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Use of correlated x-ray scattering (CXS) to track protein motions during enzyme catalysis by means of an x-ray free-electron laser.

Sebastian Doniach[1], Vijay Pande[2], John Spence[3], Daniel Herschlag[4], Sébastien Boutet[5] and Jerome Hastings[5]

Departments of Applied Physics[1] and Physics[1], Chemistry[2], and Biochemistry[4], Stanford University, Department of Physics, Arizona State University[3], and Linear Coherent Light Source[5], SLAC national Accelerator Lab.

The combination of x-ray flux in an FEL pulse and the recent advances in control of droplet formation and transport into an xFEL beam make possible the measurement of correlated scattering events in which a given molecule in the ensemble can scatter 2 photons within the same x-ray pulse. The incidence of such CXS events is greatly amplified by the number of macromolecules in the droplet. Then by correlating scattering events at different pixels in an area detector, defining a pair of scattering vectors, q and q', these 2-photon events define a 4-point correlation function *C4* which may be extracted by evaluating

$$C4(q, q', \cos\theta) = < I(\vec{q}) I(\vec{q}\,') > - < I(q) >< I(q\,') > \qquad (1)$$

where *I(q)* measures the number of photon counts in the pixel defining a scattering vector *q* and the correlated average is over all pairs of pixels subtending a fixed relative angle θ. *<I(q)>* represents the usual 2-point SAXS scattering averaged over all orientations. Since the exposure to the x-ray pulse happens for times of order 10's of femtoseconds (fs), the molecules in the droplet do not have time to undergo significant brownian motion during the exposure. Hence two photon scattering from any molecule in the ensemble happens for a given molecular orientation and internal conformation. We estimate that some 10^7 correlated 2-photon CXS events could be expected from a single 1micrometer (μm) sized droplet of protein solution at a concentration of 10mg/ml placed exposed to a single x-ray pulse in an xfel beam focused to an area of $\sim 1(\mu m)^2$. An advantage of CXS relative to the usual single photon SAXS measurement is that background subtraction is self-generated by the act of correlating the data, with subtraction of the product of average scattering in the 2 wave vector channels $|q|$ and $|q'|$ represented in equation (1).

In order to illustrate the kind of structural data available for non-crystalline samples from this kind of correlated measurement we show an estimate of changes in C4 for closed versus open states of the enzyme dihydrofolate reductase in the figure. The SAXS profile is similar to the *cos(θ)=0* slice in C4, while changes at large *cos(θ)* correspond to changes in the internal structure of the enzyme not reflected in the SAXS profile.

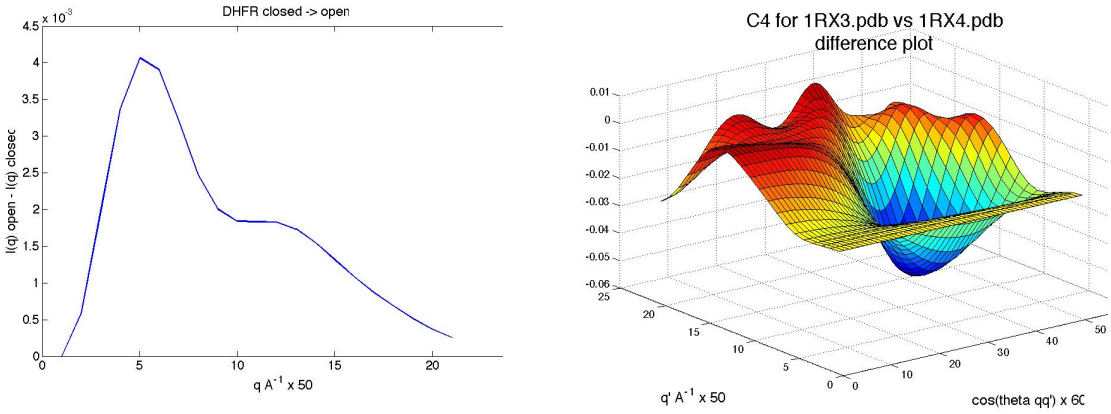

Figure: Difference in CXS for two states of dihydrofolate reductase (right) compared to the difference for one-photon scattering (SAXS) for the same pair of states (left).

We will discuss the analysis of terabytes of data, from time resolved CXS measurements on enzyme systems, using an approach in which macromolecular kinetics are simulated using Markov state models (MSMs) built from molecular dynamics simulations. MSMs are essentially discrete-time master equations, where the macrostates and rates of transitioning between them are determined from molecular simulation. The Markov states are extracted by kinetic clustering of simulation data: conformations that can interconvert rapidly are grouped into the same state while conformations that can only interconvert slowly are grouped into separate states. Such a kinetic clustering ensures that equilibration within a state, and therefore loss of memory of the previous state, occurs more rapidly than transitions between states. As a result, the model satisfies the Markov property—the identity of the next state depends only on the identity of the current state and not any of the previous states.

As an example we will examine the use of this approach to measure structural changes in Cyclophilin A. Human cyclophilin catalyzes cis-trans isomerization of a prolyl peptide bond of the HIV capsid needed to trigger a conformational change necessary for viral packaging. *cis-trans* Isomerization of prolyl peptide bonds is characterized by a very high activation energy barrier of around 16-22 kcal/mol, and the rate is in the order of tens to hundreds of seconds. Nature has provided the PPIase enzymes to circumvent this very slow kinetics by catalyzing the *cis-trans* isomerization and decreasing the time scale from seconds to the more biologically relevant millisecond time scale. In order to fully understand the catalytic mechanism of this speedup of more than 5 orders of magnitude, one has to be able to observe the *cis-trans* isomerization of the peptide bond at atomistic detail. CXS presents a revolutionary new method with which to directly observe the conformational changes essential to the catalytic function of Cyclophilin A.