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Femtosecond nanocrystallography using X-Ray Lasers for membrane protein structure determination

Petra Fromme¹ and John CH Spence²

¹Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287–1604 USA

²Department of Physics, Arizona State University, Tempe, Arizona 85287 USA

Abstract

The invention of Free Electron X-ray Lasers has opened a new era for membrane protein structure determination with the recent first proof-of-principle of the new concept of femtosecond nanocrystallography. Structure determination is based on thousands of diffraction snapshots that are collected on a fully hydrated stream of nanocrystals. This review provides a summary of the method and describes how femtosecond X-ray crystallography overcomes the radiation damage problem in X-ray crystallography, avoids the need for growth and freezing of large single crystals while offering a new method for direct digital phase determination by making use of the fully coherent nature of the X-ray beam. We briefly review the possibilities for time-resolved crystallography, and the potential for making “molecular movies” of membrane proteins at work.

Introduction

Many of the most important processes in living cells are catalyzed by membrane proteins. They play vital roles in respiration, photosynthesis, cell communication, cell import/export, cell-growth and recognition. A critical step for the elucidation of the complex processes that are catalyzed by membrane proteins is an understanding of their structure, dynamics, and function. Membrane proteins also have an overwhelming impact on human health. Over 30% of human genes code for membrane proteins and 60% of all drug molecules target these proteins. Our knowledge of the processes catalyzed by membrane proteins suffers from a lack of information concerning their structure. Whereas the structures of more than 60,000 soluble proteins have been determined, less than 300 different membrane protein structures are known at present. One rate-limiting step for structure determination of membrane proteins is the growth of large well-ordered single crystals suitable for X-ray analysis, a process which may take decades. Many membrane proteins never provide large well-diffracting crystals, despite years of effort and thousands of optimization screens.

Microcrystals of membrane proteins: Opportunities and Challenges

Structure determination methods based on microcrystals are desired not only because large single crystals are difficult to grow but also because smaller crystals may be better ordered, since they contain less long range disorder [1]. The crystallization of human membrane proteins has proven especially difficult, and just 4 years ago the first structures of a G-

Corresponding authors: Fromme, Petra (pfromme@asu.edu) and Spence, John C H (spence@asu.edu).

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protein coupled receptor were determined [2,3], [4], based on micro-crystals grown in lipid cubic phases [5]. Evidence from line-width analysis in powder protein crystallography [6] suggests that nanocrystals may be more perfect than large crystals, containing fewer of the defects which contribute to mosaicity. Nevertheless, the possibility cannot be excluded that it is the imperfections which limit growth, while nanocrystals also contain a larger proportion of atypical surface molecules. Membrane protein crystals are crystallized in the form of protein-detergent micelles or embedded in lipid phases, which generally leads to a restriction in the number of unit cell contacts. In this respect it is interesting to ask: How small can a membrane protein crystal be? It was shown in a recent powder diffraction study that 100nm protein nano-crystals of the large membrane protein Photosystem I exist,, containing less than 100 unit cells [•• 7]. Submicron crystals cannot be detected by light microscopy, so that high-throughput methods for detecting nanocrystals are highly desired. The new SONICC method does allow fast detection of crystals of submicron size [•• 8] and it has been shown recently that it is superior to all other methods for crystal detection in non-transparent media such as lipid cubic phases [•• 9].

Automated data collection on frozen microcrystals at Synchrotron sources has progressed steadily, while automated screening of protein crystals at microfocus beamlines has also been improved recently [10],[11]. However, the quality of data collected from microcrystals is limited by the inherent problem of X-ray-induced radiation damage, which occurs even if crystals are frozen [• 12]. X-ray induced radiation damage has a strong dependence on the type of protein and the packing of the protein in the crystals. As membrane proteins are crystallized in the form of protein-detergent micelles, bicelles or in lipid cubic phases, they often have fewer crystal contact sites than soluble proteins and are therefore more prone to radiation damage. It is not possible to simply expose a microcrystal for a proportionally longer time since the required X-ray dose causes structural modification before enough scattering can be acquired. This problem, and that of initial crystal quality, set the limits to resolution in conventional crystallography of membrane proteins. The methods developed for freezing the crystals have reduced, but not eliminated, the damage problem [13], [14]. Another hurdle for X-ray structure determination based on microcrystals is that the screening for the optimal cryo-protectant must to be done at synchrotron sources and is a time-consuming process. In summary, difficulties with crystallization, together with resolution limits due to crystal quality and radiation damage have become a serious bottleneck in the structural biology of membrane proteins.

Origins of femtosecond nanocrystallography

Femtosecond X-ray lasers open the way for a new approach to the collection of diffraction data from nanocrystals. The first femtosecond soft-X-ray FEL operating in the world was FLASH at DESY in Germany. The key experiment that showed the potential of the new technology for structure determination was the experiment by Chapman and his colleagues in 2006 [15]. They demonstrated the “diffract-before-destruction” principle [16] for a target etched into a silicon-nitride film. It had also been shown at FLASH that diffraction can be obtained from biological nanoparticles [17]; however, with an X-ray wavelength of $> 80 \text{ \AA}$, FLASH did not allow the determination of molecular structures.

The first hard X-ray free-electron laser in the world, the Linac Coherent Light Source, at SLAC in Stanford, was completed and started operation in 2009 [•• 18]. The short wavelength at LCLS opened the way to structural studies of molecules at atomic resolution, as proposed in 2004 [19]. LCLS provides a peak X-ray flux that is 10^9 higher than a 3rd generation synchrotron pulses [•• 18], [20], [21]. The focussed beam destroys any solid material, producing temperatures that are higher than those inside the sun. It was understood that the data acquisition rates depend on source, scatterer and detector (any of which can

provide a bottleneck), and that methods of hydrated sample delivery in vacuum would be challenging. The “serial crystallography” approach [22] was therefore adopted, with a fresh supply of samples fed continuously across the pulsed beam. Unlike single particles, these samples need not be identical, allowing data from nanocrystals of different sizes to be merged.

The first crystallography experiments at LCLS were performed in December 2009 using one of the largest membrane proteins that has been crystallized to date, Photosystem I, [23]. The results provided an impressive proof-of-principle for this radically new approach [•• 24]. Millions of single crystal diffraction snapshots were collected from a liquid water microjet of fully hydrated nanocrystals of Photosystem I in their crystallization mother liquor at room temperature. As shown in figure 1, the nanocrystals run in single file across the pulsed X-ray beam, producing one snap-shot diffraction pattern per particle, which is read out before the arrival of the next pulse. The repetition rate for the first experiments was 60 Hz, but has been increased in 2011 to 120Hz so that thousands of patterns can be collected in a few hours. The diffraction patterns were indexed, and accurate structure factors determined based on Monte Carlo integration of the partial reflections from more than 10 000 patterns [•• 25]. The resolution of the structure determined at 8.4 Å was solely limited by the wavelength of the X-ray beam and the detector [•• 24]. The large interdisciplinary collaboration which led to these results spans many fields of science, from the new data analysis methods [• 26], the development of new fast-readout detectors [•• 27], X-ray laser development [•• 18], to the design of the CAMP vacuum chamber [•• 27] and the development of the crystal-stream injector.

We now discuss in more detail the nanocrystal delivery system, the new methods of data analysis required as well as new phasing methods, and review the literature on the mechanisms of the destructive-readout process at the atomic level. We end with an outline of the future research opportunities which this exciting development offers for membrane protein structure analysis.

Delivery of the nanocrystals: the injector

Hydration is crucial for biological significance in structural biology, so that the essential problem to be solved for particle-delivery is that of synchronizing the arrival of wet particles into a micron-sized volume, in vacuum, with a repeating X-ray pulse focussed to micron dimensions. A highly collimated “high brightness” single-file particle beam is required. The particles will freeze at about 10^6 °/s in vacuum by evaporative cooling as they travel. The injector must run for days without clogging, and must provide sub-micron positional stability to match the X-ray beam and particle size. The highest possible “hit rate” (mean number of patterns per shot) is required, which depends on particle concentration. For micron-sized protein nanocrystals, neither a gas-phase, gas-focussed injector [17] (with perhaps 25 micron focus and 10% hit rate) or the electro-spray are suitable. The unsynchronized liquid jet used in late 2010 at LCLS [•• 24] provided a hit rate of about 40%, however all the protein flowing between X-ray pulses was not used for data collection. This injector, which is now fitted with a pump laser and an in-vacuum microscope, is described in detail elsewhere [28], [•• 29]. The microscope is essential for alignment purposes, since flashes of light could be seen in the control room on a large screen from the plasma formed as particles are hit and vaporized. A droplet stream of submicron dimensions [• 30] has been produced, well matched to future planned X-ray beam diameters. The design is based on a gas-dynamic virtual nozzle (GDVN), in which the nanocrystal/buffer solution flows along a hollow fiber-optic within a larger concentric capillary tube. The interstitial space is filled with high pressure helium, which emerges at the fiber-optic tip to focus the liquid from the 40 micron internal diameter of the fiber to micron dimensions or less, depending on the

conditions of gas and liquid pressure. In this way a large nozzle, which does not clog, may be used to form a much finer droplet stream. (The gas speeds up the liquid, which -to conserve mass flux- shrinks in diameter). The fiber carrying the liquid can be fed from an HPLC system through a fluidic switching device providing alternate samples [•• 24]. New versions of this injector, including a synchronized “droplet-on-demand” type, and versions based on slower viscous host media, such as the lipid cubic phase or a sucrose solution, are under development. These will drastically reduce the amount of protein needed for the collection of a full data set.

Evaluation of fs crystallography data

The snapshot diffraction patterns contain “partial” Bragg reflections [31] unlike those at a synchrotron, where continuous rotation by a goniometer provides angular integration across the Bragg condition. The indexing and merging of millions of snapshot diffraction patterns therefore involves new challenges involving many terrabytes of data. Nanocrystals as small less than 10 unit cells on a side must be considered, for which the energy-and-momentum-conserving Ewald sphere will generate scattering in non-Bragg directions, complicating the use of auto-indexing software and the use of conventional mosaicity, energy-spread and beam-divergence corrections. A new “Monte-Carlo” approach has therefore been developed, [•26] in which it is first assumed that all nanocrystal orientations occur with equal frequency. Then the intensity within a spherical volume in reciprocal space around the same reflection from different nanocrystals is summed to give a quantity proportional to a squared structure factor, under the assumption that the nanocrystal is smaller than one mosaic block, and smaller than the X-ray beam. The results of this approach have been compared with data from large crystals of Photosystem I obtained at a synchrotron, and gave R-factors of about 20% for the data collected at 1.9 kV at the LCLS in 2009 [•• 24]. The LCLS data of Photosystem I crystals was phased by molecular replacement. This data was solely limited to about 8Å resolution by the X-ray wavelength. For a nanocrystal, the scattered intensity around a Bragg condition takes the form of a “shape transform”, whose width (inversely proportional to the crystal size) differs in each pattern (see Fig 2). It is important to show that this summation converges to structure factors for a range of crystal sizes. For details of the method as applied to the PSI data, see [•• 25] where the effects of particle size distribution and flow alignment are discussed. The energy of the LCLS has recently been increased to 9 kV, which has allowed data from nanocrystals to be recorded at higher resolution using this liquid stream flow-injector for sample delivery, indicating that the method of femtosecond nanocrystallography extends to near atomic resolution.

New solutions to the phase problem

The use of “oversampling”, or scattering between Bragg peaks, to solve the phase problem has a long history going back to early work by Perutz [32] and Sayre [33]. A practical iterative solution to the non-crystallographic phase problem was first described by Feinup, whose methods were used to phase the first “lensless” X-ray image of a non-periodic object [34]. From that work has grown the field of X-ray diffractive imaging (CXDI), using “continuous” X-rays to provide three-dimensional, tomographic, lensless images at about 10nm resolution [35], [36], (see [37] for a review). All these studies provided the groundwork for solutions of the phase problem for XFEL scattering from non-periodic (or nanocrystalline) particles, where scattering is not confined to Bragg peaks. For the PSI nanocrystals, the particle sizes varied between about 0.1 microns and 2 microns, with a corresponding variation in the width of the shape transforms around Bragg peaks. Since the molecular transform modulating the Bragg peaks is the same for every nanocrystal (because each one has the same unit cell contents), whereas the shape transforms around the Braggs (which provide the size distribution) vary, these effects may be separated. The size

distribution may thus be “divided out”, to isolate the molecular transform, and allow it to be phased by iterative methods [•• 38]. Phasing by more conventional methods (such as anomalous diffraction (MAD) and isomorphous replacement) may also be possible, and theoretical work on the computation of time-dependent absorption coefficients for MAD is under way.

Overcoming the radiation-damage problem in X-ray crystallography

The idea that radiation damage may be avoided by using very brief pulses of radiation is evident in the basic physics of photoelectron spectroscopy. The suggestion to use this effect in structural biology was made by Solem and Baldwin [39] for X-ray holography, and by Neutze et al [40]. This “diffract-and-destroy” principle was first demonstrated experimentally using soft X-rays for a macroscopic object in 2006 [15]. The underlying theory for this phenomena has been treated by several groups [41], [• 42], [• 43]. For sub-10 fs pulses (which contain about 1E^{13} photons at the LCLS), elastic scattering terminates before the damaging photoelectron cascade gets under way. A comparison of merged diffraction data for several pulse durations is shown in [•• 24] indicating that high resolution (sub nm) detail begins to be lost for pulse durations longer than about 40 fs, when significant elastic diffraction from the disturbed charge density can occur. The detailed form of this “spot fading” is being simulated using both molecular dynamics and plasma models in much current research. For nanocrystals smaller than the photoelectron mean free path, photoelectrons escape, and a Coulomb explosion results, while for large nanocrystals the photoelectrons thermalize, dumping so much heat energy into the crystal that it vaporizes at very high temperature.

In summary, the method of fs crystallography may overcome the problem of X-ray damage in crystallography by using a very brief pulse of X-rays with a duration of a few tens of femtoseconds (shorter than the period for atomic vibration). The detection of elastically scattered photons terminates before radiation damage, due to ejected photoelectrons, develops, so that damage is greatly reduced despite the use of a very high dose.

Conclusions

Femtosecond nanocrystallography provides the exciting potential for structure determination of difficult-to-crystallize proteins. The method uses nanocrystals, that are too small to be seen under an optical microscope, in their native crystallization-buffer solution, for structure analysis. This allows structure determination of proteins which may only produce showers of nanocrystals that resemble amorphous precipitate. The question now arises as to how many nanocrystals of membrane proteins may already be out there in labs all over the world? The method of SONICC [8,44] allows the detection of nanocrystals, and we predict that systematic screens with SONICC will reveal thousands of nanocrystal of different membrane proteins. Our group routinely uses reversible precipitation/crystallization as the last purification step for our membrane proteins [45]. A small test sample from our lab of “reversible precipitate” of membrane proteins of unknown structure characterized with SONICC (in collaboration with G. Simpson) indicated that up to 30% of the precipitates consisted of nanocrystals. We expect that developments of the injector will further drastically reduce the amount of sample needed, and we predict that in the next few years full fs X-ray data sets of nanocrystals consisting of several hundred thousand patterns will be collected from 10ul of a crystal suspension at a protein concentrations of about 1mg/ml. Developments are already underway towards high throughput data collection where the nanocrystal samples are injected into the jet using a modified HPLC autosampler injection system. Nanocrystal samples are currently being injected into the jet using a modified HPLC autosampler injection system. The number of patterns that must be collected for a full data

set depends on the method of data evaluation. Currently, structure factors are determined using the Monte Carlo integration of all partial reflections with the same Miller indices over the full data set. Reconstruction of the peak profiles based on estimates of partiality of the reflections may further drastically reduce the amount of data needed for all but the smallest nanocrystals.

Nanocrystallography also suggests a new solution to the phase problem. Because the X-ray laser beam is fully spatially coherent, new diffraction effects are seen from nanocrystals, interference fringes running between Bragg beams (Fig 2), which provide a new solution to the phase problem. Many of these developments, including the higher repetition rates of new XFELs, will require further developments in detector technology, in readout speed, dynamic range and pixel density/detector area. The new CXI beamline at LCLS, which started operation in February 2011 uses a new type of detector developed by researchers at Cornell University, which is based on a modular design with high dynamic range [46].

A major limitation of conventional X-ray structure analysis is that it shows a static picture of the molecule. Pump-probe femtosecond nanocrystallography experiments open the possibility of collecting molecular “snapshots” of different conformational states of membrane proteins, with the final goal of obtaining molecular movies of molecules “at work”. A spectroscopic pump-probe experimental setup has been established at the AMO beamline of LCLS [• 47]. In June 2010 we conducted the first pump probe experiments on photoactive membrane protein crystals at LCLS, using a pump laser to excite nanocrystals in the liquid jet, before taking their X-ray snapshot, and the results indicate that large conformational changes occur in the crystals upon light excitation (publication in preparation). Time-resolved X-ray structure analysis using fs crystallography is especially interesting for the membrane protein complexes involved in photosynthesis, and opens new avenues to unravel the molecular mechanism of water splitting in Photosystem II. These experiments will be conducted at LCLS by two research teams in August and September 2011.

In addition to the use of optical triggers to initiate a reaction, this experimental arrangement also suggests the possibility of “snapshot chemistry”, in which a substrate fluid is used to drive, for example, an enzyme through its catalytic cycle. Using two fluids mixing at the tip of the liquid jet it will be possible to record snap-shot diffraction patterns at different points along the liquid stream as the reaction proceeds. We also envision that proteins locked in one conformation by a photo-labile substrate analog can be activated by light, upon which the conformational change is followed by fs nanocrystallography. These possibilities suggest an exciting future for time-resolved imaging using XFEL sources in future,

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Research highlights

- Femtosecond nanocrystallography is based on Free Electron X-ray Lasers
- The diffract-before-destroy- principle overcomes damage problem in crystallography
- Nanocrystals are delivered at RT in stream by new type of gas-focusing injector
- Coherently illuminated nanocrystals provide interference effects solving the phase problem
- The method opens new avenues for time resolved studies showing proteins at work.

Figure 1.

Principle of the experimental setup used for femtosecond crystallography.. The stream of crystals is provided by the injector with the gas focusing nozzle. The femtosecond X-ray beam intersect with the continuous crystal stream; diffraction patterns are recorded with the frequency of the Free Electron Laser at LCLS (60 or 120 Hz) using either a one or two CCD detector setup. In the two detector setup, which was used in the CAMP chamber at the AMO beamline at LCLS for the first fs crystallography experiments reported in [•• 24], the high order reflections are detected on the front detector. The back detector, which records the low order reflections, allows the resolution of the shape transforms of the reflections. The figure has been modified after [•• 24].

Figure 2.

Example of a diffraction pattern from photosystem I which shows the shape transforms. Note the fringes between the Bragg peaks which indicate the number of unit cells in that direction. Thus the evaluation of the shape transforms can be used to determine the size of the crystals [•• 24], and for phase determination [•• 38]. This Figure has been modified after [•• 24].

Figure 3.

Electron density map of Photosystem I at 8 Å resolution, derived by fs crystallography. The phases were obtained using molecular replacement as described in [• 24]. The section of the electron density in this picture shows transmembrane helices of the large PSI subunits PsaA, PsaB as well as helices of PsaK and PsaF. This Figure has been modified after [• 25].