Mapping protein dynamics at high spatial resolution with temperature-jump X-ray crystallography

Time-resolved crystallography (TRX) is a powerful method for investigating protein conformational dynamics with high temporal and spatial resolutions [1]. In the early stages of TRX development, the technique focused on photoactive proteins in combination with photoexcitation as the reaction initiators. The applicability of TRX has been widened by incorporating techniques, such as photocaged ligands [2] and rapid "mix-and-inject" methods [3]. However, universally triggering protein dynamics in time-resolved structural studies remains challenging.

Recent multi-temperature crystallography experiments have demonstrated that modifying the temperature allows tuning of the conformational ensemble sampled at equilibrium, facilitating temperature-jump (T-jump) TRX experiments using infrared (IR) lasers [4]. In such experiments, a mid-IR laser excites the O–H stretching mode of water molecules, resulting in rapid heating of the sample. The proteins exhibit various conformations at different temperatures, with more high-energy states at elevated temperatures. This method has also been used to study the thermal denaturation of enzymes. However, previous studies lacked detailed time-resolved observations of the motions induced by rapid heating.

We applied the T-jump method as a reaction-induced method for TR serial femtosecond crystallography (TR-SFX) with X-ray free electron lasers (XFEL). Lysozyme microcrystals suspended in hydroxyethyl cellulose carrier media were transported from the injector to the intersecting area using an XFEL and a nanosecond IR laser (Fig. 1). In the interaction zone, the crystals were rapidly heated by the IR laser and then exposed by X-ray pulses from SACLA **BL2** at specific intervals after the IR laser heating (20 ns, 20 μs, and 200 μs). Algebraic tools leveraged from previous solution X-ray scattering experiments were used to detect the induction of a T-jump, which was corroborated by observations of unit cell thermal expansion and increased atomic displacements.

Our analysis of the experimental maps supports a rapid increase in the atomic displacement parameters (B-factors) within 20 ns after IR laser irradiation, which suggests an increase in harmonic motion due to heat transfer from the excited solvent to the protein. The uneven distribution of difference electron density features, which are especially noticeable at shorter pump-probe intervals (Fig. 2), implies that heat flows more readily into certain regions of the lysozyme molecule than into others. We also modeled high-energy conformations directly from the TR difference electron density maps and refined them against the extrapolated structure factor magnitudes (ESFMs). This analysis revealed motions such as rotamer flips on fast timescales, and larger motions, including correlated shifts of loops spanning multiple residues, on slower timescales. The most significant of these movements includes a short loop covering residues 97–100, which lies at the end of an α-helix adjacent to the active site and is known to be mobile during lysozyme hinge bending.

We performed T-jump SFX in the presence of chitobiose to assess whether our analysis could detect functional protein motion. This compound is an inhibitor of lysozyme and shifts the conformational equilibrium of the enzyme toward the more compact ‘closed’ state in which the two lobes of the enzyme clamp down on the carbohydrate in the active site. We observed that the difference electron density maps corresponding to 20 ns time delay were similar for both the apo- and chitobiose-bound datasets. However, for a time delay of 200 μs, the difference electron density maps calculated from the chitobiose-bound data did not indicate the dissipation of short amplitude motions into larger-scale conformational changes, as seen for the apo enzyme. Instead, they exhibited similarities with the maps corresponding to the 20 ns time delay. The inhibitor restricted larger movements, such as hinge bending related to substrate binding and catalysis, locking it in a ‘closed’ conformation. Therefore, its presence did not affect the initial rapid

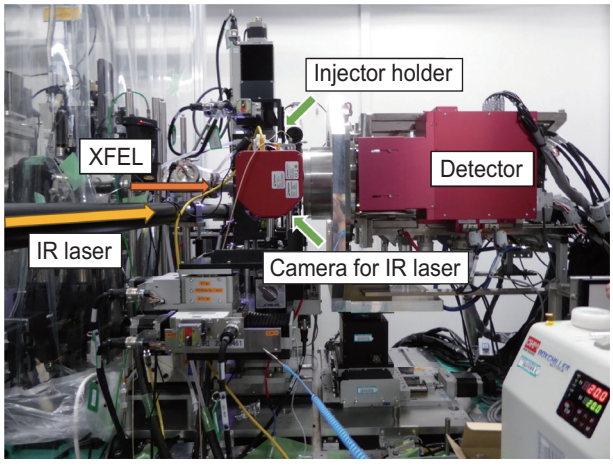

Fig. 1. Photograph of the T-jump TR-SFX experimental setup. The sample injector is installed behind the camera for IR laser detection.

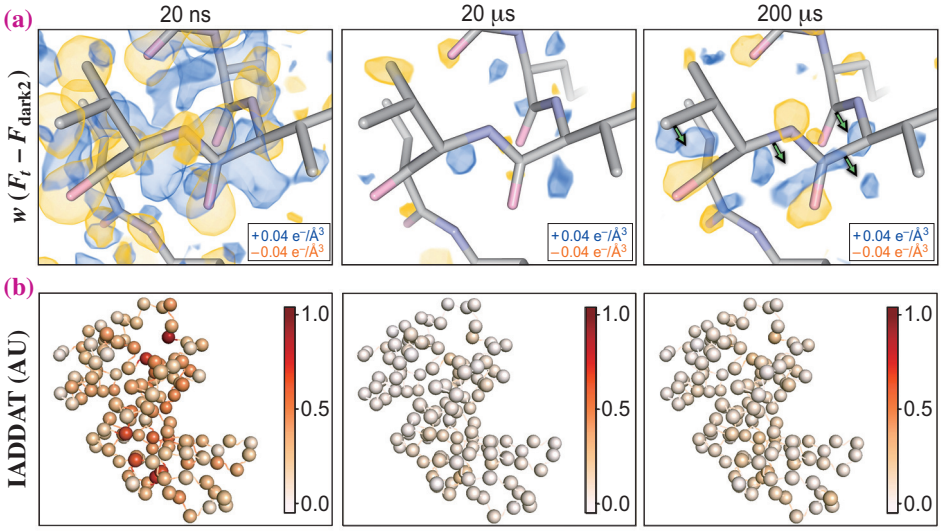

Fig. 2. Temporal evolution of the difference electron density observed during T-jump TR-SFX. **(a)** Comparison of weighted difference electron density maps for each pump-probe time delay, centered around residues 97–100. Maps were visualized at an absolute contour level of ±0.04 $e^-/Å^3$ alongside the corresponding refined models. Although the model coordinates appear stable across pump-probe time delays, difference maps reveal time-resolved changes in the T-jump induced signal, with evidence for coordinated motions (green arrows) apparent by 200 μs. **(b)** Integration of the absolute difference density above a noise threshold (IADDAT) was calculated as an average value per residue for each pump-probe time delay, then mapped onto Cα positions (spheres) of the respective model, and plotted as a function of the residue number. [5]

thermal movements through the enzyme, although it suppressed the changes attributed to microsecond functional motions (Fig. 3), probably shifting them to millisecond or longer timescales.

In this study, we revealed that rapid heating using a nanosecond-pulsed laser tuned to the mid-IR region excited the intrinsic dynamics of lysozyme crystals and demonstrated that this method is an effective perturbation for measuring protein conformational dynamics.

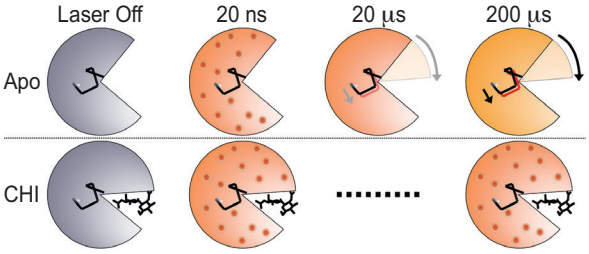

Fig. 3. Schema of the time-resolved conformational changes in the lysozyme after the T-jump. The schematic illustrates the closure of the active site cleft upon chitobiose binding, with subsequent representations of structural changes following the T-jump. For a time delay of 20 ns, atomic vibrations (red dots) occur in both the apo- and inhibitor-bound structures. These vibrations persist in the inhibitor-bound structure but dissipate into more complex motions in the apo-structure. [5]

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