*Unveiling Functional Protein Motions with Picosecond X-ray Crystallography and Molecular Dynamics Simulations.*

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We have developed the method of picosecond time-resolved Laue crystallography and used this technique to investigate structural dynamics in biological macromolecules at ambient temperature. Time-resolved snapshots of myoglobin following flash photolysis of the CO adduct were determined with 150 ps time resolution and < 2 Å spatial resolution. The structures reveal numerous sites in which CO becomes transiently trapped, as well as correlated motion of the protein side chains. When a single point mutation was introduced in a position near the binding site (L29F), the departure of CO from the primary docking site was significantly accelerated. Dramatic differences in the correlated protein displacements in wild-type vs. L29F Mb were found, which provide a structural explanation for these kinetic differences. The time-dependent structural changes have been stitched together into a movie that literally allows us to watch this protein as it functions. This movie depicts the average structure of an ensemble of intermediates, not a single molecule. To gain single-molecule insights into mechanisms of protein function, a joint analysis of all-atom molecular dynamics (MD) calculations and picosecond time-resolved X-ray structures was performed. Ensemble-averaged MD simulations of the L29F mutant of myoglobin following ligand dissociation reproduce the direction, amplitude, and timescales of crystallographically-determined structural changes. This close agreement with experiments at comparable resolution in space and time validates the individual MD trajectories. From numerous single-molecule trajectories, we identify and structurally characterize a conformational switch that directs dissociated ligands to one of two nearby protein cavities. This unique combination of simulation and experiment unveils functional protein motions and illustrates at an atomic level relationships among protein structure, dynamics, and function.