Time-Resolved Dimerization of a PAS-LOV Protein Measured with Photocoupled Small Angle X-ray Scattering

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Here, we report the coupling of photoexcitation with time-resolved SAXS to monitor conformational changes accompanying light activation of the blue-light signal transduction protein Vivid (VVD). Solution small-angle X-ray scattering (SAXS) reports the size and shape of soluble biomolecules. Previously, SAXS was used in conjunction with rapid mixing techniques to time resolve macromolecular folding.[1,2] SAXS studies have also been used to determine large scale differences between the dark and light excited states of proteins.[3,4] Flash-flow devices have been used for time-resolved IR spectroscopy;[5] integration with SAXS expands the technique, enabling exploration of global kinetics. While time dependent processes in different blue-light sensors have been revealed by a variety of techniques,[6,7] this method elucidates previously unknown association dynamics of the long-lived VVD light-adapted state.

VVD, a so-called LOV (for Light Oxygen Voltage sensing) protein of the PAS family, regulates blue-light responses in the filamentous fungus *Neurospora crassa*.[8] Photon absorption by the VVD flavin cofactor drives conformational changes within the LOV domain. In the absence of light, the protein is monomeric. Recent measurements suggest the establishment of a rapidly exchanging monomer−dimer equilibrium in the light-activated state;[9] thus VVD (like other LOV domains[10,11]) changes association state in response to light-stimulated structural modifications. PAS−PAS dimerization is believed to be a key regulatory event in signal transduction[12] and is likely important for a close VVD homologue, WC-1, to activate transcription.[13] Because of the challenge of characterizing structural intermediates in these processes, little is known about how the cofactor chemical state relates to the association mode, and ultimately, the engagement of targets.

To time-resolve changes in the structure of VVD resulting from photoexcitation, we employed a microfluidic cell coupled to both laser and X-ray sources (Figure 1). The cell consists of a thin wall polyester tube (Advanced Polymers, VT)[14] which is optically transparent and scatters X-rays minimally. A 473 nm laser beam (Holograms & Lasers International, Texas) is directed at 90° to a focused X-ray beam. The location where the X-ray and light beams intersect defines the time-equals-zero position for the experiment. Offsetting the two beams creates a controllable delay between laser excitation and SAXS measurement. This average delay time is calculated by dividing the distance between the two beams, x, by the flow speed of the protein solution, u (Figure 1). Use of a flow cell eliminates radiation damage to the protein and enables long X-ray exposures, which increase signal-to-noise without loss of time resolution. Temporal precision is restricted by the laminar flow boundary condition at the channel wall where $u = 0$ but can be improved by employing a flow of sheath buffer which confines the protein-containing solution to a thin jet that travels along the tube axis with relatively uniform speed. The transit time of each molecule through either the laser or X-ray incident spot was 160
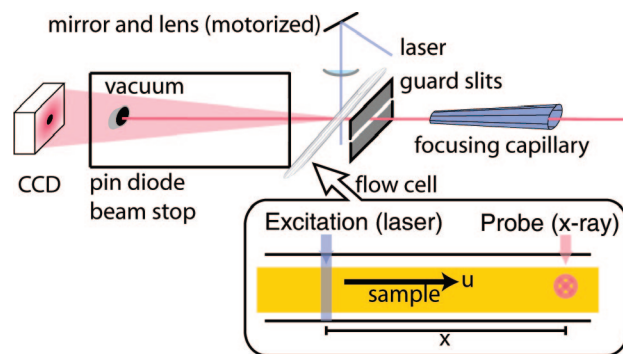

Figure 1. Schematic of the continuous flow setup that enables time-resolved SAXS measurements following photoexcitation of protein. An X-ray beam, incident from the right, passes through a focusing capillary[15,16] to obtain sufficient X-ray flux in a small spot. Guard slits are used to decrease the scattering background. The excitation laser beam is directed perpendicular to the axis of the flow cell and the X-ray beam. A magnified illustration of the flow cell demonstrates how time resolution is achieved.

ms on average. The laser spot was continuously illuminated for all light-state measurements, and the X-ray exposure time ranged from 20 to 30 s.

The intensity of the scattered X-rays is typically plotted as a function of the momentum transfer $q = 4\pi \sin(\theta)/\lambda$, where θ is half the scattering angle and λ is the X-ray wavelength. Following standard analysis procedures[17] the zero angle intensity, $I(0)$, and the radius of gyration, R_g, were extracted from the low q data (see Supporting Information). A Kratky plot[18] places emphasis on data acquired at large q, which provides detail about macromolecular conformation.

SAXS measurements of the dark state of VVD are in good agreement with the computed signal from the monomeric crystal structure 2PD7 (Supporting Information).[4] Upon light-illumination, the low angle scattering intensity from VVD is increased relative to the dark state (Figure 2 and Supporting Information). The increase occurs rapidly and appears finished by the earliest time detected, 20 ms after photoexcitation. Such an increase in $I(0)$ is consistent with dimerization. Inline multilight scattering (MALS), dynamic light scattering (DLS), size exclusion chromatography and equilibrium ultracentrifugation confirms the presence of a rapidly dissociating dimer in the light adapted state.[9] Complete dimerization in 20 ms is consistent with a diffusion controlled association rate constant in the range 10^5–10^6 M^{-1} s^{-1}[9] and demonstrates that the conformational change which precedes dimerization is unlikely to be rate limiting. This association rate exceeds that reported for other LOV domain proteins.[11] Importantly, we also evaluated the time-dependent SAXS profile of a VVD point mutant (Cys71Ser) that cannot undergo light-induced dimerization but otherwise has normal photochemical properties.[4] Cys71Ser VVD undergoes no change in scattering upon illumination; thus, laser heating or
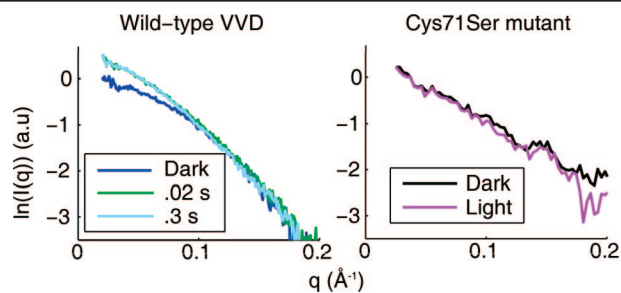
Figure 2. Left: Scattering profiles for wild-type VVD before (blue) and 0.02 s after excitation (green) display an increase in zero angle intensity, consistent with light induced dimerization. No further change is measured 0.3 s after excitation (cyan). Right: Scattering profiles of the inactive Cys71Ser mutant are not changed by light excitation.
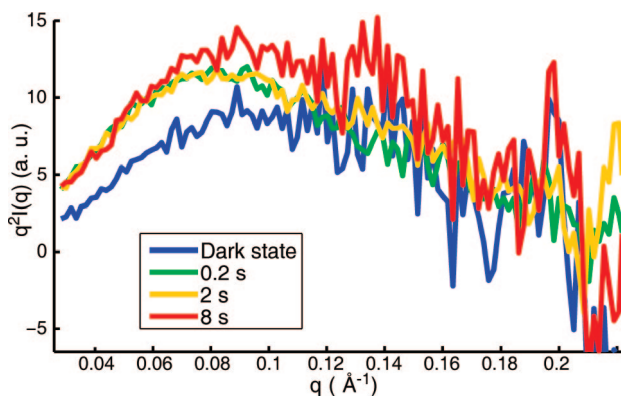

Figure 3. Kratky plots of data collected hundreds of milliseconds to seconds after photoexcitation. Variations in scattering profiles become evident at $q > 0.08 \ \text{Å}^{-1}$ after 8 s indicating conformational changes of the dimer.

radiation damage cannot be the cause of the scattering changes we observe with wild-type VVD.

Scattering data acquired at longer times after photoexcitation show variation in the high q region of the profile, suggesting that the conformational changes continue for several seconds after dimerization is complete (Figure 3). As VVD remains in the light-adapted state for several hours,[19] this phenomenon reflects additional changes *post*-dimerization that may be essential for engaging targets and propagating signals.

While these time-resolved SAXS measurements show a consistent pattern of oligomerization after light excitation, the magnitude of the change varies depending on the specific sample and experiment. The affinity of the VVD dimer is known to be highly sensitive to modifications at the N-terminus as well as protein oxidation at currently unspecified sites.[9] We suspect differences in dimer yield result from such subtle changes in protein chemistry; we are exploring the phenomenon in more detail. Measurements on millisecond (or submillisecond) time scales will be required to directly detect light-induced conformational differences in the monomeric state which must precede and gate access to the dimeric state.

Time-resolved measurements of global structural states are critical for unraveling the protein dynamics that underlie LOV protein light sensing; SAXS is an ideal tool for studying both the conformational changes and protein association that accompany such events. Application of a continuous flow cell to couple SAXS and photoexcitation, as demonstrated here, can elucidate approximately millisecond conformational changes without signal averaging by photocycle repetition, thus avoiding the sample damage associated with repeat exposures and facilitating measurement of samples with slow photocycles. Notably, this method will resolve diffusion-limited association rates, and it is generally applicable to many photoreceptors, even those with transient light-adapted states much less stable than that of VVD.

Supporting Information Available: Quantitative results of a Guinier analysis on the data and comparison of scattering data to an existing crystal structure.This material is available free of charge via the Internet at http://pubs.acs.org.

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