchrotrons. Results are of comparable quality to our previous time-resolved studies at XFELs on the same crystals (**Fig. S5**), even though the resolution was limited by the available photon flux. However, the resolution is compensated by high levels of activation from the millisecond long light exposure, more accurate data due to the use of a photon counting detector and a more stable monochromatic beam as previously observed during *de novo* phasing (13). Due to the slow extrusion rate, less than 20 μ l of prepared microcrystals were necessary to obtain these data instead of milliliters needed for the XFEL experiments. The reduced sample consumption significantly lowers the entry barrier for time-resolved crystallographic studies. While the simple TR-SMX setup implemented at the Swiss Light Source delivers excellent results, there is great potential to improve the method further. Pink beams (17) and diffraction limited sources (18) optimize beam characteristics for faster data acquisition. The Hadamard transform method (19) and continued software development (20) are means to improve data analysis. Together with stronger nanosecond lasers as well as new fast and accurate detectors (21) these improvements will allow the time resolution to be pushed from milliseconds into the lower microsecond regime to cover the majority of biologically relevant protein dynamics.

Completing the bR pumping cycle

The molecular movies of structural changes in bR are an impressive example for how XFEL sources can be used to understand biology (7). Our TR-SMX measurements extend the dynamic view of bR into the second half of the photocycle (**Fig. 2**), where the proton substrate is reloaded.

Refining the closed-state structure obtained by steady-state SMX against extrapolated data from the 0-5 ms timepoint (**Table S2**) we obtained a good fit to M-state structures obtained by cryo-trapping (22) or TR-SFX (8, 9) (r.m.s.d. 0.46 Å for the cryo-trapped structure 1IW9, 0.40 Å for 1.7 ms structure 5B6Z and 0.27 for the 8.3 ms structure 6G7L). Difference maps obtained from 10-15 ms onwards however showed clear additional features at the cytoplasmic side of the protein (**Fig. 1**). When refining the open-state structure obtained by steady-state SMX against extrapolated 10-15 ms data we obtained a better fit in the dark-state structures of the constitutively open Asp96Gly/Phe171Cys/Phe219Leu triple mutant (r.m.s.d. of 0.59 Å for the X-ray structure 4FPD (23) and 0.85 Å for the electron diffraction structure 1FBK (24)), which are considered analogous to the wild-type N-state obtained by photoactivation (**Fig. S6**). The structural rearrangements in the open-state (10-15 ms) with respect to the dark- and closed-states (0-5 ms) occur in the cytoplasmic ends of helices E, F, G. The largest change in helix F corresponds to a movement of 9 Å measured at the C α position of Glu166 (**Fig.1, Movie S2**), which resembles the movement of helix 6 upon activation of structurally related G-protein coupled receptors (**Figure S7**). The magnitude of the motion is consistent with transient movements of helices F and G in the millisecond range observed by time-resolved spin labelling studies in purple membranes (25), suggesting that bR can complete the motion inside the crystal lattice (**Fig. S8**). The final O-state does not accumulate well in bR crystals (16), possibly because crystal contacts which are changing upon formation of the open-state (**Fig. S8**) affect the kinetics of the N-state to O-state transition inside the crystal. However, the O-state is in a fast equilibrium with the N-state (26), suggesting that no large structural rearrangements occur in the transition. The small changes upon relaxation of retinal from the *cis*-isomer back to the *trans*-isomer are spread out in time with associated difference map features disappearing around the 35-40 ms time point (**Movie S1**). Our TR-SMX

experiment thus completes the crystallographic view of the bR photocycle, by revealing the structural rearrangements over the time-frame in which the M-state to N-state transition happens inside crystals until the dark-state is recovered.

Structural changes in helices E and F involve large side chain motions. The isomerizing retinal pushes against Trp182 in helix F already at earlier timepoints (9). This motion continues in the transition from the closed-state to the open-state, driving the displacement of helix F and allowing the aromatic ring of Phe156 to move 9 Å and replace the ring of Phe171. This alters the hydrophobic interaction network holding helix F in its original position. Moving helix F goes hand in hand with the disordering of the C-terminus starting from Arg227 at the end of helix G (**Fig. 3**).

In contrast, the structural changes in helix C are dominated by smaller sidechain rearrangements. The largest conformational change in helix C is a rotamer change of Leu93 matching a displacement of the nearby Phe219 sidechain in helix G (**Movie S2**). These changes create space for a chain of water molecules (Wat404, Wat453, Wat454) observed as strong positive difference map peaks ($F_o(10-15\text{ms}) - F_o(\text{dark})$ above 3σ) connecting Asp96 on the cytoplasmic side with the retinal SB (**Fig. 2**). Additional experimental evidence for such waters originates from a combination of Fourier transform infrared spectroscopy, site directed mutagenesis and molecular dynamic simulations (27, 28). Our experimental results are consistent with molecular dynamic simulations showing that three waters are sufficient to close the gap and enable the proton transfer step to the SB (28). The positions of the three water molecules are different from the four waters observed in a freeze trapping structure of the Val49Ala mutant (29) and the state trapped by mutagenesis lacks the large conformational changes on the cytoplasmic side. The constitutively open triple mutant Asp96Gly/Phe171Cys/Phe219Leu on the other hand allowed to trap N-like features without light activation (23). However, altering the side chains of these residues prevents

formation of the water chain in the X-ray structure of the triple mutant (23) (**Fig. S5**). Such controversies have long hindered the emergence of a consensus concerning large conformational changes in the late bR photocycle (10). The useful mutations by their very nature had to target functionally critical regions which complicated interpretation. Our study resolves these discrepancies by showing the relevant arrangements in light-activated native bR measured at physiological temperatures and with real-time resolution.

Molecular mechanism of proton uptake

The structural transition fits well with the molecular mechanism of vectorial proton pumping (**Movie S2**) (30). Mechanistically, bR can be divided into an extracellular half and a cytoplasmic half with retinal positioned at the center (**Fig. 2**). Light-induced isomerization of the retinal chromophore is the first principal step in the reaction and provides the energy for proton pumping. The energy is used in the second principal step to drive protein conformational changes on the extracellular side to transfer a proton from the retinal SB towards the extracellular release group via a hydrophilic interaction network of residues and water molecules. Transferring the proton to the release group removes the substrate from the center of the membrane.

In the third principal step, the protein has to adopt a different conformation to allow the SB to accept a proton from the intracellular side of the membrane. Preventing proton back leakage to the cytoplasmic side is critical for vectorial transport against a concentration gradient. In bR, this is achieved through a hydrophobic barrier between the primary acceptor of protons in the center (the SB) and the proton donor on the cytoplasmic side of the membrane (Asp96). This 12 Å barrier has to be opened at the right time during each cycle to allow the SB to accept a new proton and recharge the system. Such a reprotonation switch has been suspected to involve larger conformational

changes of the protein chain (31) in the late photocycle (i.e. M to N transition). Electron crystallography on bR mutants suggested opening on the cytoplasmic side and formation of a water channel to the retinal (24, 32). The formation of a water chain, oriented by the protein environment, could in principal act as a Grotthuss proton wire (33), bridging the hydrophobic barrier to deliver a new proton to the SB. Yet despite numerous molecular dynamics simulations and structural studies (10) it remained unclear whether proton transfer happens via rapidly exchanging waters in a channel (23) or via distinct water molecules rearranged by the protein environment (28). Beside differences in methodology discrepancies originate from the use of different starting models (**Fig. S5**). The transition from the closed to the open bR conformation dominating the 10-15 ms timepoint provides direct evidence for the transient formation of such a chain of water molecules (**Fig. 3**). The rotation of Leu93, together with Phe219 creates space for three water molecules that connect the SB with the intracellular proton donor Asp96, opening the hydrophobic barrier by altering the hydrophobic interaction network. Transferring the proton to the SB replenishes the substrate at the center of the membrane.

The structural rearrangements that we observe leave Asp96 covered by a single hydrophobic lid formed by Phe42, making it accessible for reprotonation. Furthermore, structural changes to a cluster of residues acting as a proton funnel at the cytoplasmic side of the protein aid reprotonation of Asp96 (**Fig. 3**) (34), which may transiently proceed via an intracellular uptake cluster (35).

Time-resolved serial crystallography has revealed the structural reorganizations during proton uptake and release in bR with astounding detail and over many orders of magnitude in time. The alternating rearrangements on the extracellular and intracellular sides during the pumping cycle of bR agree with an alternate access model and may provide a template to understand the principal transport steps in other membrane transport proteins. The large-scale motion of helix F further

resembles the outward movement of transmembrane helix 6 in G protein-coupled receptors. Bringing time-resolved serial crystallography developed at XFELs to the broader scientific community using synchrotrons should provide tools to answer many other pressing questions in structural biology.

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Competing interests:

The authors declare no competing interests.

Author contributions:

T.W., P.S., M.W. and J.S. conceived the research with suggestions on laser setups from D.J. and F.D.. Protein and microcrystals were prepared by A.F. and P.N.. Slow crystal extrusion for the use at synchrotrons was optimized by P.S. and D.J.. Synchronized diode and detector triggering was implemented by E.P.. The pump-probe setup was built by D.J. and F.D. with suggestions from M.W.. Data collection was done by T.W., P.S., D.J., A.F., D.K., S.B., F.D., D.O., P.N., M.W. and J.S.. Data processing and structural refinements were done by T.W. The manuscript was written by T.W. and J.S. with contributions from all authors.

Data and materials availability:

Coordinates, light amplitudes, dark amplitudes and extrapolated structure factors for light activated data have been deposited in the PDB database under accession codes 6RNJ (TR-SMX closed-state), 6RPH (TR-SMX open-state), 6RQO (Steady-state SMX activated-state). Coordinates and
5 dark state structure factors (steady-state SMX dark) have been deposited in the PDB database under accession code 6RQP.