Time-resolved Macromolecular Crystallography in Practice at BioCARS, Advanced Photon Source: From Data Collection to Structures of Intermediates

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1. Introduction

Time-resolved macromolecular crystallography is one of several "kinetic crystallography" methods (Bourgeois and Royant, 2005; Bourgeois and Weik, 2009). In kinetic crystallography experiments, genuine biological function is triggered in the crystal with a goal of capturing molecules in action and determining structures of intermediate states. In time-resolved experiments in particular, short and intense X-ray pulses are used to probe intermediates in real time and at room temperature, in reactions that are initiated synchronously and rapidly in the crystal. In other kinetic crystallography approaches, physical or chemical trapping has been used (such as trapping by freezing or chemical modifications) to extend the lifetimes of intermediate states. These states can then be studied by the more conventional and less technically challenging static crystallography.

During the last two decades, essential advancements have been made in the development of high-intensity synchrotron X-ray sources, synchrotron instrumentation, as well as methodology and software for Laue data processing and analysis. This facilitated successful time-resolved crystallographic experiments with 100ps time resolution (Ren et al., 1999; Schmidt, 2008; Schmidt et al., 2005a). Time-resolved macromolecular crystallography at synchrotron X-ray sources is in its mature phase today. Structural changes as small as 0.2-0.3 Å are detectable with 100ps time-resolution and even if the reaction is initiated in a relatively small fraction of molecules in the crystal (Ihee et al., 2005; Jung et al., 2013; Knapp et al., 2006; Schmidt et al., 2005b; Schotte et al., 2003, 2012; Srajer et al., 1996; Wohri et al., 2010). In addition, determination of time-independent structures of intermediate states from measured time-dependent structure factor amplitudes has been successfully demonstrated (Rajagopal et al., 2004a; Schmidt et al., 2003).

We provide here an overview of time-resolved crystallography as implemented today at the BioCARS beamline 14-ID at the Advanced Photon Source (Graber et al., 2011), with an overview of future directions and challenges.

2. Time-resolved Crystallographic Experiments: General Considerations

2.1 Reaction Triggering To examine short-lived intermediates in real time, it is critical to initiate the reaction in the molecules in the crystal in a time period that is significantly shorter than the lifetimes of such intermediates (Schlichting and Goody, 1997; Schmidt, 2008; Schmidt et al., 2005a). The fastest method of reaction initiation by far is the use of ultra-short laser pulses. This method is readily applicable to molecules that are inherently photosensitive and undergo a reversible reaction, like ligand photo-dissociation in heme proteins (Srajer et al., 1996) or chromophore isomerization in photoactive yellow protein (Ihee et al., 2005). Alternatively, other molecules can be rendered light sensitive by chemically attaching photosensitive groups to substrates, cofactors or important protein residues (Bourgeois and Royant, 2005; Bourgeois and Weik, 2009; Corrie et al., 1992). Reactions can also be initiated by diffusion of small molecules into the crystal (such as substrates, cofactors or redox agents) but this is a slow process that requires seconds or minutes (or even longer). It is therefore suitable as a trigger only for slow reactions (Bourgeois and Royant, 2005; Bourgeois and Weik, 2009).

Protein crystals contain a very large number of molecules, on the order of 10^{13} -10^{14}, and therefore have high optical density (OD) in the wavelength regions where the chromophore absorbs significantly. In order to photo-initiate the reaction by laser pulses throughout the crystal, smaller (thinner) crystals have to be used and the laser wavelength tuned to a spectrum region where OD<<1. Typical laser power densities used for reaction triggering are 2-5mJ/mm^2.

2.2 Pump-probe Method In time-resolved X-ray diffraction experiments laser pulses (or other triggering methods) are used as “pump” pulses that trigger the reaction, while X-ray pulses are used to “probe” the reaction, at various time delays following the pump pulse. Due to slow readout time of large area X-ray detectors (10-100ms at best), single-pump/single-probe is typically employed, unless a reaction is slow and single-pump/multiple-probe is possible, where one collects several diffraction images following a single reaction initiation.

2.3 Time-resolution Time-resolution of the experiment is determined by the duration of the pump or probe pulses, whichever is longer. At synchrotrons, best time resolution is ultimately limited by the ~100ps duration of the X-ray pulses, although somewhat better time-resolution is also possible in principle (~10ps) at the expense of the reduced signal-to-noise ratio (Haldrup et al., 2011, 2012).

2.4 X-ray Source A critical requirement for time-resolved experiments is the high X-ray flux per pulse, typically available only at third generation synchrotron

sources, such as Advanced Photon Source (Argonne National Laboratory, USA), European Synchrotron Radiation Facility (Grenoble, France) and SPring 8 (Japan). Flux greater than 10^{10} photons/pulse is needed when a single 100ps X-ray pulse is used to record a diffraction image from a protein crystal. If such high flux is not available or crystals are smaller in size (<200μm) and/or weakly-diffracting, the pump-probe sequence needs to be repeated a number of times prior to the detector readout. Such repeated pump-probe cycles are clearly possible only for fully reversible reactions. For irreversible reactions, single X-ray pulse data acquisition is necessary, where each image requires a single pump-probe sequence and a new crystal volume. For slower reactions with less demanding time resolution requirement, an X-ray pulse train of longer total duration and higher overall intensity can be used as a probe pulse, resulting in diffraction images with improved signal-to-noise ratio.

2.5 Single X-ray Pulse Isolation An X-ray shutter train is necessary to isolate single 100ps X-ray pulses or longer pulse trains from the continuous stream of synchrotron pulses (see Fig. 1 and Graber et al., 2011).

2.6 Pump/probe Synchronization Accurate and precise synchronization of the arrival of the X-ray probe pulse at the sample with respect to the laser pump pulse has to be achieved for time-resolved experiments (Graber et al., 2011).

2.7 Laue Diffraction Mechanical crystal rotation used in the standard monochromatic oscillation method is much too slow to probe fast, sub-second reactions. Laue diffraction with stationary crystals therefore has to be used. Laue diffraction employs significantly wider energy bandwidth than the standard monochromatic diffraction. It has been shown that undulators are best X-ray sources for time-resolved Laue diffraction experiments (Bourgeois et al., 2000; Srajer et al., 2000). The typical bandpass of undulators used today for time-resolved experiments is 3-5% at 12-15keV. Specialized software for Laue data processing is required, for example CCP4 *Daresbury Laue* Software Suite or Precognition/Epinorm (Ren et al., 1999).

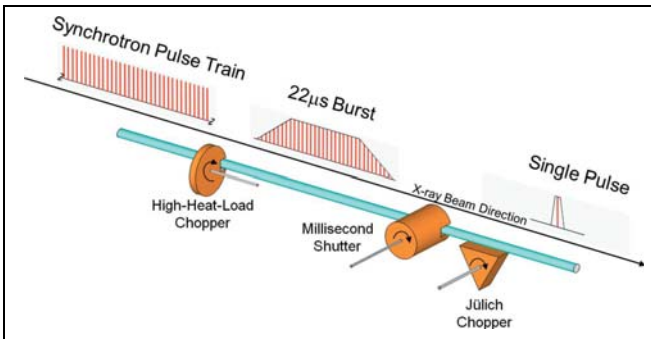

Figure 1 X-ray shutter train for time-resolved experiments at BioCARS 14-ID beamline. (Graber et al, 2011. Reproduced with permission of the International Union of Crystallography.)

2.8 Crystal mounting Crystals for time-resolved crystallography are typically mounted in thin-walled glass or quartz capillaries or using MiTeGen RT style mounts.

This is because data has to be collected at or near room-temperature rather than at cryo temperatures to facilitate complete protein mobility as the reaction in the crystal proceeds.

3. Time-resolved Crystallographic Experiments at BioCARS

3.1 Upgraded BioCARS 14-ID Beamline BioCARS 14-ID beamline at the Advanced Photon Source (Argonne National Laboratory, USA) was originally designed for time-resolved crystallography. It has been completely upgraded in 2007-2008. The new beamline layout, specifications and operation are described in detail in Graber et al (2011). In short, the X-ray source consists of two in-line undulators with periods of 23 and 27 mm, providing high pink-beam flux at 12 keV, as well as first-harmonic coverage from 6.8 to 19 keV. A high-heat-load chopper (Fig. 1) is implemented to reduce the average power load on downstream components to less than 1% of the original power in the X-ray beam. This chopper typically produces a 22 μs burst of X-rays at a repetition rate of 82.3 Hz (although it can also be operated at 1 kHz). A second, high-speed chopper (Jülich chopper; Fig. 1) isolates single 100ps X-ray pulses at 1 kHz in both hybrid and 24-bunch modes of the APS storage ring. Since these two choppers are continuously rotating, an additional, millisecond shutter is used for on-demand exposures of the samples to X-ray pulses.

In hybrid APS mode, each X-ray pulse delivers up to ~4×10^{10} photons to the sample at 14-ID. X-ray beam is focused by a new KB mirror system to a minimum spot size of 90 μm (h) × 20 μm (v). A new high-power and widely tunable picosecond laser system was also installed, covering the wavelength range of 450-2000 nm. These laser pulses are synchronized to the storage ring RF clock, with long-term stability better than 10 ps rms. A portable nanosecond laser is also available.

3.2 Typical time-resolved Crystallographic Data Collection A complete time-resolved data set spans four-dimensions: three traditional reciprocal space dimensions and time. Similar to a standard monochromatic data set, a Laue data set at each time delay contains images collected at a number of different crystal orientations, except that crystal is stationary during each X-ray exposure. Integrated intensities are nevertheless recorded due to the polychromatic nature of the X-ray source. With 14-ID undulator sources, the angular step in crystal orientation needed is typically 2–3°. The number of time points required to characterize the reaction kinetics depends on the number of intermediates and their lifetimes. A good starting point is to collect five points per decade in time, equally spaced in logarithmic time.

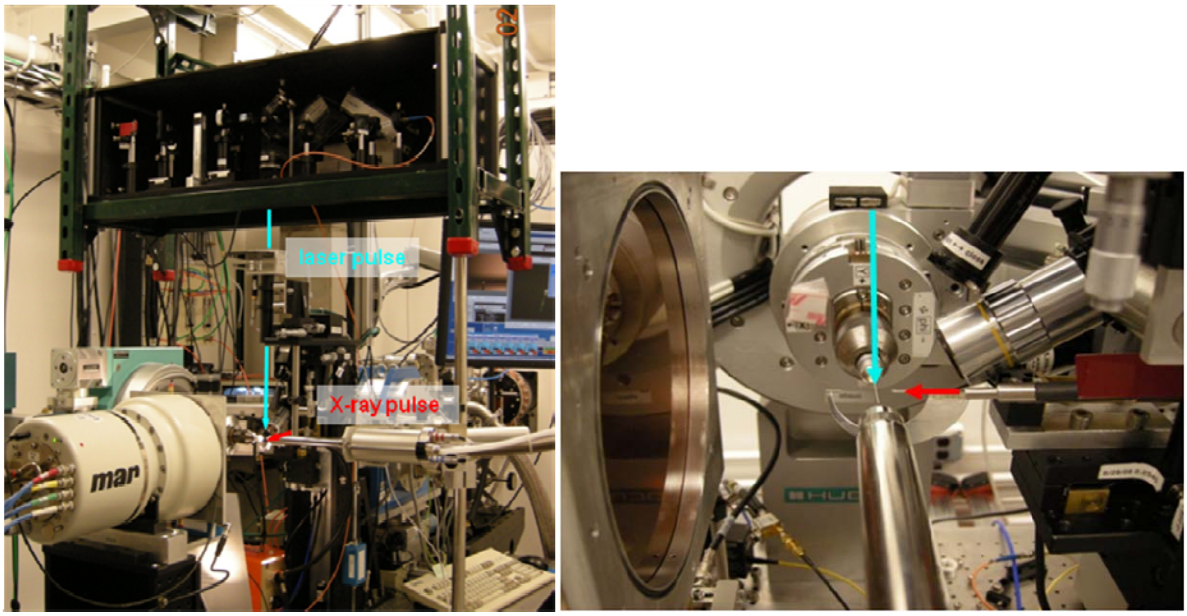

Figure 2 BioCARS 14-ID experimental hutch. Laser light is delivered to the sample via optical fiber (ns laser) or via mirrors (ps laser). The complex ps laser system is located in a separate laser lab. Left panel: experimental table is shown. Above the table is a box with ps laser conditioning and pulse stretching optics. Current Mar165 X-ray detector will be replaced in 2013 by a Rayonix MX340 HS detector. Significantly larger area will permit studies of crystals with larger unit cells. Faster readout time of the new detector (0.1-0.01s) will also permit using single-pump/multiple-probe style of experiments with sub-second time resolution (readout time of the current Mar165 detector is 3-5s). This is particularly important in studies of irreversible reactions where a new sample is needed after each reaction initiation. A single-pump/multiple-probe method will reduce the number of needed samples.

Since the signal of interest is the difference in intensity of diffraction before and after the photo-activation, it is best to collect both data sets on the same crystal, with interleaving the laser-on and laser-off images for each crystal orientation. To minimize errors across the time domain, time delay is typically a fast variable (Schmidt, 2008; Schmidt et al., 2005a): time delay is scanned for a given crystal orientation, starting with the laser-off image and collecting a series of laser-on images that span the desired time domain. The time scan is then repeated for a new crystal orientation. Several crystals might be required for a complete space-time data in case of radiation-sensitive samples.

3.3 Reaction Initiation Optimal reaction initiation is critical for the success of time-resolved experiments. Laser light is delivered to the sample either by optical fibers (ns laser) or directly (ps laser) and focused at the sample. Picosecond laser pulses are typically stretched from 1ps to ~30ps for a better match to the X-ray pulse duration (100ps) and for minimizing laser-induced crystal damage. Variable laser focusing is

available to best match the crystal size. Both elliptical (as shown in Fig. 3) and circular focus shape is available. Given the fact that crystals are very optically thick, laser wavelength is typically tuned to the part of the spectrum with lower absorption but even then light penetration into the crystal may not be very deep. A perpendicular laser/X-ray geometry is therefore employed (Fig. 3), where X-ray beam probes only the laser-illuminated surface layer of the crystal (Graber et al., 2011). To properly position the crystal in the X-ray beam, crystal is scanned vertically through the X-ray beam (edge scan) and X-ray diffraction is used for determining correct crystal position. This is repeated for each significantly different crystal orientation. Diagnostics tools for on-line monitoring of the laser beam position and intensity, as well as the laser-to-X-ray time delay, are essential to detect and correct any positional, intensity and timing drifts during the time-resolved data collection.

3.4 Data Collection Software Time-resolved data collection software used at BioCARS, LaueCollect, was developed by Friedrich Schotte (NIH/NIDDK). The emphasis was on data collection automation as well as on flexibility in data collection strategies. The edge scan and crystal translation (to minimize radiation damage) are incorporated into the data collection protocol. Software allows users to log important information such as measured laser and X-ray pulse intensities and time delay for each diffraction image. Laser position and laser-X-ray timing can also be checked and corrected automatically at regular time intervals during data collection.

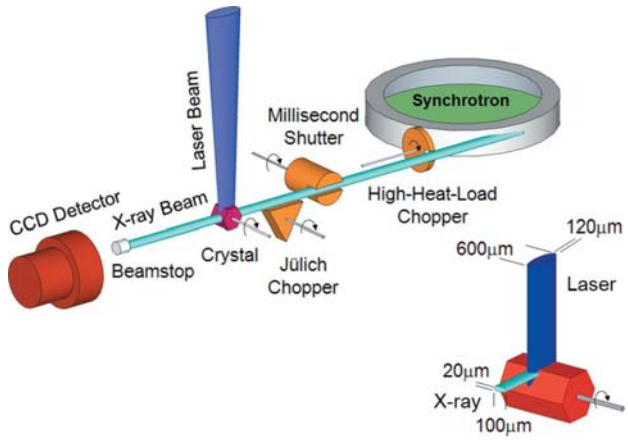

Figure 3 Schematic layout for a typical pump-probe experiment at BioCARS 14-ID beamline. (Graber et al, 2011. Reproduced with permission of the International Union of Crystallography.)

4. From Laue Data to Structures of Intermediates

Analysis of time-resolved crystallographic data involves three steps: 1) Laue data processing to derive time-dependent structure factor amplitudes from recorded diffraction images; 2) calculation of time-dependent difference electron density maps and analysis of such maps; 3) determination of structures of intermediate states.

4.1 Laue Data Processing Several problems specific to the Laue diffraction method are addressed and resolved by software: spatial overlap of diffraction spots in typically crowded Laue diffraction patterns, wavelength normalization and resolving the

harmonic overlaps. The so-called wavelength normalization is necessary due to polychromatic X-rays used and associated wavelength dependence of the incident X-ray intensity, scattering power of the crystal and detector sensitivity. A reflection or its symmetry mate may be stimulated by different wavelengths depending on the crystal orientation. Measured intensities therefore need to be brought to a common scale before data can be merged. The resulting λ-curve combines all wavelength-dependent effects in diffraction intensities as recorded at the detector. Harmonic or energy overlaps result from reflections that lie on a radial line (starting at the origin) in the reciprocal space. Such reflections are stimulated by different energies but scatter in exactly the same direction and overlap exactly at the detector. Program *Precognition-Epinorm* used at BioCARS for Laue data processing (Renz Research Inc) deals successfully both with wavelength normalization and harmonic overlaps. Another commonly used Laue package is *Daresbury Laboratory Laue Processing Suite* (Arzt et al., 1999; Campbell,1995), http://www.ccp4.ac.uk/cvs/viewvc.cgi/laue/doc/laue_install.txt?revision=1.1&view=markup. A version with improved indexing for sparse diffraction patterns is available from CHESS (ftp://waterline.chess.cornell.edu/pub/).

4.2 Difference Electron Density Maps Time-resolved crystallography method is based on a difference measurement and time-dependent difference electron density maps $\Delta\rho(t)$ are calculated and analyzed. Experimentally-determined structure factor amplitudes (SF) of the initial, dark state $|\mathbf{F}^D(hkl)|$ and corresponding time-dependent structure factor amplitudes $|\mathbf{F}(hkl,t)|$ are used to calculate time-dependent difference amplitudes, $\Delta F(hkl,t) = |\mathbf{F}(hkl,t)| - |\mathbf{F}^D(hkl)|$, for each time point t. In addition to measured difference amplitudes $\Delta F(hkl,t)$, phases derived from the known dark structure, ϕ^D_{hkl}, are used for calculating difference maps $\Delta\rho(t)$. In order to improve the signal-to-noise ratio of difference maps, several weighting schemes for difference SF amplitudes have been used (Ren et al., 1999; Srajer et al., 2001; Ursby and Bourgeois, 1997). When SF amplitudes are given on the absolute scale, difference electron density in selected regions can be integrated to provide the information about the total number of electrons displaced from or into a particular volume in space as a function of time. The program *Promsk* (Schmidt et al., 2005b), for example, can be used to integrate difference density in the region of interest within a specified mask, generated by supplying atomic coordinates and a radius of integration around the coordinates. Time evolution of such integrated densities can provide important information on formation and decay of structural intermediates (Knapp et al., 2006; Schmidt et al., 2005b).

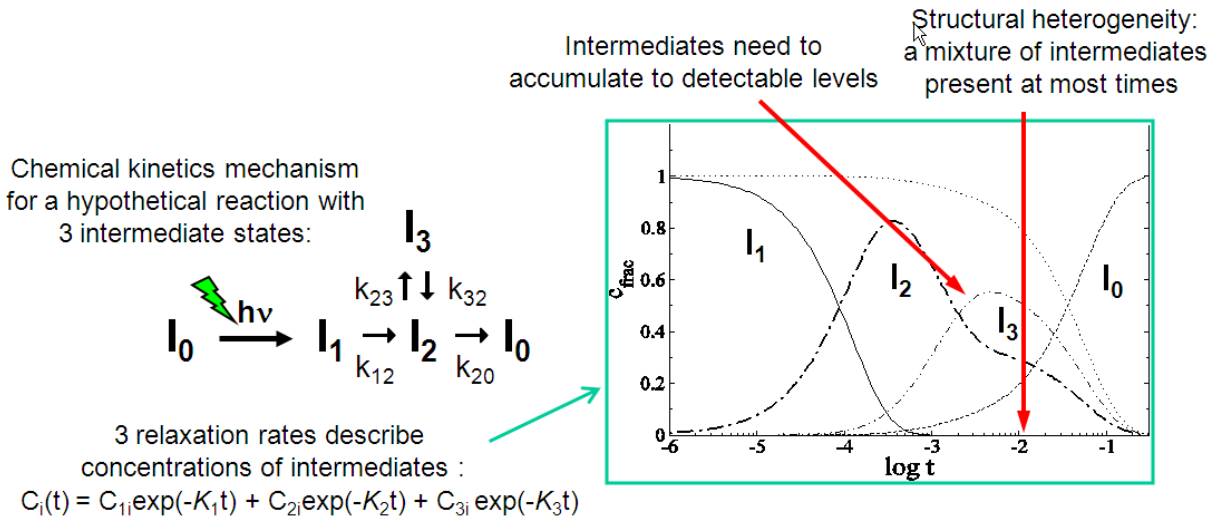

Figure 4 A hypothetical photo-initiated reaction is shown, with three intermediates, I_i (i=1,2,3), and a branched chemical kinetics mechanism. I_0 is the initial state. Three global relaxation rates K_m (m=1,2,3) govern the time dependence of concentrations $C_i(t)$ of all three intermediates. The concentration amplitudes, C_{mi}, as well as these macroscopic, observable rates K_m are functions of four rate coefficients, k_{ij}, which in turn depend on the activation energy barriers between I_i and I_j states. Notice that at most times, a mixture of several states is present. Notice also that, in order for an intermediate state to be detected, rate coefficients need to be such that this intermediate accumulates to a detectable level.

Measured time-dependent difference electron density maps are very likely to represent a mixture of intermediate states at any point in time and as such can be extremely difficult to interpret (see Fig. 4). A method such as Singular Value Decomposition (SVD) is therefore needed to decompose a series of measured mixed-state, time-dependent maps into time-independent maps corresponding to individual structural intermediates.

4.3 Singular Value Decomposition (SVD) SVD in combination with posterior analysis is a powerful method to analyze time-resolved crystallographic data. The number of intermediates, time-independent structures of such intermediates, rates of their formation and decay, and the overall chemical kinetics mechanism of the investigated reaction can be determined (Ihee et al., 2005; Rajagopal et al., 2004a, 2005; Schmidt et al., 2003, 2004). A comprehensive description of these methods is provided in Schmidt et al., 2005a, 2008. In short, SVD is a method of global analysis that is applied to a data matrix composed of time-dependent difference electron density maps, $\Delta\rho(t_i)$, i=1...N, where N is the number of time points. Each map consists of M grid points and constitutes a column in the MxN data matrix. The SVD procedure decomposes such a matrix into N time-independent $\Delta\rho$ maps or left singular vectors (lSV) and corresponding N temporal variations or right singular vectors (rSV). In

essence, SVD separates space and time variables. A singular value associated with each vector-pair represents contribution of the lSVs to the experimental difference maps. In matrix form this decomposition can be described as:

$$A=USV^T$$

where A is a MxN data matrix, U is a MxN matrix containing lSVs (maps) as columns, S is an NxN diagonal matrix containing singular values, and V is a NxN matrix, with rows of the V^T matrix containing rSVs (temporal variations).

The actual difference signal is typically contained in only few significant vectors with large singular values. The remaining singular vectors contain only noise. The SVD analysis therefore acts an effective noise filter. The input series of maps can be approximated by S/N improved maps $\Delta\rho'(t)$, reconstituted from significant singular vectors only.

It is important to note that the lSVs (time-independent difference maps) do not represent the actual difference maps of the intermediate states. They are instead their linear combinations. Similarly, the rSVs (temporal variations) are linear combinations of the actual time courses in the observed time-dependent maps. rSVs therefore provide information about relaxation processes and can be used to determine macroscopic, relaxation rates K_m in the reaction (Fig. 4). From a global fit of all significant rSVs by a sum of exponential functions, the number of relaxation processes and associated rates K_m are determined. The number of relaxation processes is the lower bound on the number of intermediates.

4.4 Structures of Intermediates With the number of relaxations and associated rates determined from the rSVs, the time-independent difference maps, $\Delta\rho_{Ij}$, corresponding to I_j intermediates can be determined (Rajagopal et al., 2004a, 2005; Schmidt et al., 2003, 2005a). At this stage, a candidate reaction mechanism has to be assumed (serial, parallel, branched etc) for the given number of intermediates (Fig. 4). For the assumed mechanism, time dependent concentrations are calculated for each intermediate by solving a system of coupled differential equations. The concentrations are functions of the actual rate coefficients k_{ij} that govern the reaction (Fig. 4). The rSVs are then fit globally by the sum of concentrations. However, scale factors are necessary since the rSVs are linear combinations of the true time courses and this linear transformation is not known. Both the numerical values of the rate coefficients and the scale factors are varied to fit all significant rSVs jointly. A scale factor E_{nj} represents contribution of the j-th intermediate concentration to the n-th rSV (Schmidt et al., 2005a).

The time-independent difference maps of intermediates can then be synthesized as a linear combination of significant ISV maps (Schmidt, 2008; Schmidt et al., 2005a). The scale factors E_{nj} from the rSV fit provide projections of ISVs onto the maps of intermediates: they are contributions of the n-th ISV to the map of the j-th intermediate. The final result is therefore achieved: the mixture of intermediates in the experimental time-dependent difference maps has been separated into the difference maps corresponding to pure intermediates $\Delta\rho_{Ij}$.

Finally, the structures of the reaction intermediates are refined. For this purpose the extrapolated, conventional maps are used instead of difference maps as they are more easily interpretable. For each intermediate, the difference map $\Delta\rho_{Ij}$ obtained from the SVD analysis described above is Fourier transformed and a multiple *f* of the resulting difference structure factor $\mathbf{\Delta F_{Ij}}$ is added to the dark structure factor $\mathbf{F_D}$ by a vector summation. The resulting structure factor is used to calculate the extrapolated map. The factor *f* is determined so that the extrapolated map has no contribution from the dark state. The structures of intermediates are then modeled and refined using these conventional maps. Alternatively, difference refinement against $\mathbf{F_D}$+ $\mathbf{\Delta F_{Ij}}$ can be done (Terwilliger and Berendzen, 1995).

To be acceptable, the maps of the intermediate states have to be interpretable by a valid and unique single atomic structure. If not, the particular mechanism must be discarded and another mechanism needs to be evaluated.

4.5 Analysis of Kinetic Mechanism At this stage of the analysis, several candidate kinetic mechanisms can lead to similar, interpretable maps for the intermediates. Plausible mechanisms are then compared by the posterior analysis. Given the models of the initial state and the intermediates, time-dependent difference electron density maps, $\Delta\rho(t,k)calc$, are calculated for each plausible mechanism, where concentrations of intermediates for this mechanism are determined by the set of rate coefficients k_{ij} (Fig 4). A fitting procedure is used to adjust mechanism-dependent rate coefficients k_{ij} to best match the measured time-dependent difference maps, $\Delta\rho(t)$. Since electron densities, when on absolute scale, are directly proportional to populations of intermediates, the only scaling factor that needs to be adjusted is the extent of reaction initiation and others scaling factors are not needed. The residual maps $\Delta\Delta\rho(t) = \Delta\rho(t) - \Delta\rho(t,k)_{calc}$ are then inspected. If there are no significant residual densities at any time-points, the mechanism is considered compatible with the data since it has generated concentrations that reproduced the observed difference electron densities. However, if significant residual densities $\Delta\Delta\rho(t)$ exist, the mechanism is considered inconsistent. Although this procedure helps to narrow down possibilities, several mechanisms may still be compatible with the data. To further eliminate inconsistent mechanisms, one can

conduct 5D crystallography experiments, where in addition to time, temperature is also a variable parameter (Schmidt et al., 2010).

Software for SVD and posterior analysis SVD4TX/GetMech (Zhao and Schmidt, 2009) is available and can be obtained from the lab of Marius Schmidt, University of Milwaukee

(web-page link: http://www4.uwm.edu/letsci/physics/staff/marius_schmidt.cfm).

5. Future of Time-resolved Macromolecular Crystallography: Major Challenges

Great advances both in hardware and software in the last two decades enabled successful realization of a challenging goal: watching biological molecules in action with 100ps time resolution and at the atomic-resolution detail. Never the less, time-resolved crystallography faces a number of challenges in becoming a tool that is used more broadly in studies of dynamic properties of biological macromolecules. A few of major challenges are briefly described below.

Rapid reaction triggering is essential for the success of time-resolved experiments. However, it is specific to each protein and associated reaction. In other words, there is no universal reaction initiation method that can be applied to all molecules. Rather, one needs to determine a suitable method for each particular case. Light can be readily used for initiating fast reactions given widely available pulsed lasers. However, this method can only be applied to naturally photo-sensitive proteins or when proto-sensitivity can be conferred to otherwise photo-inert proteins. In this second category, caged compounds hold a particular promise (Bourgeois and Royant, 2005; Bourgeois and Weik, 2009; Corrie et al., 1992). They are light-sensitive but biochemically inactivating groups that are chemically attached to substrates, cofactors or catalytically important residues. Activation is then triggered by photo-releasing the active reagent from the caged compound. However, improving characteristics of caged compounds, such as quantum yield and speed of photo-release, is necessary for this method to become more widely applied. Reactions can also be initiated by diffusion of small molecules such as substrates, cofactors, ligands or redox agents into the crystal. This is a simple method in principle but diffusion is a slow process (it could take seconds to days, depending on the crystal size and size of the diffusing molecule). Such method is therefore applicable only to relatively slow reactions. Luckily, many enzyme reactions are slow (<100 s^{-1} turnover rate). In addition, when using small crystals (<10μm), time resolution of <ms might be achievable in some cases (Schmidt, 2013). Also, to facilitate diffusion-based reaction initiation suitable and improved flow cell designs are needed.

Another challenge for time-resolved crystallography is relatively slow readout speed of large-area X-ray detectors. Ideally, one would like to follow a reaction at a number of time delays after a single reaction trigger. However, with current detectors for macromolecular crystallography, only 10-100Hz frame-rate is possible. This limits the time resolution for such “pump once, probe many” style of experiments. Yet, such style is essential for irreversible reactions where each reaction trigger requires a new crystal or fresh crystal volume. One therefore needs to maximize the data collected following each trigger in order to minimize the number of crystals that is required for a complete data set. In case of reversible reactions, repetition of “pump once, probe once” cycles on the same crystal (or crystal volume) is possible, provided sufficient time is allowed between the cycles for the recovery of the initial state. It is therefore possible to collect complete a complete data set (sampling both reciprocal space and time) using one or only a few crystals. In case of irreversible reactions a large number of crystals is necessary for comprehensive time-resolved data. Any means of reducing the required number of crystals, such as a fast-readout detector, is welcome. Also, when a large number of crystals is required, suitable methods for rapid crystal introduction into the X-ray beam (“serial crystallography”) are very important and need to be developed.

Dealing with measured heterogeneous structural states is essential for the analysis of time-resolved data. As mentioned above, at most time points a mixture of state is most likely measured. Both an SVD-based analysis, described above, and a cluster analysis (Kostov and Moffat, 2011; Rajagopal et al., 2004b) have been used in attempts to determine structures of intermediates from measured mixtures of states. More recently, a new method has been proposed (Ren et al., 2013), where structural and occupancy refinement of multiple structures across multiple data sets is conducted, based on real space approach and aided by the SVD analysis. In addition to analysis of time-resolved data, such method can be applied to any standard, static crystallographic data whenever several structures are common to multiple data sets. The new method addresses the limitation of current refinement procedures where multiple but common structures cannot be refined against multiple data sets. The method has been applied to a cryo-trapping experiment where a mixture of three common structures was observed at ten temperatures in the range from 100K to 180K (Yang et al., 2011).

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