An on-demand, drop-on-drop method for studying enzyme catalysis by serial crystallography

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Serial femtosecond crystallography has opened up many new opportunities in structural biology. In recent years, several approaches employing light-inducible systems have emerged to enable time-resolved experiments that reveal protein dynamics at high atomic and temporal resolutions. However, very few enzymes are light-dependent, whereas macromolecules requiring ligand diffusion into an active site are ubiquitous. In this work we present a drop-on-drop sample delivery system that enables the study of enzyme-catalyzed reactions in microcrystal slurries. The system delivers ligand solutions in bursts of multiple picoliter-sized drops on top of a larger crystal-containing drop inducing turbulent mixing and transports the mixture to the X-ray interaction region with temporal resolution. We demonstrate mixing using fluorescent dyes, numerical simulations and time-resolved serial femtosecond crystallography, which show rapid ligand diffusion through microdroplets. The drop-on-drop method has the potential to be widely applicable to serial crystallography studies, particularly of enzyme reactions with small molecule substrates.

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Serial femtosecond crystallography (SFX) techniques are relatively new and are undergoing rapid development. Synchronization of several femtoseconds long X-ray free-electron laser (XFEL) pulses with visible light pump lasers has been applied to time-resolved SFX (tr-SFX) studies on several systems over sub-picosecond or longer timescales. Most biological systems, however, are not light-dependent, necessitating the development of methods that could broaden the applicability of tr-SFX methods to a larger variety of biological samples. While some systems can be photosensitized by the generation of light-sensitive caged substrates or post-translational modifications of the protein itself, these strategies are sample-specific and do not offer a routine, general solution.

Studying enzymatic interactions by tr-SFX at conditions similar to physiological is emerging as a method that helps to advance our fundamental understanding of enzyme catalysis and offers great opportunities to improve the efficiency of therapeutic strategies. For enzyme-catalyzed reactions, triggering can be achieved by mixing protein crystals with substrate and collecting diffraction data after different time delays. Theoretical calculations predict that, using appropriately small crystals (~15 μm), millisecond diffusion times are achievable, which is sufficiently faster than many enzyme-catalyzed reactions (~70 ms) and should enable the substrate to occupy the majority of active sites before significant reaction occurs. Based on these findings, a number of approaches to investigate enzyme reactions are currently being pursued, both at XFELs and synchrotron X-ray sources. These have progressed rapidly in recent years to cover time delays from multisec to sub-second timescales.

Mixing approaches are, however, not easy to implement as they require rapid, reliable, and controlled mixing without damaging the crystal lattice. In this context, time-resolved serial synchrotron crystallography (tr-SSX) experiments can be particularly challenging, since much longer X-ray exposure times (e.g., ~1 ms to several tens of microseconds) are convoluted with reaction initiation strategies that together limit the overall time resolution achievable for the reaction under investigation.

The most established technique used in mixing experiments involves the sample being presented to the X-ray beam in the form of a liquid jet, which is characterized by a considerably high sample and ligand flow rate and is therefore not suited to samples with limited availability. Because of the large volumes of microcrystal slurry and ligand solutions demanded by continuous flow, sample consumption is of particular concern, especially since tr-SFX/SSX experiments typically need to cover a broad range of time delays necessary to create a molecule movie across the reaction cycle. Recent promising designs include samples being deposited onto a moving tape as a continuous stream and the liquid application method for time-resolved studies (LAMA), where substrate solution is applied from a piezoelectric dispenser onto a chip preloaded with microcrystals.

Here, we show an alternative on-demand droplet-based mixing strategy for time-resolved mixing experiments. This system builds on the drop-on-tape method, which has been shown to retain fidelity of crystalline order, provides low sample consumption, high hit rates and, above all, very high levels of versatility, especially since tr-SFX/SSX experiments typically need to cover a broad range of time delays necessary to create a molecular movie across the reaction cycle. Recent promising designs include experiments using the same calcium-sensitive fluorescent dye system and compared these results with the simulations (Fig. 1b, Supplementary Note 1, Supplementary Figs. 2–4, Supplementary Tables 1–3). In a three-dimensional reaction–diffusion system, the diffusion and binding of the calcium ligand from stationary picoliter droplets through a nanoliter-sized droplet containing fluorescent dyes takes considerably longer than the average enzymatic reaction (1–2 s, Fig. 1b). The reaction-mass-transfer simulation revealed, however, that the high-frequency and high-velocity collision of several small droplets distributed across the surface of the larger drop will decrease the equilibration time to <1 ms and to a point where the overall drop size and diffusion are less restrictive. To test how much the mixing is accelerated in the drop-on-drop approach, we performed proof-of-principle experiments using the same calcium-sensitive fluorescent dye system and compared these results with the simulations (Fig. 1b, Supplementary Note 1, Supplementary Figs. 5 and 6). The observations show that the rise of the experimentally measured fluorescence components, with rise times of <100 to 150 ms, is notably faster than that expected based on the simulated diffusion data (one order of magnitude). This confirmed that the mixing process induced by a substrate droplet colliding with the enzyme-carrying droplet is accelerated relative to simple diffusion, to a point where saturating the enzymatic active sites faster than the catalytic reaction progresses becomes possible.

Drop-on-drop enables tracking enzyme-catalyzed reactions with serial X-ray crystallography. To demonstrate the applicability of the drop-on-drop method to enzyme-catalyzed reactions in crystals, we used two different enzyme systems as case studies: hen egg white lysozyme (HEWL) and a serine β-lactamase (SBL), CTX-M-15, as two case studies to demonstrate the application of this method for enzyme-catalyzed reactions in crystals. Our results show that the drop-on-drop mixing strategy is capable of achieving sub-second time resolution with dramatically reduced ligand consumption and should be therefore readily applicable to explorations of a wide range of enzyme-substrate systems, including those such as the SBLs that are relevant to health and active or potential drug targets.

**Results**

**Drop-on-drop experimental setup enables accelerated mixing in droplets.** In the drop-on-drop mixing system design, substrate drops are added with piezoelectric injector (PEI), each carrying ~120 pl, by collision at a relative velocity of 1–2 m s⁻¹ with the main, crystal-bearing ADE drop. The drops are transported (30–300 mm s⁻¹) by the Kapton tape to the interaction region, which for SFX experiments was the location of the X-ray beam at SACLA (Fig. 1a, Supplementary Fig. 1, Supplementary Movie 1 and 2). To investigate whether collision-driven mass flow caused by the substrate drops arriving with a velocity of 1–2 m s⁻¹ at high frequency can accelerate mixing when compared to diffusion only, we carried out two sets of simulations on calcium-sensitive fluorescent dyes (see the “Methods” section, Supplementary Note 1, Supplementary Figs. 2–4, Supplementary Tables 1–3). In a three-dimensional reaction–diffusion system, the diffusion and binding of the calcium ligand from stationary picoliter droplets through a nanoliter-sized droplet containing fluorescent dyes takes considerably longer than the average enzymatic reaction (1–2 s, Fig. 1b). The reaction-mass-transfer simulation revealed, however, that the high-frequency and high-velocity collision of several small droplets distributed across the surface of the larger drop will decrease the equilibration time to <1 ms and to a point where the overall drop size and diffusion are less restrictive. To test how much the mixing is accelerated in the drop-on-drop approach, we performed proof-of-principle experiments using the same calcium-sensitive fluorescent dye system and compared these results with the simulations (Fig. 1b, Supplementary Note 1, Supplementary Figs. 5 and 6). The observations show that the rise of the experimentally measured fluorescence components, with rise times of <100 to 150 ms, is notably faster than that expected based on the simulated diffusion data (one order of magnitude). This confirmed that the mixing process induced by a substrate droplet colliding with the enzyme-carrying droplet is accelerated relative to simple diffusion, to a point where saturating the enzymatic active sites faster than the catalytic reaction progresses becomes possible.

**Drop-on-drop enables tracking enzyme-catalyzed reactions with serial X-ray crystallography.** To demonstrate the applicability of the drop-on-drop method to enzyme-catalyzed reactions in crystals, we used two different enzyme systems as case studies: hen egg white lysozyme (HEWL) and a bacterial serine β-lactamase (SBL), CTX-M-15. HEWL is a widely used model system for crystallographic methods development, while CTX-M-15 is a class A, extended spectrum SBL that is distributed worldwide in multiple bacterial pathogens where it is responsible for resistance to a range of β-lactam antibiotics (for example, ertapenem) and the mechanism of inhibition is currently not well understood, largely due to the lack of structural information. Using the drop-on-drop system at the SACLA XFEL source, we first obtained a series of time-resolved HEWL structures recorded at
0.2, 0.6, and 2 s after addition of two to six drops of an inhibitor, N-acetyl-D-glucosamine (GlcNAc, 16.7–43.7 mM final concentration, Supplementary Tables 4 and 5). As expected, the resting state control model shows an active site with ordered solvent molecules but devoid of organic ligands. Although the structure determined at the 0.2 s time point is essentially identical to the resting state model, the isomorphous difference and polder OMIT electron density maps for the 0.6 and 2 s delay times display strong evidence for the presence of a ligand in the active site despite ligand affinity in the mM range23 (Fig. 2a, Supplementary Fig. 7). The ligand can be unambiguously assigned as GlcNAc, in a position corresponding well to that reported in other studies24–26 (Fig. 2b–d, Supplementary Note 2).

Based upon the limited set of conditions tested at SACLA, the HEWL results clearly demonstrate that the drop-on-drop mixing method can be used for mix-and-diffuse studies of ligand binding to protein crystals on timescales down to at least 0.6 s after ligand addition to the protein microcrystal suspension. This encouraged us to collect a similar time-resolved series of structures for CTX-M-15 and a β-lactam antibiotic, ertapenem, with 0.6 and 2 s time delays between the drop-on-drop ligand addition and the X-ray exposure (Supplementary Tables 4 and 6). Although clear changes in the active site of the HEWL structure are visible after 0.6 s, no apparent changes in the active site of CTX-M-15 are present at the same time point after addition of four drops of ertapenem (110.3 mM final concentration, Fig. 2e, Supplementary Figs. 8 and 9, Supplementary Table 7). This is despite the similar crystal dimensions and crystal solvent content of the two systems (Supplementary Fig. 10), a greater molar excess of the ligand used with CTX-M-15 and higher protein-ligand affinity (11.6 μM for CTX-M-15–ertapenem [Supplementary Table 8] vs. 47.6 μM for HEWL-GlcNAc25). Successful binding of ertapenem in the 2 s time point data is apparent in the $F_o(2 s) - F_o$(resting) isomorphous difference map, since clear difference density adjacent to the nucleophilic Ser70 appears (Supplementary Fig. 9), which allows modeling of an acyl-enzyme complex into the electron density (i.e., with the β-lactam ring open by reaction with the nucleophilic serine, Fig. 2f, Supplementary Figs. 11 and 12). This was further confirmed by calculation of polder OMIT maps which show electron density consistent with ring-opened ertapenem bound similarly to that in a control presoaked dataset (Fig. 2g, Supplementary Note 3).

Discussion

In conclusion, the data presented here demonstrate that the on-demand, drop-on-drop method developed from the drop-on-tape XFEL sample delivery system, can indeed overcome the limitations associated with ligand diffusion through droplets. Our successful observation of ligand complex formation in our tr-SFX data at timescales below 0.6 s shows that the drop-on-drop method enables generation of enzyme complexes with small molecule ligands/substrates on timescales similar to the kinetics of enzyme-catalyzed reactions. The versatility and efficiency of drop-on-drop sample delivery substantially expands the currently very limited set of low sample consumption delivery systems able to achieve reaction times suitable for studying enzymatic reactions. Indeed, our six structures required only 84–258 μL of ligand solutions, and 162–420 μL of microcrystal slurry. For example, the 0.6 s tr-SFX HEWL dataset consumed a total of only 0.7 μmol (9.5 mg) protein and 0.18 mL of 0.226 M ligand (41 μmol total). In addition, by adding humidity and temperature control and varying the position of the PEI, the system can offer easy access to a wider range of delay times (from 50 ms to 10 s) relevant for many enzymatic reactions. Also, a large range of crystal sizes (5–100 μm) can be accommodated by this system, as the droplet size can be adjusted, and no limiting orifice is used for generation of the crystal-containing droplets. This allows to choose optimum conditions for each individual protein system, balancing needed diffraction signal strength (often increased with larger crystal size) and desired minimum diffusion equilibration time (decreased with smaller crystal size, also highly dependent on buffer viscosity).

It is important to realize how ready access to such technologies will make a major contribution to exploiting the potential of tr-SFX/SSX to transform our understanding of protein-ligand interactions and enzyme catalysis. Drop-on-drop technology can be in principle easily transferred from the XFEL to the...
synchrotron environment, which will help alleviate restricted access to XFEL facilities that is limited by global capacity. Our method has the potential to increase the popularity of dynamic structural biology studies becoming particularly applicable at next-generation MX beamlines that offer brighter/pink photon beams with sub-millisecond exposure times that will help ensure adequate temporal resolution.

Methods

Crystal preparation. Hen egg white lysozyme (HEWL) crystals were grown by the rapid-mixing batch method. In brief, 2 mL of 50 mg mL⁻¹ HEWL (Sigma L4919) dissolved in 20 mM sodium acetate pH 4.6 was pipetted to a 15 mL polypropylene tube and mixed with an equal volume of crystallization buffer (1 M citric acid, 20% (w/v) NaCl, 5% (w/v) PEG 6000, pH 3.0) with vortexing at 22 °C. Crystals nucleated almost immediately and were left for at least 1 h to reach the maximal size (3–5 µm). The data for 0.6 and 2 s datasets were collected from the original crystal slurry (~10⁷ crystals mL⁻¹) whereas the data for the resting and 0.2 s datasets were collected from the same crystal batch, concentrated threefold. Recombinant CTX-M-15 (in the expression vector pOPINF19) was expressed in SoluBL21 (DE3). E. coli cells and the His-tagged protein was purified by passage over Ni-NTA resin (Qiagen) in 50 mM HEPES (pH 7.5) and 400 mM NaCl and elution with the addition of 400 mM imidazole. The His-tag was then removed by over Ni-NTA resin (Qiagen) in 50 mM HEPES (pH 7.5) and 150 mM NaCl. Peak fractions were concentrated to 20 mg mL⁻¹ by centrifugation. CTX-M-15 crystals were nucleated with seeds generated from crushed ~500 µm sized crystals grown at 20 °C, by sitting drop vapor diffusion in CryoChem 24-well plates (Hampton Research). Drops comprised 1 µL of CTX-M-15 (20 mg mL⁻¹) in 50 mM HEPES pH 7.5, 150 mM NaCl and 1 µL of crystallization solution (0.1 M Tris pH 8.0, 2.4 M (NH₄)₂SO₄) and were equilibrated against 500 µL of crystallization solution. For the seed stock, crystals were crushed, mixed with crystallization solution, and stored at ~80 °C until needed. Micromolecules were then grown by sitting drop vapor diffusion in CryoChem 24-well plates at 20 °C. Drops comprised 2 µL of crystallization solution (2.0 M (NH₄)₂SO₄, 0.1 M Tris pH 8.0), 1 µL of seed stock and 2 µL of enzyme (20 mg mL⁻¹) and were equilibrated against 500 µL of crystallization solution. Rod-shaped crystals grew within 24 h, with a maximum width of 5 µm and length of 10–20 µm. They were then harvested and left to settle to increase the crystal concentration to ~8 × 10⁵ crystals mL⁻¹.

Sample injection and mixing. Mixing experiments were performed in July 2019 under proposal 2019A8088 at the BL2 instrument at SACLA, Japan. During these experiments, the hatch temperature was typically between 34 and 36 °C. The conveyor belt was used in combination with acoustic droplet ejection (ADE) as described in detail in Fuller et al. with some modifications. The ADE transducer was used to deposit ~3 nL crystal slurry droplets at 30 Hz deposition frequency onto the surface of the Kapton belt (Fig. 1a, Supplementary Fig. 1). The microcrystal suspension was fed into the ADE well from a reservoir through a 250 μm ID fused silica capillary attached to a syringe held in a rotation motor that was programmed to rotate back and forth by 180° to prevent crystal settling. The XFEL master clock running at 30 Hz was used to trigger ADE droplet ejection, which in turn generated a TTL (transistor–transistor logic) signal to trigger a second, piezoelectric injector (PEI) head (PolyPico Technologies, Cork, Ireland) mounted downstream with respect to the belt movement and above the tape. The PEI was used to add an aqueous solution of substrates to the crystal-containing drops in the form of ~120 µl droplets dispensed from a disposable plastic cartridge (100 µm orifice), which was continuously refilled from a syringe pump through a 200 µm ID fused silica capillary. Substrates, N-acetyl-D-glucosamine (GlcNAc, Sigma A8625) and ertapenem (MedChemExpress, USA) were dissolved in water to a final concentration of 50 mg mL⁻¹ (226 mM) and 398 mg mL⁻¹ (0.8 M), respectively, and filtered through a 0.22 µm syringe filter. Crystal suspensions were filtered through a 100 µm capillary. Both crystal slurry- and substrate-containing syringes were kept at 4 °C to prevent the solutions from degrading.

We tested dispensing substrate at a variety of frequencies, including the maximal achievable frequency of PEI in order to maximize the number of substrate drops hitting the crystal drop and so introducing as much substrate as possible. In order to reduce their consumption, substrates were added in the form of droplet bursts synchronized with the ADE slurry droplets. A camera viewing system was used to manually adjust the PEI trigger delay time that controlled the overlap between the crystal drop and the substrate burst. The calculated theoretical numbers of substrate solution drops merged with each crystal drop for data collected on HEWL and CTX-M-15 are presented in Supplementary Table 4.
Reaction times were varied by changes in Kapton belt velocity, bringing the sample to a fixed beam interaction location. The setup allowed for reaction times between 0.1 and 6 s (600 and 10 mm s⁻¹ tape speed, respectively). Our control datasets without substrate additions were recorded using 100 or 300 mm s⁻¹ tape speed. The number of substrate drops dispersed per burst (between 10 and 20) slightly exceeded the number required to fully cover the crystal drop. We observed that this greatly simplified the drop synchronization process and resulted in much more stable and burst-free PEI deposition. The tape speed was set such that the maximal achievable substrate concentration (50 pl droplets instead of 120 pl) and increasing the probability of clogging if unanticipated particulates are present in the substrate solution.

Fluorescence measurements of calcium-sensitive dyes. To experimentally assess substrate mixing and equilibration times through the ADE drops, the drop-on-drop setup was used to record the fluorescence of calcium-sensitive dyes in solution. A dye solution containing 1 mM Fura Red (CalIII-K₂, of 400 mM, AAT Bioquest) and 1 mM Fluo-SN (CalIII-K₂, of 90 mM, Thermo Fisher) was dispersed by ADE at 30 Hz onto the Kapton tape in the form of 4 nl droplets. One to five drops of 100 mM CaCl₂ solution were dispensed from the PEI onto the ADE drops at 0.5-1 kHz. APEI was equipped with a 100 μm orifice, which produced an average drop volume of 60 pl to yield an equilibrium concentration of 1.5-7 mM CaCl₂. The fluorescence signal was measured at the same position as the XFEL interaction point with a fiber-coupled spectrometer (Ocean FX). The samples were excited by a 455 nm 10 W LED (M455F1, Thorlabs). A long pass filter with a cutoff wavelength of 480 nm (FE50500, Thorlabs). The tape speed was varied to adjust the interaction time. The fastest interaction time measured was 200 ms, with measurements constrained by the position of the PEI head and limited by the sensitivity of the optical system. Four scans of 300 ms integration time were averaged per measurement (signal from 10 drops on average).

Enzyme assays. All reactions were carried out in 10 mM HEPES pH 7.5, 150 mM NaCl at 25°C using Greiner half area 96-well plates and a POLARstar Omega (BMG LabTech) plate reader28. Kinetic parameters were calculated and analyzed using GraphPad Prism 6.0.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com26. Steady-state parameters kcat and Kₘ for ertapenem hydrolysis were calculated by measuring initial rates of ertapenem hydrolysis with 5 mM CTX-M-15 and plotted against ertapenem concentration. Under the same experimental parameters, CTX-M-15 hydrolyses nitrocefin (a β-lactam reporter substrate) with a k₅₀ of 3 × 10⁻⁷ M⁻¹ s⁻¹. 5 mM CTX-M-15 was required to initiate the very slow hydrolysis of ertapenem for k₅₀ and Kₘ determination. k₅₀ and kcat/Kₘ were calculated using direct competition assays29,30 (under steady-state conditions without preincubation) against the chromogenic reporter substrate nitrocefin44. The reciprocals of the initial rates of nitrocefin hydrolysis (at a fixed concentration of 50 μM) by 1 nM enzyme were plotted against ertapenem concentrations ranging from 156 nM to 20 μM and corrected to account for the Kₘ of nitrocefin45.

Diffraction data collection. Data at SACLAC were collected at 34–36°C (similar to the hutch temperature) using ~450 μm X-ray pulses of 10 fs length at 10 keV and with wavelengths focused to a spot of 1.4 μm FWHM at the sample position. Data were recorded at 30 Hz on a Rayonix MX300-HS detector operating in the 4 by 4 binning mode. Over the course of the SACLAC data collection, the average ratio for crystal lattices integrated to images collected ranged from ~6 to 26%. Each dataset was collected within 30–80 min consuming between 162 and 420 μL of microcrystalline K₂SO₄ slurry sample, which is significantly lower than the consumption of 1.40 and 0.27 µg of HEWL and CTX-M-15 per integrable detector frame, respectively.

Room-temperature (21°C) datasets on reference crystals as well as crystals exposed to substrate for 10 min were collected on beamline I24, Diamond Light Source. Data were collected on detector 6 of MAXII (5.2 × 10²¹ photons s⁻¹) using a beam size of 8 by 8 μm² and an X-ray energy of 12.4 keV. Sample was mounted in the fixed-target setup52 with 100 μl of the
microcrystal slurry or 100 µL of 1:1 (v/v) mixture of the slurry and 100 mM etarpem solution loaded per single chip.

Data processing. Lysozyme data reduction was performed on frames identified as hits by Cheetah33 and achieved using ccP4.xfel16,44,45 as described previously49. HEWL data were indexed with dials.stills_process16 in space group P4422 with the target unit cell of a = b = 79.3 Å, c = 38.2 Å, α = β = γ = 90° (subsequently refined as detailed below). Data were merged using cifmerge33,46,47 with PDB entry AETA15 serving as the initial reference model. The resolution cut-offs for the final datasets merged with cifmerge were determined in a standard procedure based on a combination of several criteria, including where the data falls below tenfold multiplicity, where CC100, no longer decreases monotonically, and where the values of I/σ(I) do not uniformly decrease any more14. The resolution cut-offs were also confirmed in the ‘paired refinement’ test done using the PDB_REDO platform50, which includes the implementation of the original algorithm from Karplus and Diederichs49. In the first round of merging, integrated frames from all datasets were pooled together and the unit cell parameters were allowed to refine. This produced a ‘composite’ MTZ file with the unit cell parameters representing all data (a = b = 78.8 Å, c = 38.0 Å, α = β = γ = 90°). Next, a new reference model was obtained by running molecular replacement using the same coordinates against the ‘combined’ MTZ file with Phaser50. The new reference model was then used in the second, final merging round in which the target unit cell parameters were fixed and only frames with unit cell parameters lying within 1% of these values were accepted. The reference model used in the final merging step was used as the starting point in structure refinement. The structures were refined via iterative cycles of refinement performed using Phenix51 and manual model rebuilding using Coot52. Insights into ligand binding were obtained by examination of structure-factor amplitude Fourier difference maps, calculated with Phenix by subtracting observed structure-factor amplitudes for the resting dataset from those of the time point dataset, using phases calculated from the resting state model. GlcNAc was placed in the models according to the initial Fo·(time point)−Fo·(resting) and mFo–DF, maps. Restraints for GlcNAc were generated using the Grade Web Server (http://grade.globalphasing.org, 2020) using the identifier NDG. Data collection details and refinement statistics can be found in Supplementary Table 6.

CTX-M-15 data were indexed and integrated using DIALS66 (synchrotron data) and ccP4.xfel16,44,45 (XFEL data). Images were indexed with dials.stills_process in space group P212121 with the target unit cell a = 45.3 Å, b = 45.9 Å, c = 118.5 Å, α = β = γ = 90° (subsequently refined as detailed below). Next, datasets were merged with cifmerge26,27 using PDB entry 6QW830 (CTX-M-15). Source data are provided with this paper.

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7