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Microsecond time-resolved X-ray scattering by utilizing MHz repetition rate at second-generation XFELs

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Microsecond time-resolved X-ray scattering by utilizing MHz repetition rate at second-generation XFELs

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Abstract

Detecting microsecond structural perturbations in biomolecules has wide relevance in biology, chemistry, and medicine. Here, we show how MHz repetition rates at X-ray free electron lasers (XFELs) can be used to produce microsecond time-series of protein scattering with exceptionally low noise levels of 0.001%. We demonstrate the approach by deriving new mechanistic insight into Jα helix unfolding of a Light-Oxygen-Voltage (LOV) photosensory domain. This time-resolved acquisition strategy is easy to implement and widely applicable for direct observation of structural dynamics of many biochemical processes.

Main Text

Biomolecular transformations, reactions, and interactions are at the basis of all life. Deciphering these mechanisms in a time-resolved manner and with sub-molecular precision opens a new dimension of biological understanding. Access to sub-millisecond timescales in near-native environments is particularly important, but remains challenging.

There are two primary acquisition schemes to acquire time-resolved data. In ‘pump-probe’ mode, each reaction trigger is followed by a probe pulse at a defined time delay and time-series are constructed by repeated measurement of many time points. This mode enables femtosecond time-resolution and has been used at XFELs for time-resolved protein crystallography and protein solution scattering[1–3]. In practice, this method limits acquisition rates leading to larger sample consumption. An alternative approach is to read out a series of probe pulses following a single trigger event. In this way, the efficiency of data collection is vastly improved, reducing sample consumption and suppressing experimental noise through massive averaging[4]. Here, the time-resolution is limited by the X-ray repetition and detector acquisition rates.

MHz repetition rates at second-generation XFELs now open up the opportunity to use the latter scheme for time-resolved studies in the microsecond range. The European XFEL (EuXFEL) is the first in this class and delivers trains at 10 Hz containing up to 2700 X-ray pulses with a variable repetition rate up to 4.5 MHz (Figure 1b) [5]. Thus far, the high repetition rate has posed severe technical challenges for single-pulse detection of scattering and diffraction images, due to electronic noise and nonlinear gain in the detector readout, as well as shockwaves or explosions in the jet[6]. For these reasons, this unique timing capability has only been used in X-ray microscopy, dynamic compression experiments, and X-ray photon correlation spectroscopy,[7–9], but not yet in the pursuit of biomolecular structural dynamics

through protein scattering. Here, we demonstrate the realization of this approach through time-resolved wide-angle X-ray scattering (TR-WAXS) at the EuXFEL.

TR-WAXS can resolve structural changes of biomolecules and chemicals in solution, providing an ~atomic-scale glimpse of their function under near-native conditions[4,10,11]. We investigate the phototropin LOV2 domain from *Avena sativa* (AsLOV2), which features a prototypical signaling mechanism, where a C-terminal helix (Jα, 22 residues) detaches from the core in response to photoexcitation[12,13]. This unique photoactivity has been exploited in a broad range of optogenetic applications and has been the subject of intense experimental investigation[14–19]. Despite this interest, the mechanism and timing of Jα unfolding and the structure of the unfolded state are not definitively known.

To record microsecond TR-WAXS at the EuXFEL, the sample was carried in a liquid jet via 3D-printed Gas Dynamic Virtual Nozzle (GDVN)[20] to the interaction point of the optical and X-ray beams at the Single Particles, Clusters, and Biomolecules and Serial Femtosecond Crystallography (SPB/SFX) endstation (Figure 1a)[21]. Photoexcitation was achieved with nanosecond laser pulses timed to the start of every second X-ray pulse train (Figure 1b). The scattering was recorded on the AGIPD detector for each probe pulse, covering a q-range from 2.1 $Å^{-1}$ > q > 0.08 $Å^{-1}$ (corner resolution). The 2D scattering was integrated into rings as a function of the momentum transfer (q) and delay time (t) along the pulse train. Approximately 30% of the data were excluded, because the shape of the scattering was affected by fluctuations in the jet (see online methods). After averaging over several repeats, the difference scattering $\Delta S = S_{light}(q,t) - S_{dark}(q,t)$ was computed (Figure 1c). We found that it was crucial to subtract entire laser-on from the laser-off trains from each other, reducing the effect of systematic noise in the detector (Supplementary Figure 2). An experimental time resolution of 1.77 μs corresponds to the inverse of the repetition rate of the XFEL (564 kHz) and the data span a time window of ~300 μs.

The X-ray scattering of AsLOV2 shows microsecond evolution with oscillations extending beyond q-values of 1.5 $Å^{-1}$, which translates into a spatial resolution of 4.2 Å. The data have an exceptionally low noise floor corresponding to 0.001% of the total signal, which is at least one order of magnitude lower than previous accounts for this method (Supplementary Figure 3)[22]. Deconvolution of the data using spectral decomposition with exponential conversion laws indicated that the data are best fit to a sequential model of type A→B→C, yielding base patterns for the three states (Figure 2b, Supplementary Figure 4). In TR-WAXS, large difference signals at low q < 0.15 $Å^{-1}$ typically indicate changes of the radius of gyration (Rg) of the protein[3]. From this we deduce that the structural change in state C is sizable, but that

changes in states A and B are comparably smaller. We assign state C to the unfolded state (*vide infra*), which is further underpinned by its timescale, emerging within ~300 µs (Figure 2a), in agreement with kinetics inferred from infrared spectroscopy[17,18]. States A and B could only be resolved because of the low noise floor of the new scattering method approach. State A forms within the first time point of our measurement at 1.77 µs, in agreement with previous reports of FMN-cysteinyl adduct formation[23]. We assign state B to a previously unrecognized intermediate state, which occurs subsequent to Cys adduct formation and prior to large changes in the Jα helix. Interestingly, intermediate states in Jα unfolding have been previously proposed through a long MD simulation[24], but not clearly observed experimentally.

Focusing on state C and to assess the extent of Jα unfolding, we refined structural models predicted by AlphaFold[25], where a large variability was obtained through sampling with dropout enabled inference (20000 structures predicted)[26] and a number of glycine mutations in the Jα helix. We then determined best fits against the predicted structures by comparison of the root-mean-square of residuals (R^2) between theoretical and experimental difference scattering curves (Figure 2d). Since we compare the curves on absolute scales, this selection is also based on appropriate computed activation factors of the structural pairs (further described in Supplementary Information, the boxed region in Figure 2c includes 6032 structures). All of the selected structures show unfolded Jα helices (subset shown in Figure 2f), with an increase of Rg by 5-7Å yielding the best fits (Figure 2e). Interestingly, an inspection of the best-fitting models shows that the residues directly preceding the Jα segment, which form a loop segment in the dark, form an ordered helical domain (Supplementary Figure 7). Finally, we find that the N-terminal A'α helix is unfolded in most structures. Our data establishes that (i) the Jα helix unfolds in a two-step mechanism within 300 µs, (ii) that it completely unfolds, and (iii) that additional structural changes accompany this process. This concludes a long series of investigations into Jα unfolding[14–19,24], and demonstrates the promising capability of this new time-resolved X-ray scattering method.

Our new implementation of TR-WAXS realizes the unused potential of MHz XFELs to provide unique structural information about transient states on the important microsecond timescale. The additional timing information is gained with only minor adjustments of existing XFEL acquisition schemes and is highly compatible with other methods that use short X-ray pulses, e.g. serial crystallography[1–3] or X-ray emission spectroscopy[27]. The method also exploits the high average X-ray flux at the EuXFEL resulting in exceptionally low noise levels. This enabled the identification of a new transient state in Jα unfolding, opening the door for investigating reaction dynamics with dilute samples of proteins, peptides, RNA or

DNA, especially when combined with ongoing development of ultrastable liquid jet sample injection technology[28]. It also permits detection of difference scattering signals to very high scattering angles ($q > 1.5$ Å^{-1}, Figure 1c), suggesting that time-resolved and high-resolution structural information can be obtained in crystallography[29–31] or single-particle diffraction experiments[32]. Overall, the presented method accelerates knowledge gain for dynamic enzymatic and chemical mechanisms.

Figures

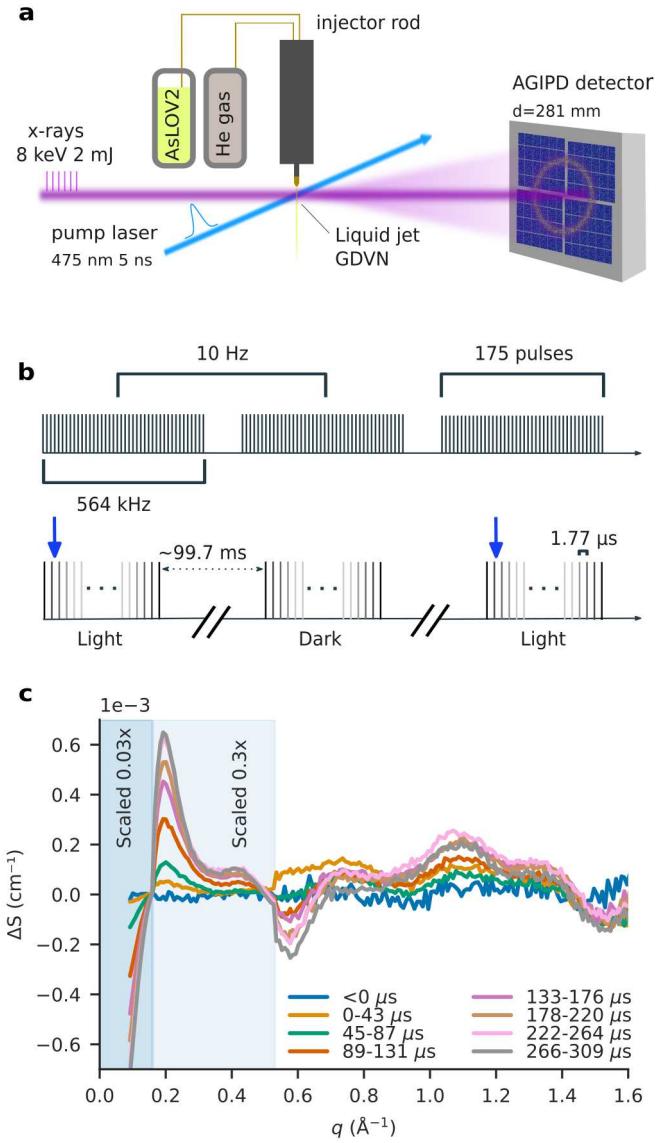

Figure 1. Microsecond TR-WAXS facilitating the MHz repetition rate at the EuXFEL. a. Schematic depiction of the X-ray and optical laser path, GDVN liquid jet, and recorded scattering with the AGIPD detector (not drawn to scale). **b.** Pulse train structure and laser excitation scheme used to obtain microsecond time-resolution. The 10 Hz trains comprise 175 pulses at 564 kHz (1.77 µs interval). The blue arrow depicts the timing of optical excitation of every other pulse train. **c.** TR-WAXS data of AsLOV2. The momentum transfer was defined as $q = 4\pi \sin\theta/\lambda$, with 2θ and λ as the scattering angle and the X-ray wavelength, respectively. The data was normalized in the q-range 1.6 Å^{-1} > q > 1.4 Å^{-1}, and scaled for better visualization as indicated in the panel.

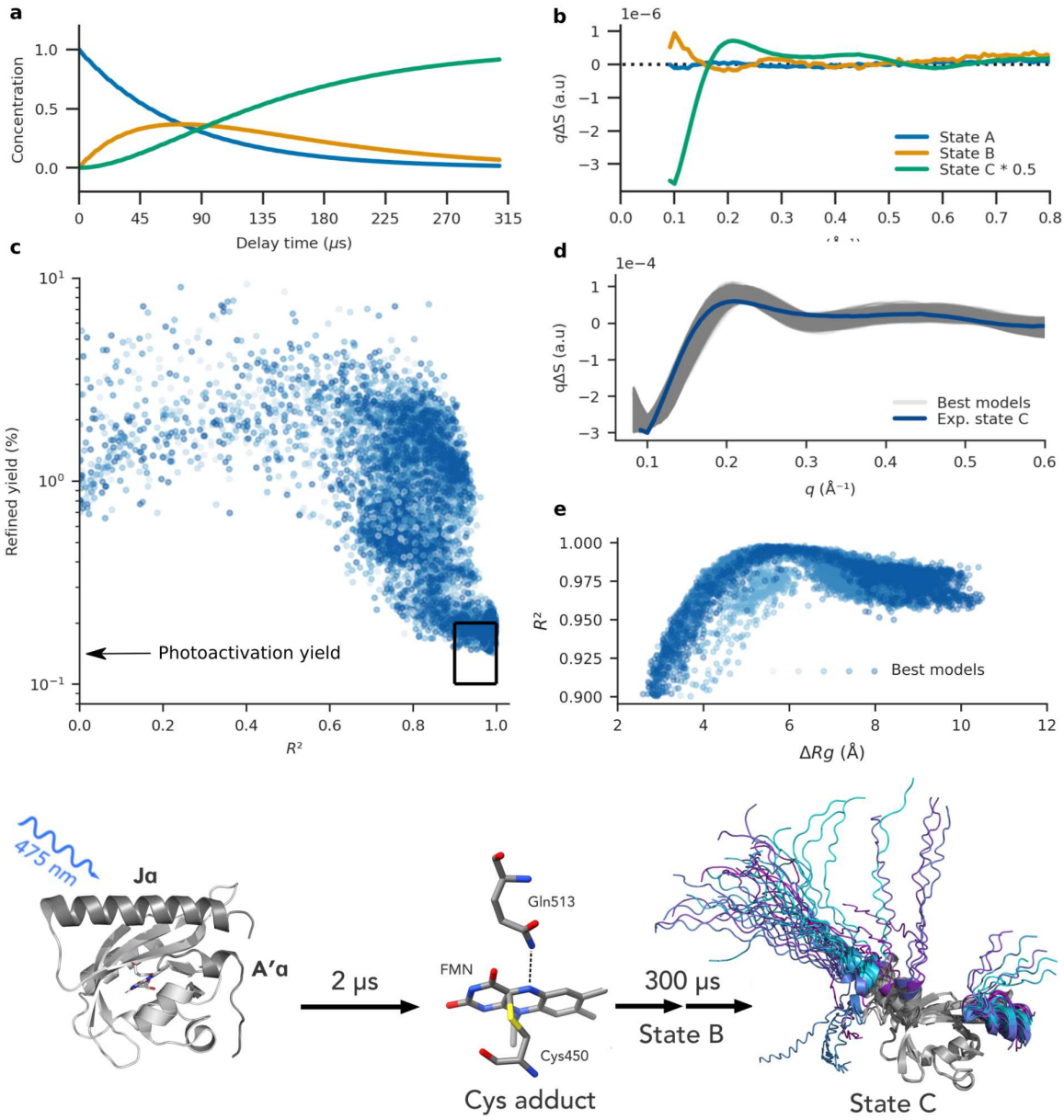

Figure 2. TR-WAXS yields a new intermediate state and the structure of the Jα unfolded state in a LOV domain protein. a. The time evolution of constituent states and **b.** their spectral components derived from kinetic decomposition of TR-WAXS data. **c.** Structural modeling results generated using our adapted AlphaFold method. R^2 is used as an indicator of a good fit between experimental and theoretical difference signals. Darker blue shades correspond to increasing numbers of mutations in the Jα helix. Structures with mutations in the N-terminal helix are also included. The best models were selected by choosing those that have both a photoactivation yield of 15 ± 5% (as derived in supplementary Figure 6) and $R^2 > 0.9$, resulting in 6032 candidate models (black box). **d.** The theoretical difference scattering of the best fits (gray) and the scaled experimental scattering profile of state C (blue) are shown. **e.** R^2 of the top candidate structures versus change in radius of gyration (*ΔRg*). **f.** The new structural dynamics results are shown in the canonical photoactivation mechanism of AsLOV2. This work finds that Jα unfolding occurs in a biphasic manner

within 300 µs. The structure of the unfolded state C is depicted as a subset of 50 selected from the best fits.

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Data availability

The raw experimental data is available at the EuXFEL repository: https://doi.org/10.22003/XFEL.EU-DATA-003046-00. The refined protein structures are also provided within this database.

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