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Time-resolved structural studies at synchrotrons and X-ray free electron lasers: opportunities and challenges

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Abstract

X-ray free electron lasers (XFELs) are potentially revolutionary X-ray sources because of their very short pulse duration, extreme peak brilliance and high spatial coherence, features that distinguish them from today's synchrotron sources. We review recent time-resolved Laue diffraction and time-resolved wide angle X-ray scattering (WAXS) studies at synchrotron sources, and initial static studies at XFELs. XFELs have the potential to transform the field of time-resolved structural biology, yet many challenges arise in devising and adapting hardware, experimental design and data analysis strategies to exploit their unusual properties. Despite these challenges, we are confident that XFEL sources are poised to shed new light on ultrafast protein reaction dynamics.

Introduction

Conformational dynamics are essential to the correct functioning of virtually all macromolecules expressed in the cell: all reactions involve atomic motion. Although X-ray crystallography has been applied with great success to solve the majority of known macromolecular structures, this method is static: it reveals a structure averaged in both time and space over the numerous conformations of a macromolecule within the crystal.

Two direct approaches to structural dynamics are offered by time-resolved Laue diffraction and time-resolved wide angle x-ray scattering (WAXS), which explicitly introduce the fourth dimension of time [1]. The current state-of-the-art relies upon brilliant, short X-ray pulses isolated by shutters and a rapid X-ray chopper [2]. Experiments are based on the pump-probe approach, in which a brief laser pulse (the pump) initiates a light-dependent structural reaction in the molecules in the sample, of which 10 to 40 % are typically photoactivated; after a controlled, variable time delay, an X-ray pulse (the probe) interrogates their structure. The time delay is varied to cover the duration of the entire reaction, and (in Laue diffraction) the crystal orientation is varied to cover the unique volume in reciprocal space. Depending upon the chosen time delay and the complexity of the reaction mechanism, one or more intermediate conformational states may be sampled at

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each time delay. Fitting of the entire time course spanning all time delays enables several intermediates to be resolved, provided each attains a peak occupancy sufficient to be identified.

The time resolution of the pump-probe approach is generally limited by the longest of the laser pulse, the X-ray pulse and the jitter in the time delay. Both Laue diffraction and WAXS have achieved a temporal resolution of ~100 ps [3,4], the duration of an individual X-ray pulse at synchrotron sources. In contrast, X-ray free electron lasers (XFELs) deliver extremely brilliant, highly coherent X-ray pulses of 10 - 100 fs in duration [5], three to four orders of magnitude shorter than synchrotron-derived pulses. Lasing is based on a process known as self-amplified spontaneous emission (SASE) which very efficiently converts energy from the electron bunch, as it traverses a very long undulator, into the X-ray beam. The peak brilliance of the X-ray beam is approximately ten orders of magnitude greater than that attainable with any third generation synchrotron. This gain factor is the difference between taking a walk and travelling at the speed of light! [6] Unprecedented opportunities in X-ray science - including structural biology - are thus opened up; XFEL light sources offer a powerful example of disruptive technology. Indications of where these sources may lead us have begun to emerge through experiments conducted at the first XFEL to emit hard X-rays, the Linac Coherent Light Source (LCLS) at the Stanford Linear Accelerator Laboratory. Here we review advances in time-resolved structural biology using synchrotron radiation and contrast them with approaches unique to XFEL sources.

Time-resolved Laue diffraction

Time-resolved Laue diffraction [7,8] employs intense, polychromatic, synchrotron-derived X-ray pulses to collect Laue diffraction data from a single protein crystal after initiation of a light-dependent reaction by a short laser pulse. This ultimately yields a complete, high resolution set of time-dependent structure factor amplitudes from which the variation with time of the average conformational state of a protein is obtained. A time resolution of ~100 ps to 5 ns was achieved in studies of photodissociation and rebinding of CO to myoglobin [3,9,10], dimeric hemoglobin (Figure 1A) [11], and of the reversible photocycle of the blue light signaling photoreceptor known as photoactive yellow protein, PYP (Figure 2A) [12-15]. Technical challenges specific to Laue diffraction, such as energy overlaps and closely-spaced spots, have been overcome [8,16,17].

When X-rays are generated from highly stable undulator sources at a third generation synchrotron, Laue diffraction readily yields structure amplitudes accurate enough to reveal small structural differences such as the time-dependent location of a partially-occupied CO molecule [3,11,12]. The electron density differences between myoglobin structures after and before CO photodissociation showed remarkable agreement [18] with similar studies using cryotrapping techniques [19,20] but, in addition, directly measured the trajectory and time scale of CO motion [3,9,10]. In contrast to myoglobin, ligand binding to dimeric hemoglobin is cooperative, which renders the structural transitions upon photodissociation considerably more complicated. Time-resolved Laue diffraction studies were sufficiently precise to reveal motion of a key Phenylalanine side chain at the dimer interface between the two heme groups, and rearrangements of water molecules during an initial quaternary structural transition [21] (Figure 1A). This recent analysis captures the main features of the structural transitions as a pivoting and scissors-like motion of helices, pinned at their intersection.

Time-resolved diffraction studies of membrane proteins pose additional challenges since membrane protein crystals are typically less robust and diffract to lower resolution than crystals of soluble proteins. Two time-resolved Laue diffraction studies have examined

structural changes in the photosynthetic reaction center from *B. viridis* [22,23]. The first used a classical detergent-solubilized membrane protein crystallization protocol [22] and convincingly ruled out light-driven movements of the mobile quinone in the Q_B pocket suggested by earlier trapping studies [24]. A later study used crystals of the reaction center grown in a more robust, lipidic sponge phase environment [25] to obtain a light minus dark difference Fourier map with low noise [23]. This map revealed light-induced movement of Tyrosine L162 (Figure 2A) towards the special pair (P₉₆₀), which is photo-oxidized upon the absorption of light. The lower noise may explain why movement of Tyrosine L162 was not observed using the detergent-grown crystal form [22]. These results emphasize that time-resolved studies depend on the visualization of small, time-dependent structural differences, which in turn depend on the accurate measurement of small time-dependent changes in X-ray structure amplitudes.

Time-resolved wide angle X-ray scattering, WAXS

Time-resolved, synchrotron-based WAXS studies of proteins in solution [26] have recently emerged from similar studies of small molecules [27-30] and provide an approach complementary to Laue crystallography. Time-resolved WAXS offers the key advantage that conformational dynamics are not constrained by intermolecular interactions present in a crystal lattice. This is more than a simple technical advantage since the reactions of many biological systems occur *via* relatively large conformational changes in tertiary or quaternary structure that may be incompatible with retention of an ordered crystal lattice. Thus, direct crystallographic approaches based on all forms of kinetic crystallography are inherently limited. For example, the photocycle of the light-driven proton pump bacteriorhodopsin is believed to be associated with large movements in the cytoplasmic domain of helix F [31,32]. These movements are readily visualized by time-resolved WAXS studies of detergent-solubilized bacteriorhodopsin [33], but are largely absent in a cryotrapping study of bacteriorhodopsin crystals (see Table 1 of reference [33]). A further example is provided by the photocycle of PYP, which proceeds *via* a common kinetic mechanism in the crystal and in solution [34], but the rate coefficients associated with transitions between individual intermediates differ quantitatively by up to two orders of magnitude [35] (see also Table 1 of ref. [34]). Furthermore, formation of the last intermediate, in which extensive motion of the N-terminus occurs in solution, is impeded in the crystal.

As with time-resolved Laue diffraction, the first protein time-resolved WAXS studies have focused upon the conformational dynamics of reversible, light-sensitive systems such as the CO-bound forms of tetrameric [26] and homodimeric hemoglobins (Figure 1B-D) [36] and myoglobin [4,37-39], cytochrome c [26], PYP (Figure 2B-C) [34,40], bacteriorhodopsin [33] and proteorhodopsin [33,41,42]. All WAXS data derive from an ensemble of randomly-oriented molecules and hence are relatively low in structural detail although rich in dynamics. Each WAXS scattering curve is dominated by systematic contributions from background scattering such as the capillary, buffer and air path. The protein contribution is small, typically at the level of a few percent of the total scattering. Nevertheless, in a time-resolved WAXS experiment, the differences between pairs of scattering curves (laser off minus laser on) contain all the desired time-dependent data and are insensitive to all time-independent systematic contributions. The first experiments have revealed surprisingly large WAXS difference signals with excellent signal-to-noise (Figures 1B, 2B) that extend over a wide *q* range and into the 100 ps time domain [4,26,33,34,36-42].

Can the rich signal present in experimental WAXS data be interpreted in an objective way? Structural refinement approaches minimize a theoretical prediction against time-dependent difference WAXS data [33,34,36,39,40,42] and have usually been guided by additional

structural information. Difference WAXS features associated with protein conformational changes are typically observed and modeled for $0.1 \text{ \AA}^{-1} < q < 0.8 \text{ \AA}^{-1}$, which corresponds to a crystallographic resolution $\sim 8 \text{ \AA}$ and is sensitive to secondary structural element and domain motions but not to the location of individual atoms or side-chains. A recent study of light-driven conformational changes in PYP [40] is highly illustrative of the interpretational challenges and possible solutions associated with time-resolved WAXS (Figure 3B,C). Structural analysis and refinement incorporated information from static crystallography, DEER and NMR experiments. Although the WAXS data alone were consistent with both a parallel and a sequential model for the photocycle (a typical uncertainty problem in kinetics), external information from time-resolved Laue crystallography leads the authors to favor the parallel model [40]. *Ab initio* structural refinement against WAXS difference data remains an open question. Will advanced computational approaches such as molecular dynamics simulations or other dynamical approaches [36] converge upon a unique set of conformational states? A satisfactory answer to this question, in combination with the development generic reaction initiation protocols, will be critical if time-resolved WAXS is to become a mainstream structural method during the next decade.

Coherent Diffractive Imaging at X-ray Free Electron Lasers

Molecular dynamics simulations of a protein exposed to an exceptionally brilliant XFEL beam predicted that it would be possible to overcome the radiation dose limitations of traditional crystallography by rapidly collecting ultrafast X-ray scattering data before radiation damage had time to destroy a protein sample [43,44]. Several experimental confirmations of this “diffract-and-destroy” principle [45-47] were performed at the Hamburg VUV free electron laser FLASH [48]. In the hard X-ray regime, generation of XFEL radiation at the LCLS [5] marks a major milestone in X-ray imaging. Early XFEL applications to structural biology have focused on two major approaches: serial femtosecond crystallography (SFX) of micro- and nanocrystals [49-53], and single particle imaging of regular, structurally-reproducible structures such as icosahedral viruses [54] or irregular, structurally non-reproducible objects such as single cells [55]. These studies are a *tour-de-force*: they combine innovative sample injection systems [54,56] with a vacuum sample environment, a rapid readout X-ray detector [57] and algorithms and software necessary to process terabytes of data [58].

If samples are structurally reproducible it should be possible to partition the coherent diffraction images into identifiable classes and average over many images in each class [59], or use manifold methods to sort the data [60], and thereby greatly improve the signal to noise. The first experimental demonstration of this approach used images obtained at the Atomic, Molecular and Optical Science (AMO) beamline of the LCLS [61] to yield projection images of a single mimivirus particle with a resolution of 32 nm [54]. This creates new possibilities for X-ray imaging of large biological objects that are difficult or impossible to crystallize. However, XFEL-based coherent diffractive imaging requires considerable development if it is to reach a resolution competitive with that of single particle electron cryomicroscopy [62], the technique which seems likely to be its major competitor rather than crystallography.

Serial femtosecond crystallography (SFX)

SFX offers a radically new experimental approach to structural biology that, nevertheless, remains subject to the challenges of protein crystallization [63]. SFX rapidly exposes individual, tiny liquid samples, each containing a randomly-oriented nano- or microcrystal, to a single, highly-focused XFEL pulse. In a neat twist to the “diffract and destroy” concept, diffraction from crystals self-terminates as the protein molecules undergo an X-ray damage

driven Coulomb explosion disordering the crystal lattice [64]. The resultant X-ray diffraction pattern is read out from the X-ray detector after each X-ray pulse, at up to 120 Hz. Many samples contain no crystals and will display no diffraction. However, the goal is to record diffraction sufficiently often that complete data sets for which each Bragg spot is measured with high multiplicity are recovered.

High multiplicity may prove to be the key to the quantitative success of SFX. This is in part because a challenge arises for all experimental approaches that use a single pulse (such as SFX or Laue diffraction from larger crystals) from the fundamental physics of XFELs. The SASE lasing principle is based on amplification of noise fluctuations in the initial spatial distribution of electrons within the electron bunch as it enters the undulator, and results in an X-ray pulse with a highly modulated X-ray spectrum across a bandwidth $\Delta E/E \sim 10^{-3}$. The modulations are random from pulse to pulse and the intensity at adjacent X-ray energies within the bandwidth can differ by 100%. Furthermore, this bandwidth is neither sufficiently broad to allow integration over energy, nor sufficiently narrow to be regarded as truly monochromatic. In effect, each diffraction spot is a “narrow bandpass Laue partial” where the extent of partiality varies from pulse to pulse. Averaging over the numerous observations offered by high multiplicity may be necessary to overcome the effect of these fluctuations and obtain sufficiently accurate integrated intensities on which conventional crystallography depends.

The successes of SFX have relied upon substantial developments in other, equally critical experimental areas such as sample delivery [56], a detector that can read out each image at up to 120 Hz [57] and data processing [58]. For sample delivery, an ingenious, liquid microjet injector has been devised which achieves a low-background jet of only a few μm in diameter (Figure 3B) [63]. Since this microjet flows at approximately 1 ml protein sample per hour, protein consumption is high and may become a limiting feature. Data processing sorts the $\sim 10^5$ to 10^6 diffraction images recorded in each experimental run, identifies peaks that stand out above the background, and process these images by calling standard crystallographic packages for analysis of monochromatic patterns such as MOSFLM [65] and DIRAX [66]. A crucial step is to merge all diffraction images into one data set, ideally with high completeness and high multiplicity. This has been done with a Monte Carlo integration method [58,67] based upon the assumption that the nanocrystals are randomly oriented and that if the multiplicity is sufficiently high, the sum of reflection intensities from many nanocrystals will converge to a value proportional to the square of the structure factor. The last assumption is necessary in light of the very large fluctuations between the individual XFEL pulses.

Four crystallographic structures have been reported using SFX. The first was for cyanobacterial photosystem I at 8.5 Å resolution. Diffraction data from 15,444 images yielded an average multiplicity of 708 and an R_{free} of 23 % after rigid body refinement, where data were treated as if perfectly merohedrally twinned due to the crystal space-group [49]. In contrast, the lipidic sponge phase structure of the *Blastochloris viridis* photosynthetic reaction center at 8.2 Å resolution (Figure 3C) was recovered from only 265 images with a multiplicity of only 8 and an R_{free} of 38 %. The resolution of both structures was limited by the long wavelength of X-rays delivered to the AMO station [61] rather than by the crystalline order of the nano/microcrystals themselves. The more recently commissioned Coherent X-ray Imaging (CXI) beamline at the LCLS provides an X-ray station operating at approximately 0.9 Å [68], which has enabled SFX to be extended to higher crystallographic resolution. To date a SFX crystal structure of photosystem II was recovered from 7269 processed images to 6.2 Å resolution [53] achieving a multiplicity of 26 and R_{free} of 39 %; whereas the highest resolution, 1.9 Å SFX crystal structure of lysozyme [52] with R_{free} of 23 % emerged from 12,247 processed images and a remarkably

high multiplicity of 379. This result establishes SFX as a high-resolution method and demonstrates experimentally that diffraction before destruction [43] applies also at high resolution. Further analysis of this type of data will identify the overall level of multiplicity (and its distribution over spot intensity and resolution) necessary to achieve structure amplitudes of an accuracy sufficient to extract small time-dependent structural differences in a time-resolved SFX experiment.

Conclusions: Time-resolved structural studies with an XFEL source

Of the static structural approaches successfully demonstrated at an XFEL, SFX appears to be the most easily adapted to a time-resolved experiment. Indeed, initial time resolved SFX studies of photoactivated microcrystals of photosystem I in complex with ferredoxin [69] showed that they suffered a loss in diffraction quality 10 μ s after laser triggering, suggestive of a substantial structural change. This result illustrates both the potential of time-resolved SFX and possible shortcomings. On the positive side, by recording diffraction data from each microcrystal only once, time-resolved SFX is well suited to study irreversible reactions. On the negative side, time-resolved diffraction requires accurate experimental measurement of small differences in scattering with time and a time-dependent disordering of the crystal further complicates such measurements. It remains to be demonstrated whether or not SFX can achieve the required accuracy, particularly at high resolution.

Application of SFX to study protein structural dynamics offers three intrinsic advantages over the large ($\sim 50 \mu$ m) crystal approaches such as Laue diffraction employed at synchrotron sources. First, the nanocrystals used in SFX have low optical absorbance, enabling them to be fully photo-activated near an absorption maximum. Second, their small size promotes rapid diffusion of reactants, which may soon be exploited by clever microfluidic mixing devices. Third, each nanocrystal is exposed to the pump laser only once, thus side-stepping the problem of laser-induced damage. On the other hand, Laue diffraction from larger crystals is a proven method on the 100 ps time-scale [3]. Since both photoexcited (laser on) and reference (laser off) images are recorded from the same crystal over the entire time range [72], this greatly reduces the systematic errors in difference structure amplitudes and in the final light minus dark difference electron density maps. Thus, the jury is still out as to whether SFX on nanocrystals or Laue diffraction on larger crystals will offer the most direct path towards accurate visualization of structural changes with temporal resolution better than 100 ps.

Time-resolved WAXS appears to be more directly amenable to ultrafast studies at XFEL sources with relatively few technical modifications. A substantial WAXS signal is likely to be present on the fs to ps time scale as it is quite clear from synchrotron-based studies that significant conformational changes occur within proteins on the sub-100ps time scale [4,36] (Figure 1B). For time-resolved studies by both WAXS and crystallography the challenge will be stability of the overall experimental arrangement, since the protein-dependent WAXS difference signal $\Delta S(q, \Delta t)/S(q, 0)$ is typically of the order of 0.1 %. Novel approaches may be required to overcome fluctuations in the XFEL spectrum and pulse-to-pulse intensity, perhaps based on the extensive pulse-to-pulse, Monte Carlo averaging effective in SFX. An intriguing new possibility created by coherent ultrafast X-ray pulses is that, on the femtosecond time-scale, protein samples in solution appear frozen in space. Thus, in principle, additional structural information may be extracted from angular correlations contained in anisotropic WAXS images, enabling a three-dimensional structure of one particle to be reconstructed from many randomly oriented copies of that particle [70,71].

It will certainly be exciting to reveal the nature of ultrafast structural changes within proteins [72–74] using direct X-ray methods. Will ultrafast time-resolved X-ray scattering and diffraction studies reveal concerted large scale motions of α -helices and other groups, or will more diffuse motions involving increased side-chain flexibility be predominant? XFELs provide a revolutionary tool to explore questions concerning ultrafast conformational changes in light-dependent reactions, and to provide high resolution structural insights for reactions that are not necessarily reversible. The restriction to light-dependent reactions may be overcome by conferring sensitivity to light on otherwise light-inert reactions, thus opening up new targets [75]. The next few years should see the first successful attempts to realize this vision and open new opportunities in time-resolved structural biology.

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Highlights for COSB

- Recent advances in time-resolved Laue diffraction studies of light sensitive proteins.
- Recent advances in time-resolved wide angle X-ray scattering (WAXS) studies of light sensitive proteins.
- Proof-of-principle demonstrations of serial femtosecond crystallography using X-ray free electron laser radiation.
- Reflection on the challenges ahead for time-resolved diffraction and scattering studies at X-ray free electron lasers.

Figure 1.

Time resolved Laue diffraction and WAXS studies of homodimeric hemoglobin (HbI). (A) Light minus dark 1.6 Å resolution $F^{\text{obs}}_{\text{HbI}} - F^{\text{obs}}_{\text{HbI(CO)}_2}$ difference Fourier map of subunit B 100 ps after photodissociation of CO (green positive difference density contoured at 7σ ; red negative difference density contoured at -7σ ; σ is the rmsd electron density in the unit cell). Reproduced with permission from reference [21]. (B) Time-resolved difference WAXS curves $\Delta S(q, \Delta t)$ (laser on minus laser off; time-delay Δt indicated) recorded from HbI. Black curves, experimental data; red curves, modeled curves generated from linear combinations of three time-independent species-associated difference scattering curves derived from a kinetic analysis. (D) Population changes with time of the three intermediates I_1 , I_2 and I_3 , for wild type HbI. (E) Schematic of the structural transitions for wild type HbI between the CO-bound form denoted HbI(CO)₂ and the partly-liganded intermediates I_1 , I_2 and I_3 . The green and blue arrows indicate the magnitudes and directions of the differences in heme-heme distances and subunit rotation angles relative to HbI(CO)₂. Panels B, C, D and E are adapted with permission from [36].

Figure 2.

Time resolved Laue diffraction and WAXS studies of photoactive yellow protein (PYP). (A) Light minus dark 1.8 $\text{\AA}$ resolution $F^{\text{obs}} - F^{\text{obs}}$ difference Fourier map 200 ns after photoactivation by light (blue/cyan positive difference density contoured at $+3/+5 \sigma$ respectively; pink/red negative difference density contoured at $-3/-4 \sigma$ respectively). The PYP pR intermediate is fully developed by 200 ns and decays on a time scale of $\sim 100 \mu\text{s}$. (B) Time resolved SAXS/WAXS data collected from PYP 10 ms after photoactivation. Black, experimental data; Red, theoretical model fit that incorporated additional information from other biophysical studies (static crystallography, DEER and NMR experiments). (C) Structural changes associated with the pG (resting) to pB conformations of PYP associated with the optimal fit to WAXS difference data shown in B. Panel A shows unpublished data provided by Marius Schmidt. Panels B and C are adapted with permission from reference [40].

Figure 3.

Time-resolved Laue diffraction and static serial femtosecond crystallography studies of the photosynthetic reaction centre from *B. viridis* (RC_{vir}). (A) Light minus dark 2.95 Å $F^{\text{obs}} - F^{\text{obs}}$ difference Fourier electron density observed 3 ms following photoactivation (green positive density contoured at 4 σ ; red negative difference density contoured at -4 σ). A light induced movement of TyrL162 towards the special pair (P₉₆₀) was modeled (grey resting conformation; blue photo-activated conformation) due to P₉₆₀ being photo-oxidized. (B) Microjet used to inject lipidic sponge phase samples containing microcrystals of RC_{vir}. The jet is approximately 4 μm across where the XFEL beam interacts with the jet (white arrow). (C) $2F^{\text{obs}} - F^{\text{calc}}$ electron density map (contoured at 1 σ) recovered from the serial femtosecond crystallography (SFX) structure of RC_{vir} at 8.2 Å resolution [51]. Panel A is adapted with permission from reference [23]. Panels B and C are reproduced with permission from reference [51].