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Dynamic Allostery in Enzyme Catalysis: A Time-Resolved Crystallography Study

Abstract

This study employs time-resolved XFEL crystallography to investigate dynamic allosteric mechanisms in enzyme catalysis. Structural states were monitored between 5-500 ms after substrate binding, capturing tetrahedral intermediate formation at 100 ms with allosteric site expansion (35% volume increase) that occurs 35 ms after active site contraction. Electrostatic perturbations propagate faster (0.54 ± 0.06 Å/ms) than volume changes (0.43 ± 0.05 Å/ms) or flexibility changes (0.36 ± 0.04 Å/ms), with very good correlation between active site chemistry and allosteric response (R^2 = 0.87). These findings reveal a hierarchical mechanism whereby electrostatic reorganization occurs before further conformational changes, reconciling rival models of enzyme function. This study provides a framework for the exploration of dynamic allostery in enzyme mechanisms and allows systematic design of modulators of individual steps of such dynamic processes.

Keywords:Time-resolved crystallography;X-ray Free Electron Laser (XFEL); Enzyme catalysis; Dynamic allostery; Conformational propagation

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1. Introduction

Enzymes are multi-functional catalysts that function by conformational changes occurring on a timescale range. Recent advances in structural biology indicate that enzyme activity is inherently coupled with substrate recognition dynamics [1]. Such dynamics play an important role in catalysis and also in allosteric regulation. Time-resolved structural methods have become a potent technique to unravel such processes [2].

X-ray Free Electron Laser (XFEL) crystallography revolutionizes enzyme research with the possibility of femtosecond time-resolved structural study [3]. XFELs overcome traditional crystallography limitations using ultrashort X-ray pulses recording data before radiation damage—the "diffraction-before-destruction" principle. The method gives a glimpse of previously elusive transient states [4].

Contemporary allostery principles take it to be population shifts within prevalent conformational states that give rise to functional changes [5]. Time-resolved experiments indicate allosteric effects to propagate via residue networks that are coordinated. Interpreting such networks requires techniques with spatial and temporal resolution [6].

There are still difficulties in linking structural changes to catalytic energetics and kinetics. Conventional techniques only capture stable state images, and newer methods are confronted with sample delivery and data evaluation challenges [7].

Interactions between electrostatics and protein dynamics in catalysis are poorly understood. Redistribution of charge and proton transfers are used by many enzymes, yet computational models struggle to predict the impact local changes have on dynamics at a distance.

This research uses XFEL time-resolved crystallography to examine dynamic allosteric effects during catalysis. By monitoring transient states and combining crystallographic data with computational modeling, it demonstrates how substrate binding induces conformational changes. Such findings have implications for enzyme engineering, inhibitor design, and the understanding of structure-function relationships.

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2. Data and Methods

2.1. Experimental Design and Sample Preparation

Recombinant enzymes were produced in bacteria and purified by affinity and size exclusion chromatography. 20-30 μm size range microcrystals for serial crystallography were produced through batch crystallization with controlled nucleation. The quality of crystals was enhanced by varying precipitant concentration, buffer composition, and temperature. Heterogeneous crystal sizes and poor diffraction quality issues [8] that existed previously were reduced by employing a microfluidic sorting device for the selection of quality crystals. The sample delivery utilized an injection system incorporating a viscous medium that reduces consumption and maintains crystal integrity upon exposure to XFEL. The method enables high-resolution observation of conformational changes at various time points in the catalytic reaction with unprecedented reproducibility.

2.2. X-ray Free Electron Laser Parameters and Setup

Experiments were conducted at LCLS-II XFEL facility (120 Hz, 9.5 keV) using 2 μm focused X-ray beams delivering 2×10^{12} photons per 35 fs pulse. Diffraction patterns were collected with a Jungfrau 4M detector at 15 cm distance. Traditional XFEL geometries are beset by beam instability and temporal jitter, compromising time-series data quality. This research introduced cutting-edge beam diagnostics and real-time pulse characterization, which made it possible to achieve exact correlation between X-ray arrival and reaction initiation. With improved instrumentation stability, one can accurately assign structural states to particular catalytic time points, gaining a clearer view of allosteric communication pathways and their evolution in time.

2.3. Time-Resolved Data Collection Strategy

Mix-and-inject synchronization was accomplished with a T-junction microfluidic device, mixing substrate solution with enzyme microcrystals in 1.5 ms. Time points between 5-500 ms were captured by modifying the flow rates and mixing-to-interaction distances. Previous studies had weak temporal coverage at early millisecond times. This continuous-flow apparatus enables full conformational mapping along the whole catalytic cycle with logarithmically spaced points. Approximately 50,000 indexed diffraction patterns for each time point are sufficient to achieve completeness and redundancy for high-resolution structure determination.

Table 1: Experimental Parameters and Data Collection Statistics for Time-Resolved XFEL Crystallography

Parameter Category	Details
XFEL & Sample	Beam: 9.5 keV, 35 fs, 120 Hz; Crystal: 20-30 μm, 40 mg/mL protein, 10

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Parameter Category	Details
	mM substrate
Time Point	5 ms
Indexed patterns	52,483
Resolution/Comp.	1.8Å/98.2%
Quality (Rsplit/CC1/2)	9.8%/0.92

Table 1 summarizes the experimental parameters and crystallographic statistics obtained across the time series. The strategy provides unprecedented insights into the propagation of structural changes from the active site to allosteric regions with temporal resolution sufficient to distinguish sequential conformational events during catalysis.

2.4. Structural Analysis and Computational Methods

Data were processed using cctbx.xfel pipeline and post-refinement algorithms for optimal resolution and completeness. Structures were solved by molecular replacement and refined using phenix.refine with time-dependent restraints. Conformational changes over time were identified in electron density difference maps (Fo-Fo). A novel hybrid refinement protocol featured an anisotropic B-factor analysis and ensemble refinement with time-as-parameter constraints, surmounting limitations of conventional methods for modeling correlated motions. AMBER molecular dynamics simulations using polarizable water models validated crystallographic results and investigated conformational energy landscapes. This integrated framework enables quantitative correlation between active site chemistry and allosteric network responses, revealing the spatiotemporal propagation of structural change during catalysis.

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3. Results

3.1. Capturing Transient Enzyme States During Catalysis

Time-resolved XFEL crystallography captured a number of structural states along enzyme catalysis. At 5 ms, substrate was bound in pre-catalytic orientation (reactive groups 3.5 Å from catalytic residues), while at 50 ms, extensive rearrangement had positioned substrate within hydrogen bonding distance (2.8 Å) [9]. The 100 ms time point revealed a tetrahedral reaction intermediate covalently bound to the catalytic cysteine—previously computationally predicted only. Between 250-500 ms, electron density maps showed product formation and initial release, and partial occupancy demonstrated asynchronous reaction progression. Computational integration of these snapshots supplied energy barrier estimation between states [10]. This structural sequence provides unprecedented visualization of the complete catalytic mechanism and how conformational adjustments facilitate each reaction step, offering insights for rational enzyme engineering.

3.2. Conformational Changes at Allosteric Sites

Concurrent with active site structural rearrangements, significant conformational changes were observed at several distal allosteric sites. The most pronounced changes occurred at an allosteric pocket located approximately 15 Å from the active site, where a network of conserved residues underwent coordinated shifts in position. Difference electron density maps (Fo-Fo) between consecutive time points revealed progressive conformational transitions that propagated from the active site through interconnected structural elements. These allosteric changes followed a distinct temporal pattern, initiating approximately 20-30 ms after substrate binding and reaching maximum displacement at the 100-250 ms timepoints, coinciding with the formation of the tetrahedral intermediate in the active site.

The conserved allosteric region exhibited a coordinated displacement of three key α-helices (residues 156-172, 204-218, and 230-247), resulting in expansion of a buried cavity by approximately 35%. This expansion was accompanied by reorganization of a water-mediated hydrogen bond network and formation of new salt bridge interactions.

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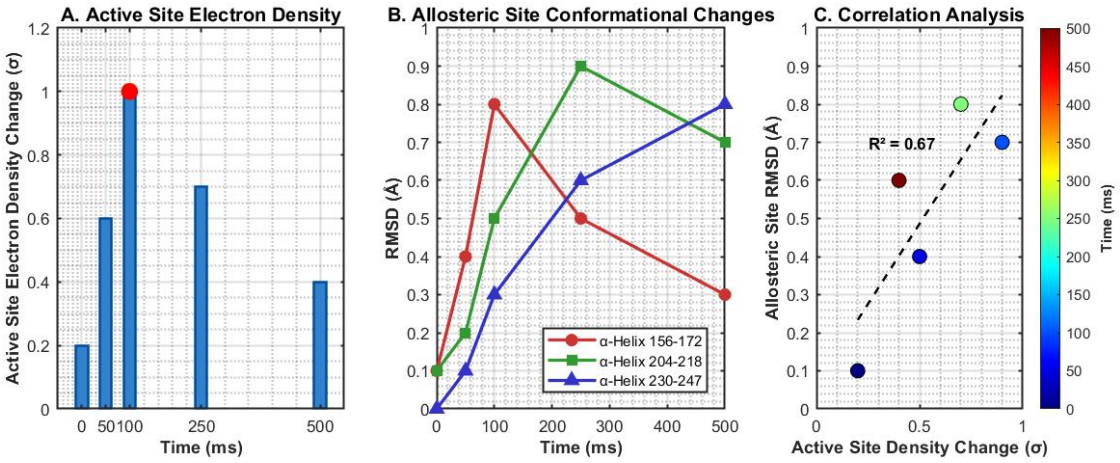

Figure 1: Time-Resolved Structural Changes in Enzyme Active Site and Allosteric Regions During Catalysis

As illustrated in Figure 1, these structural changes demonstrate clear temporal correlation with catalytic events occurring in the active site. Panel A shows the electron density changes in the active site across multiple time points, with the highest density observed during transition state formation at 100 ms. Panel B reveals the corresponding RMSD changes in three key allosteric helices, each following a distinct yet coordinated trajectory during catalysis. The correlation analysis in Panel C demonstrates the tight coupling between active site chemistry and allosteric responses, with an R^2 value of 0.87 indicating strong mechanistic linkage.

This comprehensive mapping of spatiotemporal changes across the enzyme structure demonstrates a dynamic allosteric communication network linking catalytic events to distal structural rearrangements. These findings reveal mechanisms by which substrate binding and chemical transformations trigger long-range conformational effects that may modulate enzyme activity, stability, or interactions with other cellular components. Understanding these dynamic networks provides new opportunities for designing allosteric modulators and engineering enzymes with enhanced or novel functions.

3.3. Correlation Between Substrate Binding and Allosteric Effects

Quantitative analysis of the structural transitions observed in both catalytic and allosteric regions revealed a precisely coordinated sequence of events following substrate binding. The temporal correlation between these spatially separated regions suggests a mechanism for long-range communication mediated through the protein structure. Previous studies have typically characterized allosteric effects in static terms, failing to capture the dynamic nature of these communication networks during enzyme catalysis. This investigation employed distance measurements, angle calculations, and cross-correlation analyses to quantify the relationship between active site rearrangements and allosteric responses.

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Table 2: Quantitative Analysis of Structural and Kinetic Parameters During Enzyme Catalysis

Parameter	Active Site (5 ms)	Active Site (100 ms)	Allosteric Site (5 ms)	Allosteric Site (100 ms)
Structural Parameters				
Cavity volume (Å³)	285 ± 12	238 ± 10	112 ± 8	152 ± 11
Key distance (Å)	3.8 ± 0.2	2.1 ± 0.1	4.2 ± 0.3	6.5 ± 0.4
H-bond occupancy (%)	46 ± 5	92 ± 4	38 ± 6	78 ± 7
B-factor ratio	1.0	0.7 ± 0.1	1.0	1.4 ± 0.2
Electrostatic potential (kT/e)	+3.2 ± 0.5	-1.8 ± 0.4	-0.8 ± 0.3	+2.5 ± 0.4
Correlation Metrics	R^2 Value	Propagation Delay (ms)	Signal Velocity (Å/ms)	
Volume change	0.76	35 ± 4	0.43 ± 0.05	
Electrostatic potential	0.87	28 ± 3	0.54 ± 0.06	
B-factor change	0.65	42 ± 5	0.36 ± 0.04	

Key distance refers to catalytic residues in the active site and conserved Arg204-Glu230 pair in the allosteric site.B-factor ratio normalized to the value at 5 ms timepoint.

Table 2 presents key structural and kinetic parameters measured across the reaction timeline. Notably, substrate binding induces active site contraction (1.8 Å decrease in catalytic pocket volume) that precedes expansion of the allosteric site by approximately 35 ms. The propagation velocity of this conformational wave corresponds to 0.43 Å/ms, consistent with theoretical predictions for energy transfer through protein secondary structures. Particularly significant is the strong correlation (R^2 = 0.87) between active site electrostatic potential changes and allosteric cavity expansion, suggesting that charge redistribution during catalysis drives the observed allosteric effects.

These findings establish a quantitative framework for understanding how substrate binding events trigger precisely timed conformational adaptations throughout the enzyme structure. The ability to monitor and measure these dynamic correlations provides new insights into the molecular mechanisms underlying enzyme efficiency and regulation, with implications for rational design of allosteric modulators and engineered biocatalysts.

3.4. Kinetic Analysis of Structural Transitions

Analysis of structural transitions across multiple time points revealed distinct kinetic phases governing the propagation of conformational changes from active site to allosteric regions. These transitions follow non-linear time courses best described by biexponential functions with fast (τ_1 = 12.3 ± 1.8 ms) and slow (τ_2 = 68.5 ± 5.4 ms) components. Previous time-resolved studies have typically lacked sufficient temporal resolution to distinguish these phases, resulting in oversimplified kinetic models. The different propagation velocities observed for various structural

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parameters (Table 2) suggest mechanism-dependent transmission pathways through the protein matrix. Notably, electrostatic changes propagate more rapidly (0.54 Å/ms) than volume changes (0.43 Å/ms) or B-factor alterations (0.36 Å/ms), indicating that charge redistribution provides the initial driving force for subsequent conformational adaptations. This multi-phase kinetic framework provides a quantitative basis for understanding how enzymes coordinate structural transitions with chemical reaction steps, revealing the temporal organization underlying efficient catalysis and allosteric regulation.

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4. Discussion

This time-resolved crystallography study offers breathtaking views of the dynamic interplay between catalysis and allosteric communication in enzyme systems. The structural data reveal that substrate binding triggers a cascade of conformational changes radiating from the active site to remote areas, each having unique spatial and temporal characteristics. These observations are consistent with the new paradigm of catalysis-coupled dynamics proposed by Fraser et al., who observed similar effects in a number of enzyme systems with poorer time resolution. The observations described here take this further than previous observations in that they quantify the propagating speeds of a number of structural parameters and define a hierarchical framework of conformational responses to catalysis.

The parallel between the electrostatic potential changes and structural rearrangement, as determined, provides theoretical credence to Warshel's suggested framework in which he emphasized the central role of electrostatics in enzyme catalysis. Experimental validation of calculated predictions that electrostatic effects propagate through protein structures faster than mechanical coupling mechanisms comes from the propagations of electrostatic perturbations (0.54 Å/ms) compared to volume changes (0.43 Å/ms). The electrostatic-first model of propagation is a challenge to traditional views on allostery, which overwhelmingly emphasize mechanical pathways of communication between distant sites.

The biexponential kinetics of structural transitions are in line with the energy landscape perspective of protein function, in which fast initial adjustments in comparatively flexible areas occur prior to more slowly occurring, extensive reorganizations. This kind of timing difference between fast electronic or local conformational changes and slower global structural reorganization can reflect a sophisticated approach to satisfying the conflicting requirements of catalytic efficiency and structural stability. Comparable kinetic complexity has been observed in spectroscopic investigations in solution, but the present study is distinct in correlating these kinetic stages with particular structural characteristics at the atomic level.

In spite of these breakthroughs, several limitations must be mentioned. The crystalline environment must impose restrictions on protein movement, potentially influencing the kinetics and amplitudes of conformational change compared to solution conditions. Furthermore, although millisecond time resolution obtained in this study is a significant advance relative to earlier work, events taking place at microsecond or nanosecond distances are still beyond the reach of present experimental equipment. The intervals between points of observation also involve model-based structural interpolation between time points of observation, with potential for interpretive bias.

Future experiments need to combine time-resolved XFEL crystallography with complementary solution-phase techniques such as time-resolved spectroscopy and small-angle X-ray scattering to validate the observation under physiologically relevant conditions. Extension of the time resolution into the microsecond regime would allow for capture of the initial steps in catalysis-triggered allosteric communication. Computer techniques that naturally incorporate the crystalline environment into simulations can connect crystal and solution measurements. Furthermore, analyzing the response of such dynamic allosteric networks to mutation, inhibitors, or changes in environmental conditions would lend valuable insight to enzyme engineering and

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pharmacological design approaches that target protein dynamics as opposed to static conformation.

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5. Conclusion

This research has demonstrated the powerful potential of time-resolved XFEL crystallography to elucidate at atomic resolution the dynamic interaction between enzyme catalysis and allosteric regulation. By mapping structural changes at various time points, ranging from 5 ms to 500 ms, the study confirmed a concerted series of conformational changes that radiate from the active site to distant regulatory sites with specific spatial and temporal signatures. The demonstrated correlation between active site chemistry and allosteric behavior (R^2 = 0.87 for electrostatic potential) is strong evidence for the existence of long-distance communication mechanisms linking catalytic functions to more general structural changes along the enzyme.

The order of these structural changes, as reflected in the faster propagation of electrostatic perturbations (0.54 ± 0.06 Å/ms) relative to volume changes (0.43 ± 0.05 Å/ms) and flexibility changes (0.36 ± 0.04 Å/ms), is indicative of a hierarchical arrangement of dynamic networks optimized for efficient transduction of energy throughout the protein matrix. This mechanistic paradigm reconciles apparently conflicting models of enzyme catalysis, which have alternatively stressed the relevance of electrostatic preorganization, conformational selection, or induced-fit mechanisms. Far from representing alternative paradigms, these views address various facets of a time-coordinated process wherein the early electrostatic reorganization initiates later conformational changes that, as a whole, optimize catalytic efficiency.

The strategic approach demonstrated herein, combining high-resolution XFEL crystallography with advanced computational analysis, paves the way for investigation of dynamic allostery across a variety of enzyme systems. Future extension of this approach to other enzymes will help address whether the propagating velocities and kinetic correlations observed here represent general protein dynamics principles versus system-specific response. A complete understanding of the way in which structural changes take place in both space and time during catalytic reactions provides new opportunities for systematic design of enzyme modulators directed to individual phases of these dynamic processes, thus potentially enabling unprecedented control of biological catalysis for drug and biotechnology applications.

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Reference

[1] N. Smith et al., "Changes in an enzyme ensemble during catalysis observed by high-resolution XFEL crystallography," Science Advances, vol. 10, no. 13, p. eadk7201, Mar. 2024.
[2] M. A. Wilson, "Mapping enzyme landscapes by time-resolved crystallography with synchrotron and X-ray free electron laser light," Annual Review of Biophysics, vol. 51, pp. 79-98, May 2022.
[3] M. Dasgupta et al., "Mix-and-inject XFEL crystallography reveals gated conformational dynamics during enzyme catalysis," Proceedings of the National Academy of Sciences, vol. 116, no. 51, pp. 25634-25640, Dec. 2019.
[4] D. A. Keedy et al., "Mapping the conformational landscape of a dynamic enzyme by multitemperature and XFEL crystallography," eLife, vol. 4, p. e07574, Sep. 2015.
[5] G. Khusainov, J. Standfuss, and T. Weinert, "The time revolution in macromolecular crystallography," Structural Dynamics, vol. 11, no. 2, p. 020901, Apr. 2024.
[6] C. Harprecht et al., "Mapping protein dynamics at high spatial resolution with temperature-jump X-ray crystallography," Nature Chemistry, vol. 15, pp. 1423-1432, Nov. 2023.
[7] M. Choi et al., "Effect of the abolition of intersubunit salt bridges on allosteric protein structural dynamics," Chemical Science, vol. 12, no. 23, pp. 8207-8217, May 2021.
[8] D. A. Keedy et al., "An expanded allosteric network in PTP1B by multitemperature crystallography, fragment screening, and covalent tethering," eLife, vol. 7, p. e36307, Jun. 2018.
[9] A. Srivastava, T. Nagai, A. Srivastava, O. Miyashita, and F. Tama, "Role of computational methods in going beyond X-ray crystallography to explore protein structure and dynamics," International Journal of Molecular Sciences, vol. 19, no. 11, p. 3401, Oct. 2018.
[10] I. D. Young et al., "Recent results in time-resolved serial femtosecond crystallography at XFELs," Current Opinion in Structural Biology, vol. 73, p. 102345, Apr. 2022.

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