Synchrotron radiation: micrometer-sized x-ray beams as fine tools for macromolecular crystallography

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Structural data play a central role in understanding biological function at the molecular level. At present, the majority of high-resolution structural data about biological macromolecules and their complexes originates from crystallography. In crystal structure determination, the major hurdle to overcome is the production of crystals of sufficient size and quality. High-flux x-ray beams with diameters of a few micrometers or less help to alleviate this problem as small beams allow the use of small crystals or scanning of large crystals for regions of acceptable diffraction. Using sophisticated x-ray optics and mechanics with submicrometer precision, Riek et al. [*Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 64, 158–166 (2008)], have recently demonstrated that an x-ray beam of 1 μm can be used to determine the crystal structure of a protein to a resolution of 1.5 Å. The smallest volume from which usable diffraction data were collected amounted to 20 μm^3 , corresponding to not more than 2×10^8 unit cells. In a diffraction volume of micrometer dimensions, radiation damage is expected to be reduced with respect to large volumes as a significant fraction of the photoelectrons produced by the incident radiation escapes from the diffracting volume before dissipating their energy. The possibility to make use of small and/or inhomogeneous crystals in combination with a possible reduction in radiation damage due to size effects has the potential to make many more systems amenable to crystal structure analysis. [DOI: 10.2976/1.2982661]

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With modern methods of structural biology the molecular components of living systems can be imaged in increasingly fine detail. Small-angle x-ray scattering (Petoukhov and Svergun, 2007) and electron microscopy (Orlova and Saibil, 2004) allow the determination of the shapes of molecules and their complexes, while nuclear magnetic resonance spectroscopy (Kay, 2005) and x-ray crystallography provide sufficiently high resolution for defining the positions of individual atoms.

Since the first determination of a protein structure by x-ray crystallography in the 50s of the last century (Kendrew et al., 1958), macromolecular crystallography has been used to image the atomic structures of several tens of thousands of biological macromolecules (<http://www.pdb.org>), and for many cases, the method can now be applied in a routine fashion.

Nevertheless, its limits are constantly being pushed by the determination of ever more complex systems such as the 70S ribosome in complex with mRNA and tRNA (Selmer et al., 2006) comprising more than 145,000 nonhydrogen atoms or various functional states of RNA polymerase II, a complex of ten different proteins (Kornberg, 2007).

Many factors, including the use of recombinant technologies for sample production, cryogenic cooling of crystals, availability of more sensitive detectors, and progress in computer hard- and software, have paved the way for progress in macromolecular crystallography. One enormous leap in the capabilities of the method was caused by the realization that synchrotron radiation could be used for diffraction experiments in 1970 (Rosenbaum et al., 1971). Synchrotrons provide very high photon

fluxes with a wide spectrum of wavelengths and, with appropriate optics, well-collimated beams of x-rays that are ideal for diffraction studies can be produced (<http://www.lightsources.org>). Initially, synchrotron radiation was used in “parasitic mode”; meanwhile, storage rings exclusively dedicated to the production of highly brilliant synchrotron radiation such as the PETRA III storage ring in Hamburg (<http://petra3.desy.de/>) are available.

At present, the major bottleneck in macromolecular crystallography is the preparation of large crystals of sufficient quality to achieve diffraction to high resolution. Although the probability of obtaining crystals for a given system has increased over recent years due to the adoption of high-throughput technologies in crystallization (Berry *et al.*, 2006; Mueller *et al.*, 2007) allowing the efficient screening of many conditions with small amounts of sample, the crystals obtained are often small and/or inhomogeneous in their diffraction properties.

When high-quality crystals of sufficient size cannot be made, very small x-ray beams are an invaluable tool as with such beams the quality criteria for appropriate samples become less stringent. In the following, we discuss a recent report by Riek *et al.* describing diffraction experiments on protein crystals with an x-ray beam with a diameter of only 1 μm —the smallest beam used so far for a successful structure determination of a protein structure from a crystal (Moukhametzianov *et al.*, 2008).

MACROMOLECULAR CRYSTALLOGRAPHY WITH SMALL BEAMS

In a standard crystallographic experiment, one aims to match the size of the x-ray beam to the size of the crystal. When the beam is smaller than the crystal, part of the available material is not used for the experiment, thus unnecessarily decreasing the signal. When the beam is larger than the crystal the excess radiation will interact with noncrystalline material, resulting in increased background. For beam dimensions and crystal dimensions between 0.1 and 0.5 mm, the matching between sample and beam size is well feasible by a combination of beam focusing optics and apertures confining the beam.

For achieving beam sizes below 0.1 mm, in principle, one could “slit down” the photon beam with smaller and smaller apertures. However, in practice, the fraction of intercepted photons is usually too large to be acceptable. To maintain high photon fluxes in beams of small dimensions, collimating schemes using small apertures need to be combined with highly precise and stable focusing optics. First, “microcrystallographic” experiments on macromolecular samples with focused beams of less than 20 μm in diameter were reported in 1998 (Cusack *et al.*, 1998). In recent years, several beamlines in the world succeeded in providing high-flux beams with dimensions between 5 and 20 μm . These beams have been instrumental in collecting data from a

number of systems, where otherwise no structural information to high resolution could have been obtained. Examples include the use of a 5 μm beam for the determination of the structure of a rhodopsin mutant from needle-shaped crystals of 5 μm in diameter (Standfuss *et al.*, 2007) and of an amyloid fiber structure using diffraction from crystals with a $2 \times 2 \mu\text{m}^2$ cross section (Sawaya *et al.*, 2007), and the determination of the structure of cypovirus polyhedra from crystals of $2 \times 2 \times 2 \mu\text{m}^3$ (Coulibaly *et al.*, 2007).

Small beams are not only necessary for optimum data collection from small crystals, they can also be used to selectively expose parts of large crystals. “Rastering” crystals is often key to the collection of high-quality data, as the quality of crystals can vary substantially in terms of crystalline order (Sanishvili *et al.*, 2008), or macroscopic defects such as cracks, which need to be avoided.

Also, even for a large and perfect crystal, sequential irradiation of different parts of the crystal can be useful, as by moving from an exposed part of a crystal to a “fresh” part of a crystal the effects of radiation damage on data quality can be minimized (Fig. 1) (Brodersen *et al.*, 2003; Riek *et al.*, 2005; Sawaya *et al.*, 2007).

With their experiment, Riek *et al.* (Moukhametzianov *et al.*, 2008) have now gone substantially below the 5 μm limit for x-ray beam sizes. With a 1 μm beam, they collected diffraction data from two differently sized crystals of Xylanase II, a well-known test protein for diffraction experiments with a molecular weight of ~ 22 kDa. In their study, the smallest diffraction volume giving rise to an interpretable diffraction pattern consisted of a channel with a $1 \mu\text{m} \times 1 \mu\text{m}$ cross section through a 20 μm thick part of a crystal, i.e., not more than $\sim 20 \mu\text{m}^3$ of crystalline material. Given the dimensions of the crystallographic unit cell of Xylanase II, this volume corresponds to a total of ca. 2×10^8 unit cells exposed to x rays.

SMALL BEAMS AND RADIATION DAMAGE

The many photons available in beams of synchrotron radiation hold tremendous benefits for diffraction experiments. However, there is also a major drawback of using high photon fluxes in that radiation damage usually sets in very quickly even on cryogenically cooled crystals (Owen *et al.*, 2006). Piecing together a complete diffraction data set by collecting partial data sets on different crystals, each measured until radiation damage reaches unacceptable levels, has been successful in some cases, a prominent example being the determination of the crystal structure of the 30S subunit of the ribosome (Brodersen *et al.*, 2003). However, this approach is not generally applicable as it is extremely tedious and different crystals of the same system, in many cases, display slight structural differences (“non-isomorphism”) that lead to differences in the diffraction data that cannot be handled properly with currently available structure determination software.

Figure 1. Use of a small x-ray beam to selectively expose different parts of a crystal. After a set of x-ray exposures in region “A” of the crystal has been taken, the quality of the diffraction data harvested from the diffraction pattern deteriorates (see decrease in resolution in the inset) and the crystal is translated such that a fresh region “B” will be exposed. Once region “B” is exhausted, repeated translations will move regions “C” and “D” and so on into the beam. With this strategy, the entire volume of the crystal can be used optimally for diffraction data collection while radiation damage is limited to controllable levels within the groups of exposures. Ideally, the exposure levels are adjusted such that complete diffraction data can be collected from one single crystal in order to avoid problems arising from merging data from multiple, potentially non-isomorphous, crystals. While rastering a crystal, regions of low crystal quality such as “C” here (see inset) can be excluded from subsequent analysis. It should be noted that in a typical experiment, the crystal is also rotated during the exposures. This requires mechanics with accuracy commensurate with the size of the beam and the crystal.

The current commonly made compromise is therefore to use different parts of the same crystal for data collection (Brodersen *et al.*, 2003) by translating the crystal after a set of exposures such that a fresh part is irradiated during the next set of exposures (Fig. 1). Obviously, for this strategy, the beam profile has to be significantly smaller than the crystal. This strategy works particularly well for needle-shaped crystals as, for this case, with a circular beam of the size approximately matching the “diameter” of the crystal, in every position optimum use is made of all the crystalline material.

Riekkel *et al.* have varied this approach by using an off-centered rotation (Moukhametzianov *et al.*, 2008). With this setup the channel illuminated by a thin beam will contain mostly fresh material in each exposure without any translational repositioning of the crystal. From the x-ray dose used per single exposure, significant radiation damage would have been expected in this experiment. However, interestingly, Riekkel *et al.* have not found any evidence of such damage.

To explain this observation, one can draw upon recent theoretical work by Nave *et al.* on the behavior of radiation damage in very thin crystals with the smallest dimensions on the order of micrometers (Nave and Hill, 2005; Cowan and Nave, 2008). For such crystals, Nave *et al.* predicted that, because photoelectrons produced by the interaction of the

x rays with the crystalline organic matter deposit their energy in many events along a path of several micrometers length, a thin sample should proportionally suffer less damage per photoelectron produced than a thick sample, as a substantial part of the photoelectron energy will be dissipated after the photoelectron has actually left the crystal. Riekkel *et al.* claim that a similar phenomenon occurs also inside the bulk of a crystal when a small beam traverses a large crystal: here, a significant portion of the energy of the photoelectrons will be deposited outside the illuminated volume after the photoelectron has escaped from the part of the crystal used for actually producing diffraction (Fig. 2). This is a very interesting hypothesis that should be tested in further experiments.

WILL SUBMICRON BEAMS BE USEFUL?

The beneficial effect of photoelectron escape from the diffraction volume during a single exposure should increase as the beam size decreases (Moukhametzianov *et al.*, 2008). Also, with a smaller beam, finer scans for optimally diffracting parts of a crystal become possible. Naturally, the question then arises whether one should use beams of x rays still smaller than 1 μm . As x-ray optics with foci down to 30 nm have been described (Kang *et al.*, 2006), such beams can in fact be produced.

Figure 2. Photoelectron escape. Photoelectrons produced by the interaction of X rays with the material of the illuminated volume (shown in red) dissipate their energy in multiple collisions (trajectories are depicted as gray lines originating from dots) and thereby cause radiation damage. For large diffraction volumes (left), most of the damaging collisions will occur inside the illuminated volume, causing an alteration in the sample being measured. For diffraction volumes with dimensions smaller than the typical trajectory length of photoelectrons (right), many of the photoelectrons will escape from this volume and dissipate their energy outside the illuminated volume, leading to proportionally less radiation damage in the volume of interest.

However, if one was to use a beam with a cross section $100 \times 100 \text{ nm}^2$ instead of $1 \times 1 \mu\text{m}^2$, there would be 100 times less material contributing to the diffraction, resulting in a correspondingly smaller signal to be observed.

Furthermore, if one considers a macromolecular crystal with a unit cell with linear dimension of $200 \text{ \AA}$, only 5 unit cells will correspond to the linear dimension of a 100 nm beam. Under such circumstances, where only few unit cells are exposed to the beam, the reflections will broaden (Scherrer, 1918), and when even smaller beams are used will eventually merge into one continuous transform for a single object (Sayre, 2008). This situation is certainly interesting from an experimental point of view, as one could in principle apply the technique of scanning diffraction microscopy (Thibault *et al.*, 2008) to crystals as suggested by Chapman (Chapman, 2008).

TECHNOLOGICAL IMPLICATIONS

For determining a crystal structure with a $1 \mu\text{m}$ beam, not only is an x-ray beam of this size necessary, but it is also essential that the mechanical devices for positioning and moving a sample function with submicron precision. Riekel *et al.* successfully employed an air-bearing rotation axis in combination with a piezo-micromanipulator for this purpose. Here, it should be noted that in the experiments presented, a small beam was used to investigate a large crystal. If one was to use a $1 \mu\text{m}$ beam to irradiate a sample of matching size, the requirements for alignment between beam and sample become even more stringent, i.e., for keeping the amount of diffracting material exposed to the beam constant

within 10% requires mechanical stability to better than 100 nm . Achieving such levels of stability requires massive efforts in instrumentation technologies.

To make full use of small beams, sample handling must not only be extremely precise but should be robotized as far as possible. One reason for using robotics is that, with increasing precision, the equipment becomes much more sensitive, rendering manual operation impossible. Moreover, with small high-flux beams, in principle, very many samples can be scanned in many positions on very short time scales—here, automation is the only practical solution. Automatic sample changers (Cohen *et al.*, 2002; Cipriani *et al.*, 2006), in combination with fully motorized and remotely controlled goniostats, are an important step in this direction. For making the best use of all available crystals potentially beating both radiation damage and non-isomorphism by combining many compatible partial data sets, sophisticated software for bookkeeping and planning for optimum experiments on many samples is needed (Bourenkov and Popov, 2006).

CONCLUSION

Structural biologists are moving towards larger and more complex systems for which the production of large and well-ordered crystals is becoming increasingly difficult. However, when a crystal of a macromolecule is formed, by its very nature it is chemically pure and all molecules are locked into the same conformation. This is an excellent starting point for obtaining a signal to high resolution by averaging across a large ensemble of identical objects. At present, x-ray crystallography is the most appropriate experimental method to do so.

Modern synchrotrons offer well-collimated, highly intense x-ray beams that are extremely suitable for diffraction experiments. Riekel *et al.* have shown that a beam of 1 micrometer diameter can be produced and used for diffraction experiments on small volumes of crystalline material. The technology presented is important as it makes many more systems amenable to crystallographic investigation. In addition, there are indications that radiation damage is reduced when diffraction volumes of micrometer-sized dimensions are used—this phenomenon needs to be better understood and the possible implications of size effects in crystallographic experiments need to be validated experimentally.

In terms of hardware for crystallography, combination of high-performance x-ray optics and nanometer sample positioning mechanics with low-background sample environments and high-throughput sample screening robotics will harness the potential of modern SR sources for collecting data from the “good parts of the best needles in the haystack.” This will pave the way for extracting high-resolution structural information even from small or imperfect crystals of complex macromolecular objects.

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