Time-Resolved Macromolecular Crystallography

Keith Moffat
Consortium for Advanced Radiation Sources
PI, BioCARS
The University of Chicago

BioCARS Workshop, November 20-22, 2008
Why pursue time-resolved macromolecular crystallography?

• Because ALL chemical, biochemical and biological reactions involve changes in atomic positions as the reactions proceed; intermediate structures thus differ from those of the static reactants and products.

• To understand mechanism requires that these intermediate structures, the pathways by which they interconvert and the rates at which they do so be determined experimentally. It’s all about mechanism!

• Biological reactions span an extremely wide time range, from femtoseconds (characteristic of atomic vibrations) to nanoseconds (characteristic of aromatic side chain rotations and backbone fluctuations) to microseconds (characteristic of a quaternary structural change) to milliseconds (characteristic of a single enzyme turnover), or even longer.
Static crystallographic approaches to mechanism

- Conventional macromolecular crystallography: trap and study static structures believed to resemble each intermediate; extrapolate from these to the short-lived, unobservable structures.
- Trapping by either chemical means e.g. variant protein, variant substrate, variant conditions such as pH: “chemical trapping” or by physical means e.g. cryotrapping: “physical trapping”
- Problem: assumes that a particular mechanism holds; requires that trapped states be structurally homogeneous; can’t easily trap short-lived states; may require large energetic and structural extrapolation, hard to do accurately
- But, very widely used
Time-resolved crystallographic approaches to mechanism

- Time-resolved crystallography: no explicit trapping
- Technically challenging but definitely feasible; needs a strategy for rapid reaction initiation in the crystal; yields ~100 picosecond (10^{-10} sec) time resolution
- Recognize that time-dependent structural heterogeneity exists and seek to resolve it i.e. unscramble or deconvolute overlapping structures; “analytical trapping”
- Identify whether ANY chemical kinetic mechanism holds, containing a small number of defined states; and if so, which particular mechanism
- Determine the time-independent structures of each state or intermediate
- The array of structures and the pathways by which they interconvert constitutes the chemical kinetic mechanism; provides a powerful framework for computation
How to conduct a time-resolved experiment?

• In an ideal world, all X-ray structure amplitudes - which depend on the location of the atoms in the unit cell - would be directly measured as a function of time $t$, after initiating a structural reaction in all molecules in the crystal at $t = 0$ i.e. measure $|F(hkl,t)|$
How to initiate the desired reaction?

- This is the tough part!
- Reaction can be initiated by means of a change in some variable that influences macromolecular structure/reactivity/equilibria e.g. pressure, temperature, pH, diffusion of a key reactant, electric field….or light
- Reaction must be initiated in a time that is short with respect to the lifetime of the shortest-lived, desired intermediate; uniformly throughout all molecules in the crystal, $\sim 10^{12} – 10^{13}$; and with high quantum yield
How to measure the structure amplitudes?

• No X-ray detector (presently) exists that can measure the structure amplitudes in this way, at closely-spaced points in time

• So, all time-resolved crystallographic experiments are conducted in pump – probe mode, a style of experiment common in various forms of ultrafast spectroscopies

• The pump pulse initiates the reaction at \( t = 0 \), the probe pulse occurs after a time delay \( t \) and interrogates the reaction
How to measure the structure amplitudes?

• Consider a structural reaction that can be initiated by light. A brief laser pulse illuminates a crystal of a light-sensitive molecule and thus initiates a structural reaction – the pump. After a suitable time delay t, a synchrotron X-ray pulse (~100 ps) or a pulse train - the probe - is delivered to the crystal and generates a diffraction pattern, which can be quantitated to yield values of $|F(hkl,t)|$

• A complete data set then contains $|F(hkl, t)|$ for all unique values of hkl and the entire time range over which the structural reaction occurs, with suitable redundancy and accuracy
Laue or monochromatic?

• The crystal could be stationary and the X-ray pulse polychromatic, generating a Laue diffraction pattern; or the crystal could rotate and the pulse monochromatic, generating a rotation pattern

• Laue is fast, and required for revealing structures shorter-lived than ~milliseconds; mono/rotation is slow, and restricted to revealing structures longer-lived than milliseconds
The “movie”

• From a complete data set, the variation in electron density $\rho(\text{xyz},t)$ – and structure – with time can be calculated i.e. a “molecular movie”

• Or, the differences in structure between time $t$ and time 0 can be calculated i.e. a time-dependent difference electron density map or difference Fourier $\Delta\rho(\text{xyz},t)$
"SALLIE GARDNER," owned by LELAND STANFORD; running at a 1.40 gait over the Palo Alto track, 19th June, 1878.

The negatives of these photographs were made at intervals of twenty-seven inches of distance, and about the twenty-fifth part of a second of time; they illustrate consecutive positions assumed in each twenty-seventh inch of progress during a single stride of the horse. The vertical lines were twenty-seven inches apart; the horizontal lines represent elevations of four inches each. The exposure of each negative was less than the two-thousandth part of a second.
Data analysis

• But the movie analogy is ultimately misleading; we need the time-independent structures of each intermediate, whose populations are varying with time and giving the impression of ”motion”

• Singular value decomposition is one way of identifying intermediates consistent with the crystallographic data
The diagram illustrates a sequence of reactions labeled IE1, IE2, IL1, IL2, IL3, and G, with specific time intervals indicated:

- IE1 $\rightarrow$ IE2: 300 ns
- IE2 $\rightarrow$ IL1: 2 µs
- IL1 $\rightarrow$ IL2: 200 µs
- IL2 $\rightarrow$ IL3: 20 ms
- IL3 $\rightarrow$ G: 100s ms

Additionally, the graph shows the fraction over time, with markers for $\tau(1/k)$.
It works! What do we get?

- Intermediate structures to high crystallographic resolution (~1.6 – 2.5Å), with a time resolution set by the longer of the laser and X-ray pulse lengths i.e. ~100ps with a fs laser; ~2ns with a ns laser

- Dynamics, rate coefficients for elementary steps, and a mechanism consistent with the crystallographic data
Reaction initiation

• Almost all successful experiments so far have been conducted on naturally light-sensitive, fully reversible systems e.g. photoreceptors
• But MY system isn’t photosensitive! How can you help me?
• Make your system light-sensitive by chemical approaches e.g. caging of the substrate, cofactor or the macromolecule itself; or by molecular biological approaches to “genetically encoded caging”
• Devise approaches to reaction initiation based on triggers other than light