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Watching Proteins Function with Time-resolved X-ray Crystallography

Vukica Šrajer^{1,*} and Marius Schmidt^{2,*}

¹Center for Advanced Radiation Sources, The University of Chicago, Chicago, IL, USA

²Physics Department, University of Wisconsin-Milwaukee, Milwaukee, IL, USA

Abstract

Macromolecular crystallography was immensely successful in the last two decades. To a large degree this success resulted from use of powerful third generation synchrotron X-ray sources. An expansive database of more than 100,000 protein structures, of which many were determined at resolution better than 2 Å, is available today. With this achievement, the spotlight in structural biology is shifting from determination of static structures to elucidating dynamic aspects of protein function. A powerful tool for addressing these aspects is time-resolved crystallography, where a genuine biological function is triggered in the crystal with a goal of capturing molecules in action and determining protein kinetics and structures of intermediates (Schmidt *et al.*, 2005a; Schmidt 2008; Neutze and Moffat, 2012; Šrajer 2014). In this approach, short and intense X-ray pulses are used to probe intermediates in real time and at room temperature, in an ongoing reaction that is initiated synchronously and rapidly in the crystal. Time-resolved macromolecular crystallography with 100 ps time resolution at synchrotron X-ray sources is in its mature phase today, particularly for studies of reversible, light-initiated reactions. The advent of the new free electron lasers for hard X-rays (XFELs; 5–20 keV), which provide exceptionally intense, femtosecond X-ray pulses, marks a new frontier for time-resolved crystallography. The exploration of ultra-fast events becomes possible in high-resolution structural detail, on sub-picosecond time scales (Tenboer *et al.*, 2014; Barends *et al.*, 2015; Pande *et al.*, 2016). We review here state-of-the-art time-resolved crystallographic experiments both at synchrotrons and XFELs. We also outline challenges and further developments necessary to broaden the application of these methods to many important proteins and enzymes of biomedical relevance.

1. Introduction

Biological macromolecules are molecular machines, and as such are dynamic systems. Even in their “resting” states, their structures fluctuate between numerous conformational sub-states (Austin *et al.*, 1975; Ansari *et al.*, 1985; Frauenfelder *et al.*, 1988). Such structural heterogeneity can be detected in high-resolution ensemble measurements such as X-ray crystallography and reveals itself, for example, as alternate side chain conformations and disordered regions. Some of those structural fluctuations are clearly functionally relevant. To understand how macromolecules accomplish their function, knowledge of the average,

*Corresponding authors.

“static” 3D structures, although necessary, is often not sufficient. Understanding the interplay between the 3D structure and dynamics is necessary. As reactions involving macromolecules proceed, they involve changes in atomic coordinates. Such changes can be examined in great detail and in real time, as they occur, by time-resolved X-ray crystallography, provided that a reaction can be successfully triggered and proceeds within the crystal lattice. The goal of time-resolved crystallography is to determine from the same crystallographic data both the chemical kinetic mechanism (detailed characterization of the reaction in terms of intermediate states and the rate constants for their interconversion) as well as the structures of short-lived intermediate states. In order to accomplish this, a reaction needs to be triggered rapidly compared to the lifetimes of intermediates. X-ray exposure used to probe the reaction also has to be short compared to the lifetimes of the intermediates. A number of other techniques can also provide important information about the structures of the intermediates. If a single intermediate accumulates in sufficient concentration in the kinetic equilibrium, its structure may be determined from steady state conditions. Also, various types of trapping can be used to stabilize an intermediate by prolonging its lifetimes (Bourgeois and Weik, 2009). For example, a reaction can be started in the crystal at ambient temperature and allowed to evolve to a certain point, then the crystal flash-cooled when a significant fraction of molecules accumulates in an intermediate state. Chemical trapping is also often applied by modifying the protein or solvent conditions to block the reaction at a certain point. However, in these and other similar trapping approaches, information about the rates of formation and decay of intermediates and therefore about kinetic mechanism is lost.

Time-resolved crystallography produces molecular “movies” of proteins in action. However, it is important to understand what type of movie is actually recorded. In a molecular reaction, the system evolves from an initial state, through a number of intermediate states, to the final state. Intermediate states correspond to local energy minima in the multidimensional conformational space. These energy minima are transiently occupied by molecules undergoing a reaction. Intermediates, I_i , are therefore distinct, time-independent structures (Fig. 1). Individual molecules cross the energy barriers between the intermediate states at random and independent from each other and spend a very short time at the transition states, T^* , compared to the time they spend in states I_i . As a consequence the occupancy of the transition state is very low, and transition states are therefore not captured in time-resolved experiments. This is the case for any time-resolved method, not just time-resolved crystallography. The molecular movie that is recorded therefore does not represent a movie of a structure continuously-evolving in time. The movie consists of several time-independent intermediate structures, whose concentrations are varying as a function of time. In order to capture and characterize the structure of an intermediate I_i , concentration of molecules in this state has to build up to a detectable level during the reaction, which depends on the rate coefficients in the kinetic mechanism (Fig. 1B and C). Another feature of time-resolved studies is that at any point in time, a mixture of structures is typically present, unless rate coefficients are such that intermediates are well separated in time (Fig. 1C). Experimental, time-resolved electron-density maps therefore most likely represent a mixture of states. Methods, such as singular-value decomposition (SVD) were developed to

separate this mixture and obtain maps for each individual intermediate (Schmidt *et al.*, 2003; Rajagopal *et al.*, 2004).

During the last two decades, time-resolved crystallographic studies of cyclic and reversible reactions initiated by short light pulses have been highly successful (Ihee *et al.*, 2005; Jung *et al.*, 2013; Knapp *et al.*, 2006, 2009; Schmidt *et al.*, 2005b, 2013b; Schotte *et al.*, 2003, 2012, 2013; Šrajer *et al.*, 1996, 2001; Tripathi *et al.*, 2012; Wohri *et al.*, 2010). However, some main challenges remain. There are two important challenges that have to be addressed. The technique must evolve that it can be routinely applied to non-cyclic and irreversible reactions. Other methods of reaction initiation, in addition to light, need to be developed. Progress in these two areas is under way and will lead to a breakthrough in application of the technique to many important biological molecules and in deciphering the mechanism by which they function in structural detail, at near atomic resolution.

2. Time-resolved X-ray Macromolecular Crystallography Experiments

a. Pump-Probe Method

In time-resolved pump-probe experiment a “pump” pulse, for example a short laser pulse, is used to trigger a reaction. An X-ray pulse is used to “probe” the reaction at a defined time delay following the pump pulse. Single-pump/single-probe or single-pump/multiple-probe strategies are possible. In the first case, a single pump pulse is followed by a single X-ray exposure at a particular time delay. This sequence is then repeated, in some cases several times before the detector readout. In the second case, a single pump pulse is followed by several sequential X-ray exposures, resulting in multiple detector readouts at various time delays. The first method is necessary for probing fast reactions, on sub-ms time scales, due to relatively low readout rates of large-area X-ray detectors used in crystallography experiments. Readout rates of 10Hz – 1 kHz are common. For slower reactions, single-pump/multiple-probe experiments are possible with current detectors. Newly developed higher frame-rate detectors, such as the AGIPD detector with MHz readout rate (Becker and Graafsma, 2012), will allow much faster reactions to be investigated this way. These detectors are also essential for the efficient use of the XFEL sources with serial crystallography data collection (see section 2.b). It is important to note that integrating rather than photon counting detectors are necessary for high photon rates such as those experienced in Laue experiments at synchrotrons or at XFELs (sections 2.b and d).

The time-resolution is determined by the duration of the pump or probe pulses, whichever is longer, and the accuracy (jitter) in their relative timing. The best time resolution achievable at synchrotrons is ultimately limited by ~100ps duration of the X-ray pulses. Somewhat better time-resolution of ~10ps can be achieved if a time-slicing scheme is used, where a much shorter pump pulse is scanned across the wider X-ray pulse (Haldrup *et al.*, 2011; Oang *et al.*, 2014). In this case, a part of the X-ray pulse prior to the pump pulse probes the initial state while the remaining part of the same X-ray pulse probes the photo-excited state. This method however results in smaller scattered intensities since only part of the X-ray pulse probes the photo-excited state of interest. Better, ultra-high time-resolution, down to only a few fs can only be achieved by the new XFEL X-ray sources (section 2.b) which feature fs X-ray pulses.

b. Pulsed X-ray Sources: Synchrotrons and XFELs

All synchrotrons are pulsed X-ray sources with ~100ps X-ray pulse duration. The necessary X-ray photon count per pulse to achieve 100ps time resolution in protein crystallography experiments is available only at third generation synchrotron sources, such as Advanced Photon Source (APS, Argonne National Laboratory, USA), European Synchrotron Radiation Facility (Grenoble, France) and SPring 8 (Japan). When the photon count per pulse is insufficient or crystals are small in size and/or weakly-diffracting, multiple pump-probe sequences can be repeated prior to the detector readout. Several 100ps X-ray exposures can be accumulated on the same crystal to improve the signal-to-noise ratio of the recorded diffraction pattern. Such a strategy is practically achievable only for fully reversible or cyclic reactions. For irreversible, single path reactions, accumulation of 100ps exposures is possible only if the reaction is slow enough so that the crystal can be exposed to several consecutive X-ray pulses. This of course results in degraded time resolution, determined by spacing between the X-ray pulses and the number of pulses needed. In cases where 100ps time resolution is needed, each 100ps X-ray exposure requires a new crystal or crystal volume.

Free electron lasers for hard X-rays (XFELs; 5–20 keV) that have come online recently provide largely improved time resolution. While the first free electron laser operating with micrometer wavelength radiation was built already in the 1970s, it was only in 2009 that for the first time a free electron laser, the Linac Coherent Light Source (LCLS) in Menlo Park, CA, generated hard X-rays. An XFEL is a source of ultra-short X-ray pulses which are produced by the high energy electron bunches in a process called ‘self-amplified stimulated emission’ (SASE) that occurs in long undulator banks (Schmüser *et al.*, 2014). The remarkable properties of X-ray radiation produced by XFELs are the very short pulse duration which can be as short as a few femtoseconds, the large number (on the order of 10^{12}) of X-ray photons in each of the pulses, the very large transversal coherence length on the order of 0.5 mm and a small, quasi-monochromatic bandwidth $\Delta E/E \sim 10^{-3}$. This results in a peak brilliance, photons/(s mm² mrad² 0.1% bandwidth), which is up to 9 orders of magnitude higher than that reached at the most powerful synchrotron. One of the advantages of the large transversal coherence is that it is possible to focus the X-ray beam exquisitely well, to submicron diameters, without loss of photons. Tiny specimen, from nanocrystals down to single non-crystalline particles, can be illuminated with the full amount of X-ray photons available in the pulse. Since X-rays are ionizing, they remove electrons from matter. This results in the disintegration of the specimen due to strong electrostatic repulsion. However, the X-rays scatter and produce a diffraction pattern before the sample is destroyed. This has been called the ‘diffraction-before-destruction principle’ (Neutze *et al.*, 2000). This principle was demonstrated by Chapman and colleagues by illuminating a stick figure with submicron details etched in silicon nitride with 25 fs pulses of soft (32 nm) X-rays (Chapman *et al.*, 2006). The image of the figure was retrieved from the continuous diffraction (speckle) pattern using oversampling approaches (iterative input-output algorithm) (Marchesini *et al.*, 2003), although the stick figure was completely destroyed after the X-ray pulse. There is justified expectation that this approach allows for the determination of the structure of single, uncrystallized particles at the XFEL (Munke *et al.*, 2016). The extension of the diffraction-before-destruction principle to very small crystals

with edges on the sub-micron length scale was proposed and demonstrated again by Chapman and colleagues (Chapman *et al.*, 2011) and denoted ‘protein nano-crystallography’. Since in this method the crystals are delivered one by one, in random orientation in a serial fashion, this method was renamed “serial femtosecond crystallography (SFX)” (Boutet *et al.*, 2012). It has been quickly realized that time-resolved crystallography experiments should be feasible with the SFX approach. In the first time-resolved SFX attempt, a reaction was initiated in microcrystals of the photosystem I-ferredoxin complex upstream from the X-ray interaction region by an intense 532 nm laser pulse of 10 ns duration. The crystals were then probed after a delay of 5–10 μ s by the XFEL pulse (Aquila *et al.*, 2012). Differences in the structure factor amplitudes between crystals exposed to laser illumination and those probed in the dark were observable up to 8 Å resolution. The first successful time-resolved SFX experiment with near atomic resolution has been demonstrated by Tenboer and colleagues in 2014 (Tenboer *et al.*, 2014), see details below.

c. Reaction Triggering

Reaction triggering is of critical importance for a successful time-resolved experiment. Initiation has to be accomplished synchronously in as many molecules in the crystal as possible and in a time period significantly shorter than the lifetimes of reaction intermediates (Schlichting and Goody, 1997; Schmidt, 2008; Schmidt *et al.*, 2005a).

Triggering by light is by far the fastest way to start a reaction as lasers with pulse duration down to fs are readily available. This method is most easily applied to inherently photosensitive proteins, especially those that undergo a reversible reaction, for example ligand photo-dissociation and rebinding in heme proteins (Šrajer *et al.*, 1996) or chromophore isomerization in photoactive yellow protein (Ihee *et al.*, 2005).

In naturally photosensitive proteins a chromophore which absorbs the light is embedded within the protein. The protein concentration is very high in macromolecular crystals (10–50 mM). Therefore, the optical density in the chromophore absorption region is very high and laser penetration depth is shallow. For example, even at the blue tail of the absorption band of the photoactive yellow protein, at ~390nm, laser penetration depth is only ~30 μ m (Schotte *et al.*, 2012). A careful consideration of wavelength and crystal illumination geometry is needed to achieve uniform photo-initiation throughout the crystal. Smaller (thinner) crystals have clear advantage since they are compatible with the shallow light penetration close or at the peak of the absorption and permit higher levels of reaction initiation. This was strikingly demonstrated in the recent XFEL time-resolved experiment with 1–5 μ m sized crystals (Tenboer *et al.*, 2014 and section 5). Careful assessment of the laser power is needed to assure sufficient photoinitiation yield and to avoid excessive heating or other damaging effects of the high peak power of ultra-short laser pulses (Lincoln *et al.*, 2012; Hutchison *et al.*, 2016). Laser pulse energy densities that have been used with protein crystals are on the order of 0.5–5 mJ/mm².

If molecules are not naturally photosensitive, they can be rendered photosensitive in some cases by using caged compounds as photoactivatable bioagents. A photosensitive but biochemically inactivating (protecting) group is chemically attached to a bioagent which could be the substrate, a cofactor or a catalytically important protein residue (Bourgeois and

Royant, 2005; Bourgeois and Weik, 2009; Pelliccioli and Wirz, 2002). A number of caged molecules and groups are available: oxygen, ATP, phosphate, nucleotides, metals protons, neurotransmitters etc. (Goeldner and Givens, 2005). However, only a few successful time-resolved crystallography applications have been reported. For example, caged GTP was used in studies of Ras P21 (Schlichting *et al.*, 1990), caged phosphate in studies of catalysis in phosphorylase b (Duke *et al.*, 1992), caged NADP in the investigation of the isocitrate dehydrogenase (Stoddard *et al.*, 1998) and caged choline in studies of acetylcholinesterase (Bourgeois and Weik, 2009). Several difficulties limit the broader application of these compounds in time-resolved crystallography. Substantial expertise is required to design and produce these compounds since they must be protein- and reaction-specific. For an effective photo-removable protecting group several characteristics are highly desirable. They should have high quantum yield for the photoreaction and a high absorption coefficient above 300nm where protein absorption and potential for photo-damage are lower. Byproducts resulting from the activation of the bioagent should not interfere with the photoreaction, they should not affect protein reaction or be damaging to the protein. The release rate of the bioagent has to be fast compared to the reaction that is investigated and radiolysis induced by the X-ray pulse must be minimal. These conditions are not trivial to satisfy all in a single compound. The most common limiting factors are low quantum yield and slow release of the bioagent, much slower than 1 μ s (Pelliccioli and Wirz, 2002; Goeldner and Givens, 2005). 2-nitrobenzyl derivatives have been the most commonly used photolabile protecting groups, despite their toxic and strongly absorbing byproducts and slow release rates. More recently, a number of alternatives have been developed. The p-hydroxyphenacyl (pHP) cage is one of the most promising alternatives to the nitrobenzyl derivatives. The pHP derivatives are water soluble and stable, quantum yields are high, ranging from 0.1 to 0.4, and the bioagent photorelease is very fast, on the order of 10 ns (Pelliccioli and Wirz, 2002; Goeldner and Givens, 2005).

Reactions can also be initiated by diffusion of small molecules such as substrates, cofactors or redox agents into the crystal. Although diffusion is conceptually simple, there are significant challenges to be addressed as diffusion is a relatively slow process. Depending on the size of the diffusing molecule, protein density in the crystal and crystal size, it can take as long as seconds and minutes or even longer for a compound to diffuse into a crystal (Geremia *et al.*, 2006). However, most enzymes are relatively slow. The fastest catalytic rates are on the order of 10^6 s^{-1} (e.g. for the enzyme catalase, Purwar *et al.* 2011), while most are in the 100 s^{-1} range. With the micro-focused X-ray beams at synchrotrons and with the ultra-intense XFEL sources, microcrystals can be used. With $\sim 10 \mu\text{m}$ crystals, it is possible to reach diffusion times on the order of 10 ms, while with $<1 \mu\text{m}$ crystals diffusion times of 10–100 μs might be achievable (Schmidt, 2013). For diffusion-induced reactions a flow cell was used where a crystal was immobilized in some way in a glass capillary while solution with the diffusing agent was flown over it (Petsko, 1985; Edwards, 1993). Such flow cells were typically used for driving a multi-turnover, steady-state experiment, where reaction rates are such that a rate-limited species accumulates and is investigated (Stoddard, 2001). Even for steady-state experiments, where potentially a single crystal can be used for a complete data set, there are significant difficulties with crystal mounting. Crystals need to be kept centered in the X-ray beam and immobilized as the reactant solution is flown over the

crystal. The entire cell is then rotated during data collection. New types of crystal mounting and delivery into the X-ray beam are clearly needed to facilitate more routine crystallographic studies of diffusion-triggered reactions. Promising new developments are described in section 6.b. They significantly expand the use of time-resolved crystallography to study of a wide range of catalytic reactions in enzymes.

d. Monochromatic and Laue Methods

Both Laue and monochromatic X-ray diffraction techniques can be used for time-resolved studies. Typical X-ray exposure times today at the 3rd generation synchrotron sources are in the 40–100 ms range when the monochromatic rotation method is used. Serial crystallography experiments were also conducted recently at the synchrotrons with 10 ms exposure times (Stellato *et al.*, 2014; Nogly *et al.*, 2015). In this case crystals are flown through the X-ray beam or a fixed target with crystals is scanned through the beam (see section 2.f). The idea is to expose each crystal the X-ray beam only once while crystals are virtually stationary during the sufficiently short X-ray exposure. In practice, some crystal rotation has been observed during 10–100ms exposure times (Botha *et al.*, 2015; Stellato *et al.*, 2014). At quasi-monochromatic XFELs, exposure times can be as short as a few fs. Because crystals are stationary during the fs X-ray pulse, “still” diffraction patterns are collected. All recorded reflections are “partials” meaning only a fraction of the full intensity is recorded. New methods and software packages have been developed for extracting full intensities from serial crystallography “still” images. Programs such as CrystFEL, cctbx.xfel and nXDS (White *et al.*, 2012, 2013; Sauter *et al.*, 2013; Sauter, 2015; Kabsch, 2014) are now used both with XFEL and synchrotron data. Although required number of indexed and merged frames per data set varies depending on the task (de novo phasing, molecular replacement, presence of a ligand, time-resolved difference signal etc), on the order of 5,000–50,000 frames per data set have been reported (Botha *et al.*, 2015, Nogly *et al.*, 2015, Stellato *et al.*, 2014, Coquelle *et al.*, 2015; Kirian *et al.*, 2010; Chapman *et al.*, 2011; Bublitz *et al.*, 2015, Tenboer *et al.*, 2014; Barends *et al.*, 2015).

While XFELs are quasi-monochromatic sources ($E/E=10^{-3}$ bandwidth, as compared to $E/E=10^{-4}$ for monochromatic X-rays at synchrotrons), synchrotrons are polychromatic sources. The Laue method has therefore been a method of choice for time-resolved crystallography at synchrotrons. It permits recording full intensities with stationary crystals by integrating over the X-ray energy, while reaching 100ps time resolution. It has been shown that the narrow-bandwidth undulators are the best X-ray sources for time-resolved Laue diffraction experiments due to their high peak intensity and lower polychromatic background (Bourgeois *et al.*, 2000; Šrajer *et al.*, 2000). The typical bandwidth of undulators used today for time-resolved experiments is 2–5% at 12–15keV (Graber *et al.*, 2011). Even with these relatively narrow bandwidth sources, a large volume of the reciprocal space is stimulated in a single Laue exposure. An angular increment in crystal orientation of 1–3° is typically sufficient for the reciprocal space coverage (Ren *et al.*, 1999; Šrajer *et al.*, 2000; Bourgeois *et al.*, 2000). The exact number of frames per data set depends on the crystal space group (determines the unique part of the reciprocal range to be covered), completeness that is needed (particularly at low resolution where very high completeness is more difficult to achieve with Laue method) and the extent of redundancy required (Ren *et al.*, 1999). At

the BioCARS 14-ID beamline, APS (section 2.g), Laue time-resolved data is typically collected with 2–3° angular increment and therefore <100 frames per data set are merged even for low symmetry space groups. This is an advantage when a limited amount of sample is available. There are two main disadvantages of the Laue method when compared to monochromatic methods. Laue diffraction patterns have higher polychromatic background, and the Laue method is very sensitive to crystal mosaicity. High mosaicity results in elongated Laue diffraction spots. This increases the spot overlap in already crowded Laue patterns and reduces the accuracy of the measured intensities. The first disadvantage is somewhat mitigated by using narrow bandpass X-ray sources such as undulators. The second one is a large problem since it excludes imperfect crystals of otherwise highly interesting biological systems. Careful optimization of the crystal quality is required.

e. Sample Delivery Methods: Traditional

In time-resolved crystallography as used originally at synchrotron sources, a single or a small number of large crystals were used to collect either several data sets (Šrajer *et al.*, 1996; Šrajer *et al.*, 2001; Schotte *et al.*, 2003; Schmidt *et al.*, 2005b) or a comprehensive time series of data (Ihee *et al.*, 2005; Knapp *et al.*, 2006; Schmidt *et al.*, 2013). In these experiments, crystals were mounted individually, in glass capillaries, with a small amount of mother liquor adjacent to the crystal to maintain humid environment in the sealed capillary. A somewhat easier method to mount individual crystals for room temperature crystallography is the room temperature mounting kit developed by MiTeGen, consisting of a cryo-loop that holds the crystal and a transparent polymer capillary that fits over the loop and prevents dehydration.

f. Sample Delivery Methods: Serial Crystallography

In serial crystallography a large number of small micro- or even nanocrystals are exposed once, one after the other, in a serial fashion, in random orientations. The method applies to both synchrotron and XFEL. However, only at the XFEL the method can be pushed to the limit in terms of crystal size and time resolution. Due to the diffraction-before-destruction principle (see section 2.b), tolerated doses are many orders of magnitude higher than the Henderson-Garman safe limit for synchrotron data collection of cryo-cooled crystals (Owen *et al.*, 2006; Chapman *et al.*, 2014). The diffraction-before-destruction principle does not apply to synchrotrons with the 100 ps X-ray pulse duration, although with such exposures secondary radiation damage might be outrun at ambient temperature (Owen *et al.*, 2012; Warkentin *et al.*, 2013). Somewhat larger crystals need to be employed to alleviate problems with radiation damage.

Unlike in traditional crystallography, the challenge in serial crystallography is to grow a large number of small crystals rather than a small number of large crystals, and to bring them rapidly in the X-ray interrogation region. The methods to do so needed to be developed (see Table 1 for an incomplete compilation). For well crystallizing protein the challenge is to keep the crystals small. In a typical experiment a high concentrated protein solution will be brought in contact with a highly concentrated precipitant solution. A shower of microcrystals forms whose growth is quenched rapidly by the absence of more protein. Crystals shown in Fig. 2 are grown by the batch method by vigorous mixing (vortexing)

concentrated PYP with a high concentration of malonate. The suspension is subsequently mixed once per day for 1 min. After 3 days the crystals are mature. These crystals are 10 times too large for XFEL based investigations but they can be used for serial crystallography at the synchrotron. To prepare smaller PYP crystals, the initial mixture needs to be quenched by centrifugation and removal of excess protein, or, alternatively, vigorously stirred overnight which must look like a horror scenario to the traditional crystallographer. Small crystals can also be obtained directly from cells overexpressing protein in large concentrations or from mixtures with lipids in the lipidic cubic phase (see Tab.1 for some relevant references). The advantages of small crystals for time-resolved studies are numerous. (i) They can be uniformly illuminated by laser light. As a consequence the photo-activation yield may be very large (Tenboer *et al.*, 2014). (ii) The possibility exists to chemically trigger reactions by diffusion of substrate much more rapidly than in larger crystals, which enables structure based enzymology (Kupitz *et al.*, 2016). This is due to significantly shorter diffusion times in small crystals. (iii) The small crystals might accommodate large structural changes with concomitantly large unit cell changes (Kupitz *et al.*, 2014a) that may occur uniformly through the crystal without destroying it. In large crystals such changes are likely to lead to crystal cracks due to development of strain.

Once the small crystals are prepared, they need to be exposed to the X-ray beam. Various versions of five basic methods are used to transport the crystals in the X-ray interaction volume: 1) the liquid jet (Weierstall *et al.*, 2012); 2) the electrosprayed jet (Sierra *et al.*, 2012; Sierra *et al.*, 2016); 3) the viscous jet (Weierstall *et al.*, 2014; Botha *et al.*, 2015), 4) the fixed target (Hunter *et al.*, 2014; Perry *et al.*, 2014; Mueller *et al.*, 2015; Roedig *et al.*, 2015) and 5) the drop on demand (Roessler *et al.*, 2016). The last four can also be used at the synchrotron. Simply flowing crystal suspension through a glass capillary has also been used at the synchrotron (Stellato *et al.*, 2014). All of these methods are compatible with time-resolved studies. The main differences between the methods are protein consumption and speed. Liquid jets may have the speed to even cope with the ~5 MHz pulse repetition rate of the European XFEL. Viscous media jets are suitable for the low 120 Hz repetition rate at the LCLS as well as synchrotrons where data is typically collected at 10–100Hz set by the readout rate of the detectors such as PILATUS or RAYONIX MX340-HS. With the new high frame-rate detectors such as EIGER, with the frame rate up to 3000 Hz (Casanova *et al.*, 2016), it is possible to use faster jets. Note that due to the high instantaneous polychromatic X-ray flux, photon-counting detectors such as PILATUS and EIGER are not suitable for synchrotron Laue experiments and integrating detectors such as the RAYONIX MX340-HS are necessary. Liquid jets might consume trillions of small crystals in an 8h shift of data collection which requires about 0.5 g of protein to grow (Tenboer *et al.*, 2014). Typical sample consumption with such jets is ~100–300 mg per data set (Tenboer *et al.*, 2014). On the other hand, viscous jets and electrosprayed jets might not even need 1 mg for a data set (Liu *et al.*, 2013; Kang *et al.*, 2015; Sierra *et al.*, 2016) and the protein requirements for data sets from fixed targets is really miniscule. The jet speed from gas dynamic virtual nozzles (GDVNs) is on the order of 10 m/s. 8 cm of crystal containing jet material is wasted between the 120 Hz pulses at the LCLS. With a hit rate of 10% only one out of about 1 million crystals is hit, the rest goes to waste (assuming a jet diameter of 5 μm and a crystal density of $5 \times 10^{10} \text{ ml}^{-1}$). With the 5 MHz bursts of X-ray pulses of the European XFEL, more

crystals in the liquid jets can be interrogated. Protein consumption will be substantially lower. With jets formed by viscous media such as lipids in the cubic phase (LCP) or superlube (Sugahara *et al.*, 2014) only a few μm are extruded between the X-ray pulses at 120 Hz. Consequently, by using the LCP jet at 120 Hz LCLS, protein consumption is several orders of magnitude lower compared to that achieved with a GDVN jet. With fixed target methods, crystals are loaded or grown *in-situ* on a device that is then scanned through the X-ray beam. A large number of available crystals on the device can be hit, depending on the scanning method. With an assumed crystal volume of $125\ \mu\text{m}^3$ ($5\ \mu\text{m}$ edge length) and 40,000 diffraction patterns collected, a total crystal volume of $5 \times 10^6\ \mu\text{m}^3$ is required per data set. Assuming that the protein is packed into the crystal at $1\ \text{g}/\text{cm}^3$ ($10^{-12}\ \text{g}/\mu\text{m}^3$), these $5 \times 10^6\ \mu\text{m}^3$ require $5\ \mu\text{g}$ of total protein per data set. Compared to this, a macroscopic crystal with $100\ \mu\text{m}$ edge length ($1 \times 10^6\ \mu\text{m}^3$) contains $1\ \mu\text{g}$ of protein. Meeting the MHz pulse rate at the European XFEL with fixed targets or any other ‘slow’ technology such as the LCP jet or the electrospun technique becomes a challenge if all pulses in the train are to be used. The development of suitable injectors is required for high repetition rates for the most efficient use of such MHz pulsed X-ray source. With the low repetition rates of the LCLS the slow techniques enable experiments with extremely small amounts of protein and may outperform even protein requirements of traditional macromolecular crystallography.

g. Conducting Pump-probe Synchrotron Experiments at BioCARS 14-ID Beamline at the Advanced Photon Source

BioCARS 14-ID beamline at the Advanced Photon Source (Argonne National Laboratory, USA) provides an example a state-of-the-art synchrotron facility dedicated to time-resolved X-ray crystallography and solution scattering (Graber *et al.*, 2011). The source of polychromatic X-ray beam consists of two in-line undulators with periods of 23 and 27 mm, optimized for a maximum flux at $\sim 12\ \text{keV}$. In the 24-bunch mode of the APS storage ring (Fig. 3A), each 100ps X-ray pul delivers up to $\sim 10^{10}$ photons to the sample, while in the hybrid mode the isolated 100ps pulse delivers $\sim 4 \times 10^{10}$ photons. The X-ray beam is focused by two sets of Kirkpatrick-Baez (KB) mirrors, primary and secondary, to a minimum spot size at the sample of $15 \times 15\ \mu\text{m}^2$.

A series of three X-ray shutters (Fig. 3B) is used to select the X-ray exposure time. The exposure is adjustable from a single 100ps X-ray pulse to a pulse train of N consecutive X-ray pulses (Graber *et al.*, 2011). The shutter train can isolate a single X-ray pulse even in 24-bunch mode of the APS storage ring where inter-bunch spacing is only 153.4 ns (Fig. 3A). This is the standard APS mode, available for $\sim 60\%$ of the user beamtime. The layout of the equipment in the 14-ID experimental station is shown in Fig. 4. The ALIO diffractometer enables fast rotation and translation of the sample (120 mm/s translation and $720\ ^\circ/\text{s}$ rotation rates). A fast-readout, large area Rayonix MX340-HS detector, with 10–100 Hz frame rate, facilitates rapid data collection. Precise synchronization of pump and probe pulses is accomplished by using a sophisticated timing system based on a field-programmable gate array (FPGA). The timing of X-ray shutters, laser and other triggers is synchronized to the storage-ring RF frequency signal provided by the APS.

Two pulsed laser systems are available at BioCARS. A modified 1 kHz Spectra-Physics Spitfire Pro laser coupled to TOPAS optical parametric amplifier provides 1.2ps laser pulses in the wavelength range 450–2000 nm. In addition, a compact and portable Opotek Opolette 355 II ns laser is available. It is highly tunable laser with 220–2200 nm wavelength range and ~7ns pulses at 20 Hz.

Laser light delivery at 14-ID is possible in two geometries: with the laser beam perpendicular to the X-ray beam or nearly colinear along the X-ray beam (~15° between the laser and X-ray beams). The perpendicular geometry (Fig. 4 and 5) is used when working with larger crystals. It permits to probe with X-rays only the thin layer of the crystal that matches the shallow laser light penetration (Fig. 5). In some cases, like in fixed-target or injector-based serial crystallography experiments (see section 2.f), it is not possible to use the perpendicular geometry and is necessary to deliver the laser beam along or close to the X-ray beam path.

h. Conducting Pump-Probe XFEL Experiments at the LCLS

The pump-probe experimental setup reported in the last paragraph can also be applied to the free electron laser. However, due to the small bandwidth ($\Delta E/E = 0.1 - 0.2\%$), the Laue method is not applicable. For macroscopic crystals a step-scan technique as employed in monochromatic neutron scattering (Niimura *et al.*, 1997) may be adapted (Suga *et al.*, 2015). Due to the inherent intensity and energy fluctuations of the XFEL beam and damaging ultra-high X-ray intensity, a time-resolved pump-probe experiment with macroscopic crystals is a challenge (van Thor *et al.*, 2014). Using nano- and micro-crystals with a serial crystallography approach proved to be the most suitable method for data collection at XFELs, both for static and time-resolved measurements (use of nano-crystals is an exception; most XFEL experiments used micro-crystals). A very tightly focused X-ray beam at ultra-high intensity is a good match for such small crystals, while serial crystallography facilitates rapid sample exchange after a single X-ray pulse exposure of each crystal. Microcrystals also provide additional critical advantage for time-resolved studies: laser light can easily penetrate the crystal even at wavelengths close to the absorption maximum. One can show with simple assumptions that a 4 ns laser pulse of 800 $\mu\text{J}/\text{mm}^2$ of 450 nm wavelength (the absorption maximum) may excite up to 80% of PYP molecules in a 5 μm thick layer (Tenboer *et al.*, 2014), which matches the size of the PYP microcrystals used in the LCLS experiment (the observed value was 40%, see section 5).

A successful time-resolved pump-probe crystallographic experiment requires (i) photoactive microcrystals, (ii) a short-pulsed laser tuned to the most suitable wavelength, which must be synchronized to the X-ray pulse to achieve variable time-delays and (iii) one of the five technologies to transport the crystals into the X-ray interaction volume as described in section 2.f. Ideally, the extent of photoactivation should be checked beforehand by spectroscopic means to fine-tune the laser pulse parameters. An optical path length of a few micrometers is sufficient to reach an extinction of 1.0 even at the absorption maximum which then can be conveniently used to monitor changes on the time scale of interest. For this, either a microcrystal slurry is used (Kupitz *et al.*, 2016), or macroscopic crystals can be crushed between two cover slides (Hutchison *et al.*, 2016; Purwar *et al.*, 2013). For ultra-fast

experiments on time scales faster than a few picosecond, the laser pulse to X-ray pulse jitter, which is on the order of 300 fs at the LCLS (Glowia *et al.*, 2010), becomes a challenge. This jitter is measured on a shot by shot basis using a time-tool developed at the LCLS (Bionta *et al.*, 2011; Harmand *et al.*, 2013). This measurement is then used to sort the images according to actual time-delays as opposed to nominal time delays. The resulting time-resolution of the pump-probe experiment is much smaller than 100 fs down to a few femtoseconds (Harmand *et al.*, 2013). With the laser properly synchronized to the X-rays and tuned close to the absorption maximum and with the time-tool in place, typically 20,000–40,000 indexable diffraction patterns are collected per data set to produce high quality difference maps for each time point on ultrafast time scales. It depends on the hit rate (and therefore on crystal density) how many X-ray exposures need to be collected to obtain the necessary number of indexable patterns. For example for photoactive yellow protein microcrystals, with the GDVN (Fig. 6), about 10^6 X-ray exposures at a rate of 80 Hz were collected for a reference data set in the dark, which required more than 5 hours (Tenboer *et al.*, 2014). To store these 10^6 diffraction pattern about 10 terabytes (TB) of disk space is required.

3. From Data to Structures of Intermediates and Beyond

a. Processing Laue Data

Laue data processing is more complex compared to processing of standard monochromatic rotation crystallographic data. Specialized software has to address several issues specific to Laue diffraction (Ren *et al.*, 1999). Spatial overlap of diffraction spots can be significant in typically crowded Laue diffraction patterns and therefore has to be resolved rather than excluding overlaps. The so-called wavelength normalization is necessary to account for variation of the incident X-ray intensity, scattering power of the crystal and detector sensitivity with the wavelength. A particular reflection and its symmetry equivalents are likely to be stimulated by different wavelengths with different incident intensities. Measured intensities therefore have to be brought to a common scale before data can be merged. The wavelength normalization curve, derived from the Laue data themselves, combines all wavelength-dependent factors affecting diffraction intensities as recorded by the detector. Harmonic or energy overlaps are another feature characteristic for Laue diffraction. Such overlaps result from reflections that lie on a radial line in reciprocal space and are all stimulated simultaneously by different X-ray energies. They scatter at the same angle and overlap exactly on the detector. These multiple reflections must be resolved into component single reflections rather than excluded in order to obtain improved data completeness particularly at low resolution. This is particularly important when broadband X-ray sources such as wigglers are used. With undulator sources that are used today for time-resolved Laue experiments, resolving harmonic overlaps improves completeness only marginally (Šrajer *et al.*, 2000). The program Precognition/Epiform used at BioCARS for Laue data processing (Renz Research Inc) computes the wavelength normalization and resolves spatial and harmonic overlaps. Another commonly used Laue software package is Daresbury Laboratory Laue Processing Suite (Arzt *et al.*, 1999; Campbell, 1995). A version with improved indexing for sparse diffraction patterns is available from MacCHESS, Cornell University. An older program, LaueView, is also available from University of Chicago.

b. Processing Serial Crystallography Data

The first experiments on biological macromolecules with jet-based serial crystallography were conducted right after the first light was observed at the LCLS (Chapman, 2011). It became apparent that only a small fraction of recorded patterns contain Bragg reflections. The majority of them are blank because the X-ray pulse did not hit a crystal. Those few patterns that contain Bragg reflections had to be identified among millions. Several hit finding programs have been developed, such as Cheetah, Cass and NanoPeakCell (Barty *et al.*, 2014; Foucar *et al.*, 2012; Coquelle *et al.*, 2015). All diffraction patterns with hits are still exposures, because the crystals are frozen in time during the very short, fs X-ray pulses. In addition, they are quasi monochromatic exposures since the X-ray bandwidth is on the order of 0.1% (9 eV). Consequently, all reflections are only recorded as partials. Since in serial crystallography crystals are delivered one by one, in random orientation, each diffraction pattern must be indexed anew, and the integrated reflection intensity recovered from a large number of partial reflections collected and averaged from random orientations. This required new software development. Rick Kirian in John Spence's lab at Arizona State University demonstrated a feasible approach for recovering integrated intensities, first by simulations (Kirian *et al.*, 2010) and then later with actual data (Kirian *et al.*, 2011). Crystal orientations are determined first by indexing each collected diffraction pattern independently. The diffracted "partial" intensities that fall in the vicinity of each reciprocal lattice point are then summed. Due to random size, random orientation and large intensity fluctuations at the XFEL, the accuracy of diffraction intensities depends on the square-root of the number of diffraction patterns collected. To reach the required accuracy, a large number of these patterns must be collected. Note that in addition to random errors, sources of systematic errors also need to be evaluated and such errors corrected if possible in order to improve the accuracy. With increasing sophistication of the data evaluation software packages and detector improvements, the number of needed patterns steadily decreased. The result of the Monte-Carlo-style partial intensity summation is proportional to the square of the structure factor amplitude for a given Bragg reflection. The method was refined and further developed by Tom White at DESY, Germany who produced a widely used software package called "CrystFEL" (White *et al.*, 2012). CrystFel is continuously updated and improved to tackle even the most complicated cases. Notably, the first electron density maps from X-ray FEL based macromolecular structure determination were published with the largest membrane protein crystallized to date, Photosystem I as model system (Chapman *et al.*, 2011) at a resolution of only 8 Å. Only a year later new results were published with dramatically improved resolution of 1.9 Å (Boutet *et al.*, 2012).

As discussed above, serial femtosecond crystallography can also be conducted in a time-resolved fashion (Aquila *et al.*, 2012). Two data sets are needed for this differential measurement: the reference data set usually collected without laser illumination and a time-dependent data set collected a time t after laser illumination. From these, difference structure factor amplitudes are calculated from which difference maps can be obtained (next paragraph). It has been shown by the first successful time-resolved crystallographic experiment at the X-ray FEL with near atomic resolution (Tenboer *et al.*, 2014) that the accuracy of the amplitudes derived from the Monte-Carlo approach is sufficient to generate difference maps with very strong, chemically meaningful features.

c. Difference Electron Density Maps

Difference electron density maps lie at the heart of time-resolved crystallography. They are calculated from reference amplitudes usually collected in the dark $|F_r|$ without reaction initiation and time-dependent amplitudes $|F_t|$ collected a small time interval after a reaction has been initiated. Phases ϕ^{calc} are derived from an accurate reference structural model, that has been ideally determined either from refinement against the reference amplitudes, or derived earlier. Of course, the model unit cell and the unit cell observed in the collected data must be isomorphous. From $|F_r|$ and $|F_t|$ time dependent difference amplitudes are calculated:

$F_t = |F_t| - |F_r|$. The F_t are ideally weighted with weighting factor that takes into account errors in the measured amplitudes as well as erroneously too large differences which could impact the interpretation. Consider for example a low resolution difference amplitude. Typically, both $|F_r|$ and $|F_t|$ are large at this resolution. Subtraction of two large numbers can result in appreciable erroneous differences which subsequently dominate the difference map. These large, low resolution differences will generate a 3D rolling landscape in the unit cell with deep valleys and high crests, on which the high resolution features are sitting. When contouring the difference map, fine features in the valleys are not observed and those on top of the crests are erroneously enhanced. This can be avoided by multiplying F_t with the following weighting factor (Ren et al., 2001), where the erroneously large F_t s are down-weighted by the F term in the denominator:

$$w_{t,hkl} = \frac{1}{1 + \frac{(\Delta F_{t,hkl})}{\langle (\Delta F_t)^2 \rangle} + \frac{\sigma_{t,hkl}^2}{\langle \sigma_t^2 \rangle}}$$

A typical difference map calculated from data collected recently at the LCLS (Tenboer et al., 2014) is shown in Fig. 7. There are strong features in the difference map which are interpreted by the atomic models in red (pR₂) and magenta (pR₁). The negative features are lying on top of the reference (dark) state model (pG, yellow). The dark model is shown in yellow.

d. Intermediates and Kinetic Mechanism

Chemical kinetics is governed by barriers of activation (Steinfeld *et al.*, 1985). Individual molecules overcome these barriers by thermal excitation. The probability to reach the top of the barrier is given by the Boltzmann factor. The goal of a time-resolved crystallographic experiment is the determination of the kinetics including the magnitude of the barriers of activation as well as the 3D structures of the reaction intermediates by interpretation of the features in a time series of difference maps. Without a time series, the kinetics and the structures of the reaction intermediates cannot be extracted. The fastest time points in a time-series are determined by the time-resolution. It is about 100 ps at synchrotrons and on the order of 20 – 100 fs at free electron lasers. Chemical kinetics is described by coupled differential equations (CDE) and typically exhibit exponential time-dependence of the transients. However, on very fast time-scales, this exponential behavior might break down, since the ensemble has not had time to dephase and the molecules synchronously move on their respective potential energy surface (PES). These time regimes merit different analysis methods.

SVD Analysis—As discussed in the introduction, if multiple intermediates are present in a reaction, several intermediates may be present at any measured time point during the reaction. This measured mixture needs to be separated into its constituents, namely the pure, time-independent difference electron densities from which the structures of the intermediates can be determined. The separation of this mixture is a major challenge. It is usually done in two steps. (i) First a component analysis such as the Singular Value Decomposition (SVD) separates spatial and temporal dependencies inherent in the time-dependent difference maps (Schmidt *et al.*, 2003). From an SVD analysis, the left singular vectors (lSV) contain the spatial components, which are difference maps, and the right singular vectors (rSV) the temporal components, which represent the temporal variation, the kinetics, of the corresponding spatial components. (ii) The mixture can be resolved by interpretation of the temporal dependencies by a kinetic model. For this a candidate mechanism with a set of CDEs containing reaction rate coefficients k_{ij} (Fig. 1) is assumed. The mechanism should be selected so that it reproduces the number of observable relaxation times in rSVs. This number can be obtained by globally fitting exponentials to the significant right singular vectors. The number of significant singular values, right singular vectors and the number of exponentials required for the global fit should match. (iii) By solving the CDE a concentration profiles for intermediates are obtained which are fitted to the significant right singular vectors by varying the k_{ij} . The time-independent difference maps are then recovered by a projection algorithm described in detail in (Henry & Hofrichter, 1992), and implemented for crystallography as described in Schmidt *et al.*, 2003. Please refer to these publications as well as review articles such as Schmidt, 2008 for further details.

i. Structures of Intermediates—Once the time-independent difference electron densities of the intermediates are determined, their structures can be determined from extrapolated, conventional electron density maps. To calculate these maps, first the difference maps are Fourier transformed. This results in time-independent difference structure factors ΔF_j^{ind} for all $j=1 \dots J$ intermediates. A multiple N of these are then added to, preferentially calculated, structure factors F_r^{calc} of the reference model according to:

$$F_j^{\text{ext}} = F_r^{\text{calc}} + N \Delta F_j^{\text{ind}}$$

From F_j^{ext} , conventional maps ρ_j^{ext} are calculated for all intermediates. N is varied to the extent that ρ_j^{ext} does not show electron density at positions where strong negative difference electron density is present in the time-independent difference maps. The extrapolated maps are conventional electron density maps which can be interpreted with an atomic model in a straightforward way. First, the structural model of the dark (reference) state M^{dark} is compared to ρ_j^{ext} . Since structural changes are often small, this model explains most of the map already quite well. At those locations where M^{dark} strongly deviates from ρ_j^{ext} , the corresponding residues of M^{dark} are moved interactively using a model building tool such as ‘Coot’ (Emsley, 2010). Preferentially, the model is further refined in real space against ρ_j^{ext} . The final refinement is then performed in reciprocal space using standard refinement

programs such as 'Refmac' (Murshudov, 2011) against the $|F_j^{\text{ext}}|$. This is done for each time-independent electron density map, and results in a set of intermediate structures in the context of a candidate mechanism.

ii. Post-Analysis of the Kinetic Mechanism—Once the structures of the intermediates are determined, these structures can be used to refine the candidate kinetic mechanism which is initially employed to globally fit the significant right singular vectors. The rate coefficients as well as a scale factor are the fit variables which are varied to optimize the agreement between calculated and observed, time-dependent difference electron densities. The calculation of time-dependent difference electron density maps requires several steps: (i) structure factors are calculated from all atomic models including the reference state. (ii) for each intermediate, time-independent difference structure factors ΔF_j^{calc} are calculated by subtracting the reference structure factors from the intermediate structure factors. (iii) time-independent difference electron density $\Delta \rho_j^{\text{calc}}$ is calculated for each intermediate from ΔF_j^{calc} (iv) a concentration profile $c(t, k)_j$ is calculated for each intermediate by integrating the CDE representing the mechanism. Obviously, $c(t, k)$ is largely determined by the magnitude of the rate coefficients in the mechanism. (iv) time-dependent difference electron density maps, $\rho(t)^{\text{calc}}$, which can be compared with the corresponding observed density, are calculated as the sum of $\Delta \rho_j^{\text{calc}}$ weighted by their respective $c(t)_j$ at all time-points:

$$\Delta \rho(t)^{\text{calc}} = sc \sum_{j=1}^J c(t, k)_j \Delta \rho_j^{\text{calc}}$$

The result is a time-series of calculated difference maps which depend on the rate coefficients k that define the mechanism and a global scale factor sc that accounts for the difference in scale between the calculated and the observed difference densities. The calculated difference maps are then compared to the observed difference maps at all measured time points. The rate coefficients and the scale factor are changed iteratively until best agreement is reached. The result is a set of refined rate coefficients from which a refined concentration profile for all intermediates can be calculated, and a scale factor which is a measure of the number of activated molecules in the mechanism in terms of fractional occupation. Since the observed difference electron densities are calculated using the difference Fourier approximation, sc must be multiplied by two to obtain the extent of reaction initiation. Typically, this number is small, on the order of 5 to 10%. In favorable cases, however, the extent of reaction initiation can be as high as 40 to 50%, even 100% in favorable cases (Šrajer *et al.*, 2001; Knapp *et al.*, 2009; Tenboer *et al.*, 2014; Barends *et al.*, 2016).

iii. Mapping the Energy Landscape—Macromolecular reactions are most often thermally activated. The rate coefficients k_{ij} between intermediate states I_i and I_j (Fig. 1) in the mechanism are temperature dependent. When the temperature is increased, the reaction speeds up. In simple cases the temperature dependence of the k_{ij} is described by the

transition state equation (Eyring, 1935). Here entropy $S^\ddagger$ and enthalpy $H^\ddagger$ differences to the transition state are important parameters and k becomes:

$$k = \frac{RT}{N_A h} e^{\frac{\Delta S^\ddagger}{R}} e^{-\frac{\Delta H^\ddagger}{RT}},$$

with T the temperature, R the gas constant, N_A the Avogadro number, and h Planck's constant. When time-series are collected at different temperatures, they can be analyzed with the approach discussed above. As a result, the temperature dependence of the rate coefficients in the mechanism are extracted, which can be analyzed by equation above, and

$H^\ddagger$ and $S^\ddagger$ determined from crystallographic data alone (Schmidt *et al.*, 2010; Schmidt *et al.*, 2013). The $H^\ddagger$ is a measure of the energy change on top of the energy barrier, e.g. how many hydrogen bonds are broken or whether there is a covalent bond breakage necessary for the reaction to proceed. The $S^\ddagger$ stand for the change of order at the barrier. If $S^\ddagger$, for example, is positive, the transition state is more disordered as compared to the intermediate state. It experiences a larger, or more open configurational space compared to the intermediate state. If $S^\ddagger$ is negative, the transition state is more ordered. There might be only a narrow pathway, for the reaction to proceed. Enthalpy and entropy differences are equally important to control reactions. In many cases, reactions are mainly controlled by enthalpy, hence it is unfavorable to break hydrogen bonds or to move away from an electrostatic interaction. However, if the intermediate is structurally disordered, when it is structurally heterogeneous with many possibilities to distribute in configurational space, refolding through a more ordered transition state is unfavorable. This then slows down the reaction substantially.

In photoactive yellow protein both scenarios can be found. In solution refolding from a relatively disordered state called pB to the dark state is slow. $S^\ddagger$ is strongly negative and the

$H^\ddagger$ contribution is negligible (Van Brederode *et al.*, 1996). Obviously, the reaction advances through an ordered transition state starting from the disordered intermediate state pB. There are no strong interactions that need to be eliminated in the pB state for the reaction to occur. The reaction is controlled by the entropy. The situation is different in the crystals. Here the pB state is well ordered, and the chromophore is tightly bound to Arg 52 and to water molecules. To reisomerize back, the chromophore needs to occupy an enlarged chromophore binding pocket, where it is poorly bound. $S^\ddagger$ is slightly positive. In addition, the hydrogen bonds to Arg 52 and the water molecules need to be broken. The enthalpy difference $H^\ddagger$ is very positive, hence unfavorable for the reaction. In the crystal, the reaction is controlled by the enthalpy. In solution the reaction is almost two orders of magnitude slower. Hence, the larger disorder of the pB state has a much larger impact on the reaction dynamics in solution than the $H^\ddagger$ in the crystal. However, when a water molecule is bound in the chromophore pocket, the reaction slows down significantly (Tripathi *et al.*, 2012), because the water interactions with the chromophore binding pocket need to break to free the space for the chromophore to rebind. Although no 5-dimensional crystallographic data for this situation are available, this should result in a much larger enthalpy contribution to the reaction kinetics at this final stage of the photocycle.

4. Synchrotron Example: Allosteric Action in Dimeric Hemoglobin

As an illustration of a comprehensive synchrotron time-resolved crystallographic study we review briefly here the study of a cooperative dimeric hemoglobin. Structural changes were followed in real time during an allosteric transition in a single crystal, on the time scales from 100 ps to 100 μ s (Knapp *et al.*, 2006; Knapp *et al.*, 2009; Ren *et al.*, 2012). This dimeric hemoglobin, HbI, from the mollusk *Scapharca inaequivalvis* is very well suited for such studies. Fully cooperative ligand binding was demonstrated in the crystal (Mozzarelli *et al.*, 1996). Unlike the case of tetrameric human hemoglobin where allosteric structural changes are so large that crystals crack, in this case changes are relatively localized and limited and therefore compatible with the crystal lattice (Knapp and Royer, 2003). The crystal remains well ordered throughout the transition from the high-affinity R state to the low-affinity T state. Despite limited structural differences however, the R and T states exhibit a very large, 300-fold difference in oxygen affinity (Royer *et al.*, 1996; Knapp *et al.*, 2005).

The key ligand-induced structural changes during the R-to-T transition in HbI have been determined by static crystallographic studies of R and T states. They involve (Fig. 8) the flip of the Phe 97 from the dimer interface into the proximal heme pocket, a rearrangement of the water molecules at the dimer interface, movement of the two hemes toward the dimer interface and relative subunit rotation of $\sim 3.3^\circ$ (Knapp & Royer, 2003; Royer *et al.*, 1996; Knapp *et al.*, 2005; Pardanani *et al.*, 1997; Knapp *et al.*, 2001). The time-resolved studies conducted at BioCARS addressed the question of relative timing of these key structural events involved in the R-to-T transition. What is the cascade of events triggered by the ligand release? How do these events proceed: in a sequential or concerted manner? Are there structural intermediates during this allosteric transition?

The initial ns time-resolved studies, conducted with the M37V HbI mutant with an enlarged distal pocket (Knapp *et al.*, 2006), identified an early intermediate, present at 5 ns after ligand photo-dissociation and persisting until $\sim 1 \mu$ s. The subsequent experiments with 100 ps time resolution and WT HbI confirmed that similar intermediate in fact forms within 100ps (Fig. 9A; Ren *et al.*, 2012). In this intermediate, the hemes buckle and heme irons get displaced by $\sim 0.4 \text{ \AA}$ to the proximal side, perpendicularly to the heme plane, toward the F helix. There is no significant motion of the hemes toward their T-state positions. Significant early changes in M37V HbI are observed along the F helix and CD loop, the regions that also show the largest differences between the R and T states. The F helix moves toward the subunit interface and disrupts the R-state network of water molecules. Most notably, two key water molecules are displaced (Fig. 9A). Removal of these molecules is necessary for the motion of the hemes toward the dimer interface that occurs in the next, allosteric phase of the transition.

In the second phase of the transition that occurs significantly later, on the 1–10 μ s time scale (Knapp *et al.*, 2006), Phe 97 moves from the dimer interface to its T-state position in the proximal pocket, the heme moves toward the interface and the cluster of T-state water molecules assembles. All these events seem to follow each other very closely and it was not

possible to resolve the leading, rate-limiting step. However, only a partial subunit rotation toward the quaternary T-state of $\sim 0.6^\circ$ was observed by 80 μ s.

In addition to revealing the time scales and sequence of key events following ligand photo-dissociation in HbI, these studies also provided a clue on how ligand-induced structural changes propagate from one subunit to the other and modulate ligand-binding affinity. Although the HbI is a homodimer, time-resolved results provided evidence of an actual asymmetry of the HbI dimer in the crystal: ligand photolysis was consistently higher in subunit B compared to subunit A (Knapp *et al.*, 2009). The molecules are assembled in the crystal in such a way that the side of subunit B, which points away from the dimer interface, is accessible by a large solvent channel, while this is not the case for subunit A (Ren *et al.*, 2012). A comprehensive simultaneous analysis of the 100 ps photoproduct and 70 available static R and T structures of various mutants of HbI in the Protein Data Bank revealed some common features: a) the space between the E and F helices (angle and distance between helices) is consistently larger in the T state than in the R state; b) E–F space is consistently larger in the subunit B than in the subunit A; c) this subunit A–B asymmetry is also present in the 100ps photoproduct. In the photoproduct, therefore, subunit B is more advanced toward the T-like state than subunit A. The photoproduct thus mimics the otherwise unavailable asymmetric, singly-ligated state.

The analysis revealed that the E–F space opening (clam-shell motion) in each subunit is facilitated by a “hinge” region that is located in the middle of the E helix rather than at the E–F connection (Fig. 9B). Also, a close examination of the coupling of the E–F helices of the two subunits across the dimer interface revealed important “bolt” regions between the F helix of one subunit and the E’ helix of the other subunit. Several conserved residues in this region permit slight relative rotation but prevent sliding of the helices. This tight coupling region therefore facilitates efficient transmission of motion from one subunit to the other, across the dimer interface. A cooperativity mechanism in HbI was proposed based on these findings (Fig. 9).

Ligand migration pathways through several protein cavities following photo-dissociation were also explored (Knapp *et al.*, 2009). Two protein cavities, Xe2 and Xe4 cavities, relatively remote from the closest ligand docking site in the distal heme pocket, get populated within 5ns from the ligand release. However, it was shown that although Xe4 is a docking site for the ligand, it is not on the major pathway for the ligand exit from the protein. It was proposed that in HbI, like in myoglobin, the distal histidine gate rather than a “back door” via Xe4 cavity is the major ligand entry and exit route, despite its location and interactions at the dimeric interface in HbI.

5. XFEL Example: Early Events in the Photoactive Yellow Protein Photocycle

The photoactive yellow protein (PYP) together with carbon monoxide bound myoglobin (MbCO) were the main model systems to develop time-resolved crystallography at the synchrotron. The photoactive yellow protein (Fig. 7) features one of the most common reactions in chemistry, a *trans* to *cis* isomerization reaction of its central p-hydroxy-cinnamic

acid (p-coumaric acid, pCA) chromophore. Hence the PYP offers an opportunity to observe this chemically very important isomerization in real time. Consequently, the interest in time-resolved structural results has been enormous. As a result of synchrotron-based time-resolved structural dynamics investigations with ns and 100 ps time resolution the following picture unfolds: in the reference, dark structure (called pG, G for ground state), the pCA chromophore is *trans*, the absorption maximum is around 460 nm. 100 ps after photoexcitation with a blue 30 ps laser pulse the chromophore is already near *cis*. A very early state has been determined by two different groups and called IT (Jung *et al.*, 2013) by one group and pR0 (Schotte *et al.*, 2012) by the other. The structural differences between IT and pR0 are small, however it must be noted that the torsional angle of the 4 atoms including the double bond (red line in Fig. 10A) differs between the two structures (90° in IT versus 35° in pR0). Small atomic displacements which are lying within the coordinate error of the refinement may produce these differences. Both IT as well as pR0 are not fully *cis* but were thought to represent a twisted transitional form on the pathway to *cis*. It should be noted that in the Schotte *et al.* (2012) study non-standard conditions for crystal preparation were used which might have altered the chemical kinetic mechanism. Therefore, in the following we discuss results obtained from more conventional conditions (Ihee *et al.*, 2005; Jung *et al.*, 2013; Schmidt *et al.*, 2004). IT decays in a bifurcation reaction within 1 ns to pR1 and ICT, where the R stands for a putatively red shifted absorption spectrum of this intermediate and CT denotes a twisted *cis* configuration. ICT then decays on the nanosecond time scale to pR2 which lives together with pR1 for several orders of magnitude in time, until they both decay to the pB1 intermediate at around 200 μ s (Fig. 7). The letter B in pB denotes a blue shifted absorption spectrum. Another pB state is populated (pB2) with significant displacements observed on the N-terminal helix. After this pB relaxes back to the dark state pG within about 100 ms. All structures in the photocycle starting with ICT are in the *cis* configuration with the torsional angle close to 0°. The final pB to pG relaxation includes the re-isomerization back to *trans*.

As the earliest intermediate, IT, was already near *cis*, the *trans* to *cis* isomerization reaction itself could not be time-resolved at the synchrotron. Literature evidence suggests (Nakamura *et al.*, 2007; Creelman *et al.*, 2014) that the pCA isomerization takes place on an ultrafast, sub-ps time scale. The ultrafast, fs X-ray pulses available at the XFEL were exploited to structurally characterize this transition (Pande *et al.*, 2016). To show that fs time-resolved crystallography with high spatial resolution is possible at the XFEL, two proof-of-principle experiments are necessary. (i) It has to be shown that near atomic resolution time-resolved serial femtosecond crystallography is feasible with PYP, and (ii) that the photocycle can be started effectively with fs laser pulses. The proof-of-principle experiments were successful. In a first experiment (Tenboer *et al.*, 2014), the PYP photocycle was initiated with nanosecond laser pulses (450 nm, 0.8 mJ/mm²). 64500 indexable reference diffraction patterns were collected in the dark and 31300 pattern were collected 1 μ s after reaction initiation. Data were analyzed to a resolution of 1.6 Å. Both data sets showed excellent statistics with high I/σ_1 values and R_{split} values smaller than 10%. Difference maps calculated from these data are of very high quality with features on the order of 20 σ . The extent of photoactivation was around 40%. To bring this into perspective, the best difference maps for PYP at the synchrotron did not show features higher than 12 σ . At the synchrotron

the yield achieved so far was only 10%, most likely due to probing with the X-ray beam deeper than the laser penetration depth. The next logical step was to initiate the reaction with fs (140 fs) laser pulses (Pande *et al.*, 2016) and determine how many molecules are active in the photocycle. With fs laser pulses, only one attempt at entering a photocycle for each molecule is possible, and the maximum achievable population in the photocycle equals the primary quantum yield. With longer ns laser pulses, those molecules that return to the dark state without entering the photocycle can be excited again, even multiple times, which boosts the number of molecules in the photocycle. Since with fs pulses we cannot expect more than 20% of molecules in the photocycle (the primary yield), over 100,000 indexable diffraction patterns were collected again to 1.6 Å at a time delay of 200 ns after the 140 fs blue laser pulse. As for ns laser excitation, the difference map showed exceptionally clear positive and negative features on the order of 18 σ (compare green and red electron densities in Fig. 10B with Fig. 7C). The extent of reaction initiation was 13% which agrees with the values found spectroscopically (Lincoln *et al.*, 2012; Hutchison *et al.*, 2016). With this it could be shown that fs laser excitation is feasible with microcrystals. These two successful positive control experiments open the door to the fs time scale.

On the femtosecond time scale, the time-tool as described in section 2.h was necessary to determine the actual laser-to-X-ray time delay on a shot to shot basis. The inherent X-ray pulse jitter helped to distribute the actual time delays through a wider time range while using only two nominal delay settings, 300 fs and 600 fs. In addition, drifts shifted a large number of delays from the nominal time delay of 600 fs to longer times. Overall, the fs time range actually covered from 140 fs to 1000 fs. The time-range was divided into 8 time-delay bins, each containing about 40000 indexed diffraction patterns. In addition, a time-delay of 3 ps was also measured (time jitter was negligible on this time scale) and includes 79000 indexed patterns. The difference maps show strong, chemically meaningful and contiguous difference electron density features (Fig. 10C,D) and can be interpreted by structural models. Comparing the 250 fs map in Fig. 10C with 3 ps map in Fig. 10D, there is a clear difference. In Fig. 10C positive feature β is kinked and lies on the left side, and behind, of the negative feature α. In Fig. 10D, the negative feature is crossing the positive feature, and the positive feature is much more aligned along the axis of the chromophore foot. In addition, the positive feature β points out of the plane of the drawing. The difference density in Fig. 10D is reminiscent of that of IT previously determined with 100 ps time resolution at the synchrotron, which was near *cis*. The difference electron density of Fig. 10C is new, but the kinked feature β clearly indicates a *trans* configuration. 7 of the 8 fs time delays were interpreted with structural models, the pCA chromophores of 3 were still in *trans*, and 4 were near *cis*. The *trans* to *cis* isomerization, happens between 450 fs and 800 fs. To determine the transition more precisely, the 8 fs time-delays were re-binned into 16 time delays with about 20000 diffraction patterns each, and the resulting time series of difference maps was subject to singular value decomposition. The transition was identified in the right singular vectors which globally report the dynamics of the system. Through a fit with a step-

like trial function $f(t) = \frac{A}{1 + e^{-\frac{t-\tau}{B}}}$, the relaxation time τ was identified to be about 600 fs. It should be mentioned that the duration of the transition itself (180 fs) is faster than the time-scale on which it has been measured (600 fs) (see blue bell shaped curve in Fig. 11). Simple

chemical kinetic models (Steinfeld et al., 1985) do not provide solutions for such a step-like functional form in linear time (compare solid and dashed lines in Fig. 11A, insert and Fig. 11B). Concepts of barriers of activation and the promotion of reactions by the thermal excitation that play such an important role for chemical kinetics have limitations. On ultrafast time-scales, transitions may rather be governed by electrostatic forces on the excited electronic state potential energy surface (PES) and promoted by functional modes. Here, a functional mode can be envisioned to become important when the chromophore is displaced in the first 200 fs. The chromophore can be thought to collide with the protein matrix and be rapidly reflected or thrown back by this mode which may assist the rotation of the chromophore tail towards the *cis* configuration. The identification of these functional modes should be a prime goal in ultrafast structure determination. After the *trans* to *cis* transition takes place at 600 fs in PYP, the resulting configurations continue to relax electrostatically on the ground electronic state PES. The first intermediate is an IT like intermediate which is populated within a few ps. After this, the dynamics is then governed by chemical kinetics on the ground electronic state PES until the photocycle comes to an end. One can predict that up to a few ps the dynamics is temperature independent. After this the reaction can be activated by temperature which becomes the basis for 5D crystallography (Schmidt *et al.*, 2010; Schmidt *et al.*, 2013). Overall, the PYP photocycle is now characterized from initial, fundamental motions to the end of the reaction.

6. Future Trends and Challenges

a. Irreversible Reactions and Structural Enzymology

As illustrated in sections 4 and 5, time-resolved crystallography proved to be highly successful in investigating light-induced reversible reactions, such as ligand photolysis in heme proteins and PYP photocycle. However, many other biologically and pharmaceutically interesting reactions that are important targets for studies by time-resolved crystallography are irreversible. For example, it is highly desirable to investigate enzymatically catalyzed reactions since they are widely relevant for bio-medical applications and structure based drug design. Enzymatic reactions are typically irreversible, single pass reactions. Substrate binds and will be processed to product that is finally released. With macroscopic crystals, this requires an inactive substrate called a caged substrate, which can be soaked into the crystals and which can be subsequently activated by short light pulse. After each enzymatic cycle, the product needs to be washed away and some new substrate must be loaded. This can be done in specially designed flow-cells. Alternatively, one can also use similar flow-cells for a multi-turnover, steady-state experiments where continuous supply of active substrates is used. Diffusion of the substrates into the crystal drives accumulation of a high-occupancy, rate-limiting complex in the crystal (Stoddard, 2001). Both are challenging approaches as discussed in section 2.c. Crystals are very difficult to mount, immobilize and visualize in such specialized flow-cells. As a result, the application of time-resolved crystallography to irreversible reaction and structural enzymology has had very limited progress so far. Novel methods for reaction initiation and sample delivery described below have a potential to be transformative for such studies.

b. Novel Methods of Reaction Initiation and Sample Delivery

i. Mix and Inject—The small crystals employed by serial crystallography provide an appealing way to initiate reactions chemically simply by diffusion of substrate or cofactor, change in pH etc. This will then provide a long thought after pathway for structure based investigations on enzymatically catalyzed reaction. If diffusion times are much faster than turnover times, reactions in enzymes can be conveniently initiated and observed in real time simply by mixing substrate with small enzyme crystals. The mixture is subsequently injected into the X-ray beam. Diffusion into crystals is governed by Fick's first and second laws in three dimensions. Diffusion times depend on the square of the crystal size (Schmidt, 2013). Early experiments (Hajdu *et al.*, 1987) and simulations (Geremia *et al.*, 2006) corroborate this view and also demonstrate that estimates using simplified solutions to Fick's laws describe experimental findings accurate enough to predict diffusion times. With microcrystals diffusion times may reach already 1 ms. Diffusion times into crystals with sub-micron cell edges can be even smaller than 100 μ s. With these tiny crystals there are two challenges, (i) the mixing times might become longer than the diffusion times, and (ii) the crystals are so small that with conventional methods diffraction pattern cannot be collected due to radiation damage. The first challenge can be overcome with specially designed liquid-liquid mixing injectors (Wang *et al.*, 2014; Calvey *et al.*, 2016) reminiscent of GDVNs, where a stream of microcrystals is rapidly mixed with a concentric flow of substrate.

Through gas focusing, the mixture forms a thin jet that is interrogated by an X-ray pulse. Diffusion times from the outer concentric flow into the inner, crystal containing flow may be as short as 200 μ s (Wang *et al.*, 2014). To address point (ii) intense femtosecond X-ray pulses of XFELs are required, because the 'diffraction-before-destruction' principle holds. The enzymatically catalyzed reaction proceeds undisturbed at ambient temperatures, until the FEL X-ray pulse arrives and a diffraction pattern is collected. After this, the tiny crystal is destroyed and discarded, which neither effects the chemical reaction probed nor the diffraction pattern recorded. First results using this technique were already obtained using a T-junction which allows diffusion and reaction times between 2 s (Kupitz *et al.*, 2016) and 10 s (Stagno *et al.*, 2016). Stagno *et al.* investigated the diffusion of a small molecules, adenosine, into crystals composed of an RNA 'riboswitch' and observed the binding of adenosine and a concomitant structural change of the RNA riboswitch. This structural change was even accompanied by a change of the size and the symmetry of the unit cells without destroying the crystal. Kupitz *et al.* (2016) investigated the enzyme β -lactamase (BlaC) from *M. tuberculosis*, which is responsible for the broad resistance of this organism to lactam antibiotics (Tremblay *et al.*, 2008; Hugonnet *et al.*, 2009). Thin plates of crystalline BlaC grown from ammonium phosphate were mixed with 200 mmol/L ceftriaxone, a third generation antibiotic. 2 s after mixing the suspension was injected into the X-ray interrogation zone and probed by the XFEL pulse (Fig. 13). The BlaC electron density appeared in the catalytic cleft of two of the 4 subunits that are found in the asymmetric unit (Fig. 14). Since, after 2 s, most likely a steady state is formed, the electron density can be interpreted as a mixture of three states, the enzyme-substrate complex, a covalently bound form, and a product with an open lactam ring (not shown). This mixture can only be resolved if a time-series (a movie) is available to separate the intermediate states with techniques described above. Collecting movies on enzymatically catalyzed reactions

with properly spaced time-delays is one of the prime goals at the XFEL. Structures of enzyme intermediates along the catalytic pathway may then be extracted by established methods discussed above. When applied to biomedically and pharmaceutically important enzyme targets, the mix-and-inject method will provide an invaluable tool that has a potential to revolutionize structure based drug design. Ligand binding to these enzymes and their effects on the kinetics of the catalyzed reaction can then be observed in real time. High repetition rate, superconducting X-ray FELs such as the European XFEL or the LCLS-II, which both feature MHz pulse rates, are exquisitely suited to collect a time series with a sufficient number of time delays within a small, manageable period of time. Then, the impact of a larger number of ligands on the catalytic reaction can be investigated within the allocated beamtime.

ii. Microfluidic Chips—Microfluidic platforms have been originally developed as crystallization platforms that can be used directly for *in-situ* room temperature X-ray data collection (Axford *et al.*, 2012; Guha *et al.*, 2012; Perry *et al.*, 2014; Pawate *et al.*, 2015). This approach eliminates manual crystal harvesting and mounting that can damage smaller and more fragile crystals. By eliminating manual crystal mounting it facilitates high-throughput crystallography. This is in essence the original “fixed target” form of “serial crystallography”. The crystallization chip provides a 2D array of crystals. By scanning the device across the X-ray beam, fresh crystals can be rapidly and sequentially exposed to the X-ray beam. One can expose each crystal only once but in this case, as with other types of fixed target approaches (Hunter *et al.*, 2014; Mueller *et al.*, 2015; Roedig *et al.*, 2015), one can also rotate the chip through a limited angular range and collect multiple images on each crystal at several crystal orientations.

Microfluidic platforms are often compatible with time-resolved experiments. They may be used with light triggering but also provide opportunity for chemical triggering by diffusion. One of such platforms, with multi-layered, integrated fluidic control was in fact successfully used for initial Laue and time-resolved experiments at 14-ID beamline (Perry *et al.*, 2014; Pawate *et al.*, 2015). In initial Laue experiments, Laue data was collected on PhnA protein from *Sinorhizobium meliloti* crystallized on the chip (Perry *et al.*, 2014). Align-mark-and-find identification of crystals was used in this case where crystal positions were defined and recorded first. X-ray exposures were then delivered in a series to these pre-defined crystal locations. A single Laue diffraction patterns was collected from each crystal at room temperature, using 110 X-ray pulses, each of 100 ps duration, for exposure with crystals of 50–200 μm size. Patterns from 58 crystals were merged into a Laue data set that was ~90% complete to 2.1 Å resolution. For comparison, monochromatic data were merged from 19 crystals, with 10 exposures and 10° wedge collected on each crystal at room temperature. The two data sets are of comparable resolution and quality. In the first time-resolved experiment used with the same type of microfluidic platforms (Pawate *et al.*, 2015), ns laser pulses were used to trigger the reversible photocycle in photoactive yellow protein. Small slices of reciprocal space spanning 1–3 Laue diffraction images were collected on each of 10 crystals and merged at 10 μs and 1ms following the photo-trigger. The resulting difference electron density maps were of sufficient quality to detect the intermediate states at these time delays as determined from traditional single-crystal time-resolved experiments (Fig. 15).

Since these microfluidic devices have the valve system that is used for crystallization, it is conceivable that these devices may also be used to trigger reactions by diffusion. As discussed in sections 2.c and 6.b.i, using microcrystals provides opportunity to reach sub-ms diffusion times. Data described here (Perry *et al.*, 2014; Pawate *et al.*, 2015) were collected with 50–100 μm crystals. In order to facilitate use of <10 μm crystals, background scattering from the chip has to be significantly minimized. In a great step in that direction, use of a single-layer graphene as part of an ultra-thin microfluidic device was recently reported (Sui *et al.*, 2016). This architecture resulted in a total chip material thickness of only ~ 1 μm , while still preventing water loss.

iii. Sample Delivery by Acoustic Injection and Levitation—With the success of the serial crystallography methods implemented so far, both at XFELs and synchrotrons (described in section 2.f), efforts to improve this technology are focused on reducing sample consumption and increasing the overall crystal hit rate. While fixed target platforms and viscous-media injectors already provide a number of options, additional efforts are aimed at developing methods to synchronize specimen delivery to the arrival of the X-ray pulse.

Acoustic droplet ejection method (Ellson *et al.*, 2003) uses an acoustic transducer to induce droplet ejection on demand. In a proof-of-concept experiment with protein crystals, nanoliter-volume droplets with thermolysin crystals were acoustically ejected from a sample well onto a motorized Kapton tape, creating a conveyor belt for crystal delivery into the X-ray beam (Roesler *et al.*, 2013). Droplets can be deposited on the target tape with high positional precision and repeatability. Small ($<30^\circ$) wedges of monochromatic data were collected on 22 crystals at cryogenic temperatures, and a complete dataset was assembled from three best diffracting partial datasets. In a recent, more advanced implementation of this method at the LCLS, two novel acoustic injection systems were used to provide droplet-on-demand sample delivery synchronized to the X-ray pulse arrival at the sample (Roessler *et al.*, 2016). Droplets were ejected directly into the X-ray beam rather than onto a solid target thus minimizing the background. In these initial experiments, X-ray pulses intersected up to 88% of the droplets. The method is also applicable to intense, micro-focused synchrotron beams as well as to time-resolved experiments both at XFELs and synchrotrons.

In another development, an acoustic levitator developed at the Argonne National Laboratory (Benmore and Webber, 2011), provides alternative possibility for delivering samples into to the X-ray beam. The levitator uses two piezoelectric transducers to generate sound waves at frequencies just above the audible range, at ~ 22 kHz. By aligning the top and bottom transducers, a standing wave is created and light small objects such as liquid drops with crystals can levitate when placed at the nodes. By automating the loading of the nodes as well as node shifting through the X-ray beam, such devices in principle can provide another “conveyor belt” method for crystal delivery for time-resolved serial crystallography applications. It has been demonstrated that by modulation of the acoustic nodes spatially and temporally, transport and mixing of acoustically levitated droplets is also possible (Foresti *et al.*, 2013).

Finally, a precise manipulation and patterning of protein crystals for X-ray data collection and screening was demonstrated by using surface acoustic waves (SAW; Guo *et al.*, 2015). A

multi-crystal lysozyme data set was collected using $< 20 \mu\text{m}$ crystals patterned by SAW in a capillary tube. They also demonstrated that protein crystals can also be distributed into 2D arrays, which could be scanned through the X-ray beam thereby greatly enhancing the X-ray pulse hit rates.

iv. Electric Field Jump—As noted above, reaction initiation is a critical step in time-resolved crystallographic studies and expanding the methods for reaction initiation is the key for a wider applicability of the technique. Advancing the application of caged compounds will broaden the range of reactions that can be studied by time-resolved crystallography from the initial, relatively narrow field of light-induced reaction in inherently photo-sensitive proteins. Improving methods for reaction initiation by diffusion, discussed above, will open the door for time-resolved studies of a very large class of enzymatic reactions. In addition, the exciting new results point to another promising method of reaction initiation: electric field-stimulated crystallography (EF-X) (Hekstra *et al.*, 2016).

All biomolecules contain charges, dipole and quadrupole moments. Application of an external electric field can alter spatial distribution of charges, dipole moments and polarizability and can affect any process that involves movement of charge. It therefore may represent a very general method to study structural dynamics in biomolecules. In one approach, one can use external electric field to perturb populations and reaction dynamics of processes that involve displacement of charges (such as electron or proton transfer reactions). This new method of reaction initiation is aimed particularly at understanding the mechanism of voltage gating in ion channels, a highly biomedically-relevant class of biomolecules. In another approach, an external electric field can be used to bias transitions between protein conformations sampled by biomolecules (i.e. redistribute conformations). This is a quite general approach, applicable to any protein. It can provide information about collective motions and functionally relevant conformational transitions that link protein structure and mechanics to function.

In the first EF-X time-resolved experiments conducted at BioCARS (Hekstra *et al.*, 2016), this new method for studying biomolecular machines was applied to the PDZ domain of the human E3 ubiquitin ligase LNX2. The protein was selected because it does not have known functional voltage dependence. It was therefore used to test the generality of the EF-X approach to analyze the mechanics of protein machines. Voltage pulses of 5–8 kV and 50–500 ns duration were applied to 50–100 μm thick crystals, resulting in electric field strengths of $\sim 0.5\text{--}1 \text{ MV cm}^{-1}$. Voltage pulses were synchronized with the X-ray pulses. X-ray diffraction images were collected with single 100 ps X-ray pulses, before the electric field pulse (the reference OFF data) and at specified time delays from the rising edge of the electric field pulse (ON data). The experiments demonstrated that crystals tolerated hundreds of electric field and X-ray pulses without significant loss of diffraction. In fact, a time series of four data sets was collected from a single PDZ crystal: an OFF data set and ON data sets at 50, 100 and 200 ns delays.

Comparison of the OFF and ON data, revealed that the external electric-field induced a wide range of structural changes, from side-chain rotamer flips and displacements of backbone atoms and side chains, to global motions of secondary structure elements and coordinated

changes in large regions. These first EF–X results also suggest that external electric field pulses populate low-lying conformational states, energetically close to the ground state and most likely functionally relevant.

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Summary

New ultra-high brilliant XFEL X-ray sources and micro-focusing capabilities at synchrotron sources, together with new methodologies to best utilize such sources, such as serial crystallography, spawned a plethora of new opportunities for time-resolved crystallographic experiments on biological macromolecules. Previously mainly cyclic reactions were investigated which required low mosaic crystals. Now non-cyclic reactions can be investigated using imperfect crystals. This will have a decisive impact on the characterization of biologically and biomedically important enzymatically catalyzed reactions.

Figure 1.

Molecular reaction: intermediate states and chemical kinetics. **(A)** Intermediate states I_1 and I_2 correspond to energy minima along the reaction coordinate and are transiently occupied by molecules as the reaction proceeds. Transition state T^* at the top of the activation barrier is also shown. **(B)** Hypothetical reversible photo-initiated reaction, with three intermediates, I_i ($i=1,2,3$), and a branched chemical kinetics mechanism. I_0 is the initial state. Rate coefficients k_{ij} , determined by activation energy barriers, determine in turn the observable, macroscopic relaxation rates K_m ($m=1,2,3$) and concentration amplitudes C_{mi} for each intermediate I_i : $C_i(t) = C_{1i}\exp(-K_1t) + C_{2i}\exp(-K_2t) + C_{3i}\exp(-K_3t)$. **(C)** Time dependence of concentrations $C_i(t)$ of intermediates for a particular set of rate coefficients k_{ij} . Notice that at most times, a mixture of several states is present. Notice also that, in order for an intermediate state to be experimentally detected, rate coefficients need to be such that this intermediate accumulates to a detectable level during a reaction.

Figure 2. Larger microcrystals of photoactive yellow protein in a Neubauer counting chamber (0.1 mm thickness). Crystal size and concentration can be conveniently estimated from this (bar shown in black is 50 μm). Crystals used at the XFEL are one to two orders of magnitude smaller (0.5 – 5 μm) and crystal density is 3 orders of magnitude larger ($\sim 10^{11}$ crystals/mL).

Figure 3.

Single X-ray pulse isolation at BioCARS 14-ID beamline. **(A)** The APS storage ring modes used for time-resolved experiments with 100 ps time resolution: 24-bunch mode (4.25 mA per bunch, with bunch separation of 153.4 ns) and hybrid mode (16 mA isolated bunch separated from adjacent septuplets by 1.59 μs). The two modes account for ~76% of the total APS user beamtime. 324-bunch mode is also available and can be used when time resolution of 200 ns or longer is sufficient. **(B)** 14-ID beamline shutter train can isolate single 100 ps X-ray pulses in both 24-bunch and hybrid modes.

Figure 4.

The table and instrumentation in the 14-ID end station. Laser light is brought to the station through a port above the table and delivered to the sample either along the vertical direction or nearly-along the horizontal X-ray path (not shown). Two sample viewing cameras are at 60° and -30° with respect to the X-ray beam horizontal direction (shown in dark blue in the enlarged view of the sample environment in the right panel).

Figure 5. Perpendicular laser-X-ray beam geometry at BioCARS 14-ID. A large PYP crystal is shown. The X-ray beam is perpendicular to the page and the long crystal axis in this view.

Figure 6.

Time-resolved serial femtosecond crystallographic experiment at LCLS using a GDVN jet. Crystals are loaded in a reservoir and pumped through long capillaries into the GDVN. Because the jet velocity is ~ 10 m/s, the laser pulse and X-ray pulse only overlap at short time delays. Diameter of laser spot: $150\ \mu\text{m}$, diameter of the X-ray beam: $1\ \mu\text{m}$.

Figure 7.

Photoactive Yellow Protein (PYP). (A) The overall structure of PYP. The pCA chromophore and the nearby Arg52 chromophore pocket lid is also shown. (B) The photocycle of PYP, with dark state (pG) and intermediates marked. The photocycle can be initiated by blue (450 nm) laser pulse. I_T : early intermediate, chromophore *cis*, twisted. pR_1 : first intermediate whose absorption maximum is assumed to be red-shifted. I_{CT} : chromophore *cis*, twisted (different from I_T). pR_2 : second red-shifted intermediate. pB_1 and pB_2 : late blue shifted intermediates. Red-box: part of the photocycle which is faster than the time-resolution at the synchrotron, needs XFEL. Red dotted line: 1 μ s time point used for positive controls (see text). (C) A difference map for the PYP chromophore region obtained at 1 μ s after photoexcitation at the LCLS. Red: negative difference electron density; green: positive density. Densities are contoured at $-3/3\sigma$, respectively. Structure shown in magenta: pR_1 , in red: pR_2 and in yellow: pG (=dark/reference state). The central chromophore that undergoes photoisomerization is shown as well as several surrounding amino acids.

Figure 8.

Heme and Phe 97 displacements in the R-to-T transition in *Scapharca* dimeric hemoglobin. Upon ligand dissociation both Phe 97 and hemes move from their R-state locations (orange) to the T-state locations (cyan). Hemes move closer to the dimer interface, while Phe 97 in each subunit moves from the interface to the corresponding proximal heme pocket.

Figure 9.

Allosteric action in *Scapharca* dimeric hemoglobin HbI. (A) Difference electron density map corresponding to an early intermediate formed by 100ps in WT HbI is shown for subunit B (negative density is shown in red, positive in green). Notice the plume of density extending from the heme (orange) to both distal B and proximal F helices upon release of the ligand. Some key R-state water molecules at the dimer interface, like the two circled in blue, have been displaced. (B) Proposed mechanism for transmitting ligand-induced structural changes across the dimer interface. E-F helices are shown for the two subunits. When a ligand is dissociated from one subunit, the F helix gets pushed away from helix E by the out-of-heme-plane iron motion, observed already at 100ps. A clam-shell motion "hinge" in the middle of the E helix permits part of the E helix to move with the F helix as the E-F space opens. Due to the F-E' "bolt" across the dimer interface, part of E' helix moves along with the F helix, increasing the E'-F' space in the second subunit. As the ligand in the second subunit gets released, the overall relative rotation of subunits completes the quaternary rotation in HbI during the R-T transition (Ren *et al.*, 2012, see also movies in the supplemental information in the reference).

Figure 10.

Femtosecond excitation of the PYP photocycle with a 140 fs blue (450 nm) laser pulse (Pande *et al.*, 2016). (A) Chemical structure of the pCA chromophore. The torsional angle of the double bond is marked in blue and outlined in red. (B) Positive control. 40 fs X-ray probe pulse 200 ns after fs excitation. Difference electron densities are shown in red (negative) and green (positive), both here and in (C) and (D). Magenta and red models: structures of intermediates pR₁ and pR₂. Yellow: dark (reference structure). (C) 250 fs after fs excitation. Pink: electronic excited state structure, twisted *trans*. (D) 3 ps after fs excitation. Green structure: electronic ground state structure, near *cis*.

Figure 11.

The *trans* to *cis* isomerization derived from TR-SFX data collected at LCLS. **(A)** Analysis of the difference electron density by singular value decomposition. Spheres, squares and triangles 1st, 2nd, 3rd RSVs, respectively. Black line: fit of 1st RSV with one exponential, dashed black and red line: separation into two processes (i) placement on excited electronic state PES, (ii) second relaxation to ground electronic state PES. 2nd RSV fit by a step-like function (see text) with a relaxation time of ~600 fs. Bell shaped curve: derivative of the step-like function. Duration of the transition from 470 fs to 650 fs. Insert: 2nd RSV fit by a step-like function, root mean squared deviation between observed data and fit, rmsd = 0.068, and by a simple exponential, rmsd = 0.164. **(B)** Torsional angle dynamics. Yellow sphere: torsional angle of reference state, excitation with blue laser (blue arrow). Black spheres: torsional angles after excitation. Black line: fit by a step-like function with the same relaxation time as the one used to fit the 2nd RSV in A, rmsd = 9.4. Dashed line: fit by an exponential, rmsd = 16.1.

Figure 12.

Mixing injector, picture modified from Calvey *et al.*, 2016. The crystal suspension (simulated by the central pink flow) is focused by the substrate flow (blue arrows). The mixture is further focused by a gas flow and produces a jet which is probed by the X-ray FEL pulse. The length of the diffusion region can be varied to allow for variable reaction times (= time delays).

Figure 13.

Mix and inject setup to establish enzymology at the LCLS. Ceftriaxone and enzyme microcrystals are mixed and the mixture transported through the nozzle rod to the X-ray interrogation zone. Diffraction patterns are recorded on the CSPAD detector.

Figure 14.

BlaC tetramer, subunits A–D are marked, 2 s after mixing with ceftriaxone. Only subunits B and D display CEF electron density (blue, boxes with solid lines), subunits A and C still show phosphate (orange density, dashed boxes) in their catalytic cleft because access is occluded by neighboring subunits.

Figure 15.

Difference electron density map (red/green: $-3\sigma/3\sigma$ contour level) of PYP depicting structural changes at the chromophore binding pocket at 10 μ s after photo-initiation. Maps obtained using the microfluidic approach (left) at room temperature are compared to maps obtained from a large, single crystal mounted traditionally (right) in a thin-walled glass capillary. Laue diffraction was used in both cases. Yellow: dark state; red and magenta: intermediate states.

Table 1

Various methods to grow microcrystals.

Method Exemplary	Biological System	Reference
Free interface diffusion	photosystem I and II	Hunter <i>et al.</i> (2011), Kupitz <i>et al.</i> (2014b)
Mix-and-stir photoactive	protein yellow	Tenboer <i>et al.</i> (2014)
Mix-and- centrifuge	photoactive yellow protein	Tenboer <i>et al.</i> (2014)
In-cell	Cathepsin	Redecke <i>et al.</i> (2013)
Lipidic cubic phases	G-protein coupled receptors	Liu <i>et al.</i> (2013)