Illuminating crystallography

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Synchrotron radiation is an established tool in macromolecular crystallograpghy. Its high intensity and tunability play crucial roles in many structural analysis.

In the early days, the low intensity of conventional radiation sources and the absence of efficient detectors for recording the full three-dimensional diffraction patterns posed severe problems for those wishing to collect diffraction data from protein crystals. Synchrotron radiation began to play a role in data collection for proteins only in the late 1970s, building on pioneering work a few years before (see the article by Kenneth Holmes in this supplement). Yet, synchrotron radiation has already had an enormous impact on the field of crystallography[1,2].

The problems of carrying out X-ray crystallographic analyses of macromolecules arise from the nature of the molecules themselves and of the way they pack into crystals. The fact that they are large means the repeating unit of the crystal, the unit cell, is large. Hence there are many weak reflections to be recorded. This leads to a low signal-to-noise ratio, which can be alleviated by a high intensity source and efficient detector. Another complicating factor is that the molecules are held together in the crystal by weak interactions between the proteins, while a large fraction of the crystal, on average about 50%, is disordered solvent. Thus the molecules in the crystals display substantial disorder, which means that the high resolution data are weak or indeed absent.

Advantages of SR

Several properties of synchrotron radiation make it attractive for studying proteins. Its high intensity makes the weak diffraction from macromolecules possible to record on a tractable time scale. Highly parallel rays that can be finely collimated to produce a focused beam, which are provided by synchrotron radiation, are essential for large unit cells with closely spaced reflections. An example of the resolution possible on a source such as the European Synchrotron Radiation Facility (ESRF) is shown in Fig. 1.

Unlike the radiation from conventional X-ray sources, synchrotron radiation provides a broad spectrum and the monochromatic wavelength is therefore tuneable. First and foremost this allows the use of short wavelengths, avoiding the effects of absorption and associated radiation damage, which can be severe with CuKα radiation at 1.54 Å. The spectral region of choice is 0.8–1.0 Å for most single wavelength experiments, as this minimizes the effects of absorption while retaining a good diffraction intensity. The reduction of the effects of secondary radiation damage are especially important for experiments at room temperature.

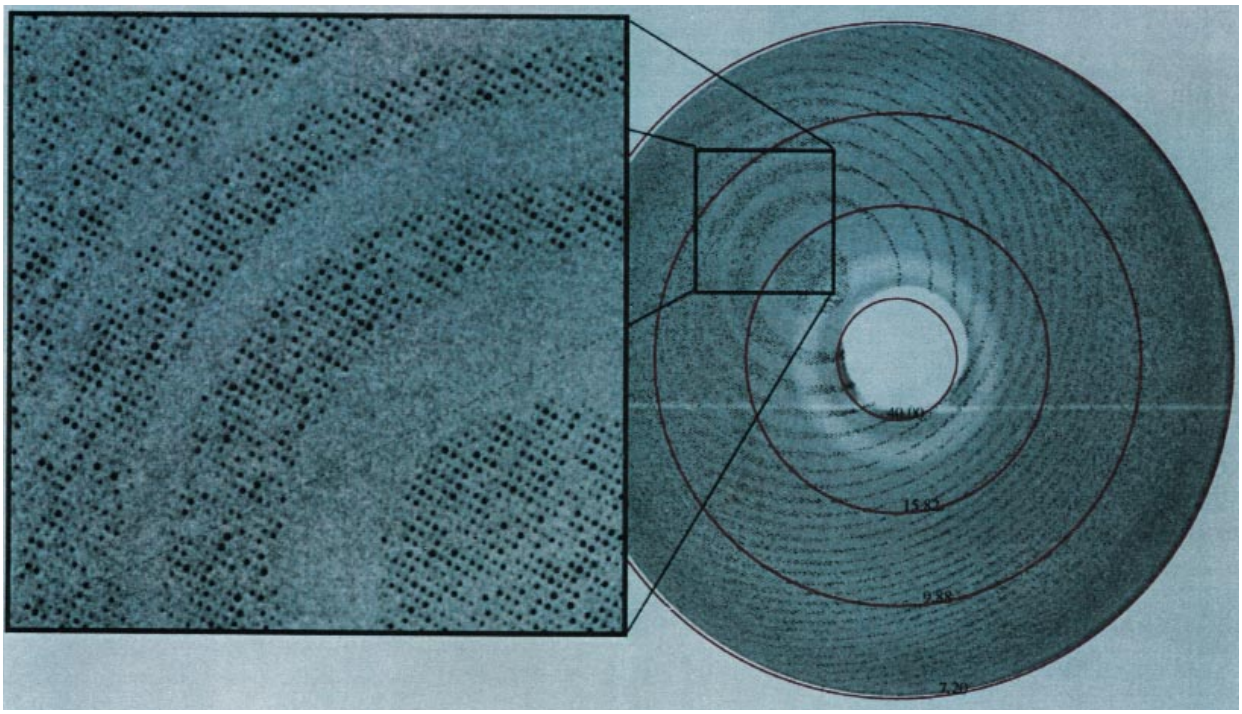

Fig. 1 A diffraction pattern from a crystal of the core particle of blue-tongue virus, BTV 10, recorded on the high brilliance beamline ID2 at the ESRF. The cell dimensions of the crystal are a = b = 1,120 Å, c = 1,592 Å. Data have been processed to 7.0 Å, the oscillation range was 0.3° with 10–20 s exposures. The current data set was collected from over 50 crystals. The detector was a 30 cm Mar-Research image plate system. The photograph was provided courtesy of D. Stuart.

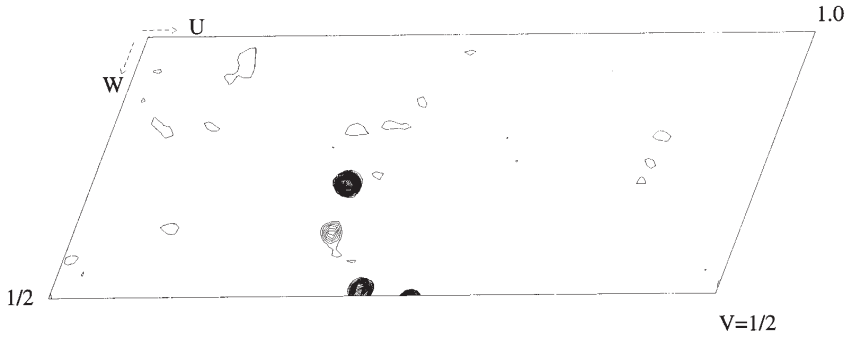

Fig. 2 Harker section v = 1/2 of the anomalous difference Patterson synthesis for a mannanase platinum derivative at 1.65 Å resolution. The peak is ~20 σ. A total of 180° of data were recorded in just 35 min on station 9.6 at the SRS, Daresbury Laboratory using an ADSC (San Diego) CCD device during a test period. Thanks are due to Miroslav Papiz (SRS) who helped with this work. The figure was provided by Gideon Davies. This emphasises the power of such detectors even on second generation sources.

The tunability of synchrotron radiation can be further exploited by using X-ray absorption edges where the anomalous scattering effects provide experimental phase information. Many experiments involve a single wavelength with optimization of the scattering at the edge of an isomorphous heavy atom derivative. There have been great advances in techniques for recording multiwavelength anomalous dispersion (MAD) data on metalloproteins, and more importantly on crystals of proteins or nucleic acids into which selenomethionine or bromine respectively, has been incorporated (see the article by Craig Ogata).

Synchrotron radiation can be applied as 'white' radiation, where the whole spectrum, or a substantial range of the spectrum, is used simultaneously to illuminate the crystal. This allows very short exposures, in principle capable of determining the structures of short lived states in the crystal through time-resolved studies (see the article by Keith Moffat). Severe problems arise here in the conflict between Bragg's and Boltzmann's laws: it is difficult to find effective triggers for enzyme catalysis, or other changes in proteins, that allow a large population of molecules to begin the reaction simultaneously. These are presently restricted to lasers with chromogenic systems. However even if an effective trigger is found, all the molecules in the crystal will not, in general, proceed through the reaction profile in an orderly manner. With the available techniques, time resolved studies are limited to a few systems. The next step in time resolved studies is the use of a single bunch of particles in the storage ring for recording an X-ray image, to shorten the time window of the state studied to that of the bunch length, often at the 100 picosecond level. However, this will pose further problems because of the current limits of detectors and sample stability .

Detectors

The use of synchrotron radiation for protein crystallography requires efficient detection of the diffracted X-ray pattern. Single counting diffractometers are clearly inappropriate. The first routine protein crystallography experiments used film as a detector. This was reasonable in terms of spatial resolution, but very limited in dynamic range and suffered from significant noise level in the background as a result of the chemical processes involved. Some attempts were made to use television or wire chamber gas-filled detectors for protein crystallography, but these met with limited success for a variety of reasons such as saturation of the count ratio and spatial resolution.

At the end of the 1980s, the situation was transformed rapidly by the development of imaging plate detectors. The first automated on-line read-out system for routine work on a beamline was developed by Jules Hendrix and Arno Lentfer at European Molecular Biology Laboratory (EMBL) Hamburg, Germany in 1990 (building on experience from groups in Japan). Within a few years, imaging plates became the synchrotron radiation detector of choice (with a commercial system installed on most beamlines) and have remained so for the last decade. With the new imaging plate technology, for the first time the user could record full diffraction images directly on computer disk. Within a short space of time data reduction software packages such as MOSFLM[3], DENZO[4] and XDS[5] had advanced such that images could be analyzed on-site, and users could return to their home laboratory with a processed and analyzed set of X-ray intensities. Data collection was thus revolutionized, and the throughput of existing beamlines advanced apace.

We are now in the middle of the next step forward. Imaging plates suffer from a major limitation. Reading out the image took a substantial time, originally two to four minutes, now reduced to 20–40 s for the latest systems. Nevertheless, this step became rate limiting on the beamlines at ESRF and APS where exposure times are down to the order of seconds (or sometimes less). Charge coupled devices (CCDs) have overcome this problem. After a long gestation period when they were being developed by a number of academic and commercial groups[6], they have at last reached maturity. The problems with CCDs were always one of size — they were too small — and, related to this, expense. The new devices are already installed on beamlines at a number of sites (for more information, see the article by Robert Sweet). The cost per device lies roughly in the range $250,000–$500,000, a small price to pay for the expected gain in effective beamline usage. The quality of data potentially available at high speed is shown in Fig. 2.

Cryogenic freezing

The development of cryogenic freezing techniques has been critical to recent advances in exploiting the potential of synchrotron radiation. Already as long as ten years ago, the stronger beamlines were causing substantial radiation damage to the specimens, even with the use of wavelengths below 1 Å and despite the rapid data collection times. In the early 1990s there was much debate about whether protein crystals were in general amenable to flash freezing, but happily flash freezing is now an established technique[7,8] and cryogenic facilities are (or should be) available on all beamlines. Today probably more than three quarters of synchrotron radiation experiments use frozen samples, with little increase in

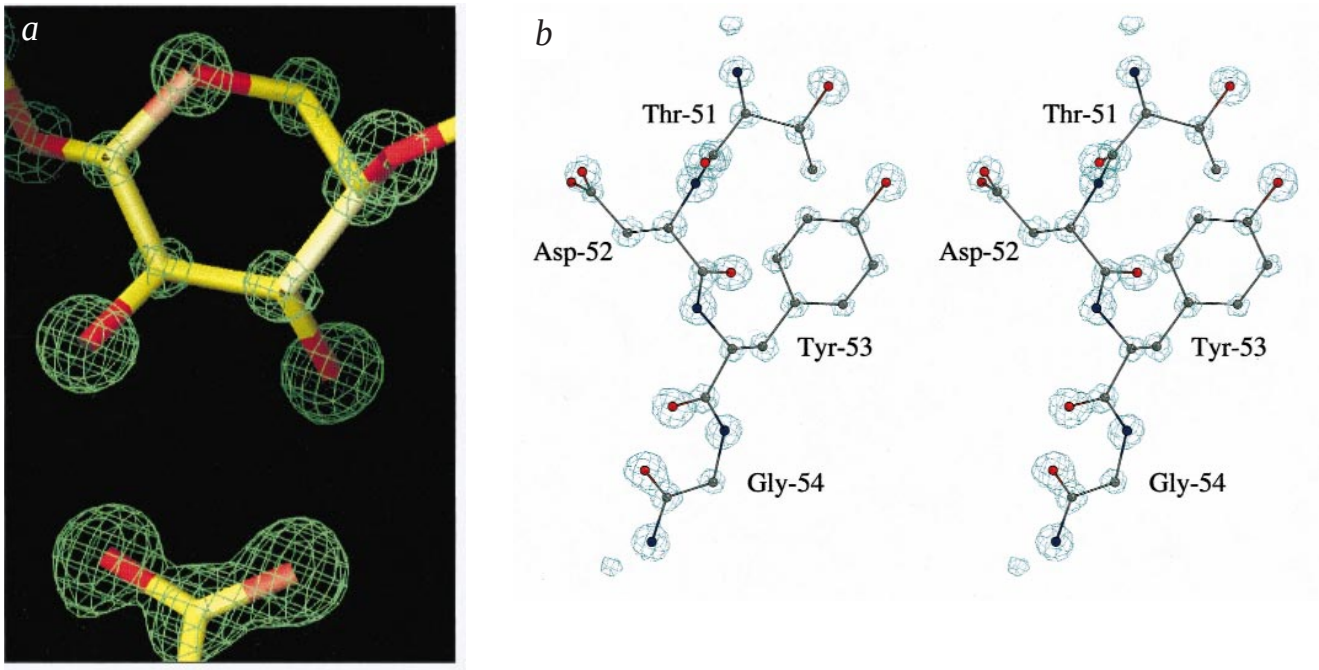

Fig. 3 ***a***, The structure of a complex between cellulase CelA from *Clostridium thermocellum* and cellopentaose has been refined to 0.94 Å resolution (D.M.A. Guerin & P. Alzari, manuscript in preparation). Extended stacking interactions on both sides of the scissile glycosidic linkage and several hydrogen bonding contacts promote a significant distortion of the sugar ring at subsite -1 which displays the skewed conformation shown here. The data were recorded on beamline BW7B at EMBL Hamburg, Germany. Figure kindly provided by Pedro Alzari. ***b***, Stereo view of a representative region of the 0.97 Å resolution $3F_o$ - $2F_c$ Fourier map contoured at 4σ (2.86 e Å^{-3}) for tetragonal hen egg white lysozyme. Data were collected on the SBC undulator beamline at the Advanced Photon Source (APS). The flux was 1.71 x 10^{12} photons s^{-1} 100 mA^{-1}. The crystal size was $0.12 \times 0.08 \times 0.07$ mm^3 with a 1 s exposure per 0.5° frame for the high resolution data and a total data collection time of 25 min. Figure produced with the program BOBSCRIPT[12] and kindly provided by Andrzej Joachimiak and Martin Walsh.

mosaic spread, usually some extension in resolution, a substantial increase in effective crystal life time and, therefore, a huge increase in the quality of the data. A more recent technical development is the transport of prefrozen crystals to synchrotron radiation sources, the diffraction quality having been checked in the home laboratory prior to the visit. This is leading to much more efficient use of beam time. However, at the most intense sources, problems of radiation damage are re-emerging, especially for small crystals and with microfocus lines. Further lowering of the collection temperature to liquid helium levels may alleviate this, although it has been known for many years that biological samples can indeed only tolerate a finite radiation dose[9].

Beamlines, insertion devices and sources

The number of sources and protein crystallography beamlines has increased substantially in the last decade. In brief, the beamlines fall into two general categories: (i) lines with horizontal focusing provided by a bent single crystal monochromator followed by a bent (often segmented) mirror and (ii) lines with double crystal monochromators and toroidal mirrors for focusing. The former are not readily tunable and tend to be operated at fixed wavelengths, usually around 0.8–0.9 Å. This is below the Pt, Hg, Au, Se and Br absorption edges, providing good measurements of the anomolous scattering for the multiple isomorphous replacement phasing. The latter are much more readily tunable and ideal for MAD experiments but the double crystal set up reduces the overall intensity of the X-ray beam, making some of these beamlines, especially on the lower energy synchrotrons, less than optimal for analysis of small crystals or large unit cells.

Many lines of the first type were built on bending or wiggler magnets on the earlier machines (see the articles by John Helliwell and Robert Sweet for a description of these terms), as were the first MAD lines. This was largely due to the uncertainty of how well undulator magnets would perform in practice. Experience at the ESRF and elsewhere indicates that undulators are indeed tunable over the energy ranges required for protein crystallography MAD work, as the physical gap size attainable in the undulator magnets has been rapidly reduced. One can anticipate that on third generation synchrotron radiation sources with energies of 2.5 GeV and above, protein crystallography lines may generally be of the second type with an undulator insertion device.

Atomic resolution

One notable development has been the extension of the tractable resolution for an increasing number of proteins to atomic resolution, roughly 1.2 Å or better[10]. This is allowing the extension of the standard techniques of small molecule crystallography to such proteins, including refinement using full anisotropic models, and improves the level of accuracy towards that required for a full understanding of the biochemical behaviour of the protein. Two examples of the level of detail that can be obtained from a second and a third generation source are shown in Fig. 3. *Ab initio* phase determination is possible with such data and the structure of at least one small protein has been solved in this manner[11].

Summary
Sychrotron radiation facilities everywhere have become increasingly user friendly with easy-to-operate beamlines, detectors with straightforward acquisition, data handling software and cryogenic freezing. The ease of use and automation of the beamlines themselves is continually evolving, and the introduction of graphical user interfaces (GUIs) is greatly simplifying the lot of the user. This means that use of synchrotron radiation has moved away from a technique being used only by a small number of specialists to one that is now routinely applied by the large majority of protein crystallography groups world-wide. Estimates of what proportion of new structures are determined using synchrotron radiation range from ~40% to >70%, but the proportion is certainly substantial and growing. All the current beamlines are considerably oversubscribed by at least a factor of two — and this is likely to be an underestimate of the real demand for synchrotron radiation.

With the advances in cloning, expression and crystallization, and the opportunities opened up by microfocus beamlines, cryogenic freezing, the speed of data collection and MAD phase determination, synchrotron radiation X-ray methods have become and will remain the work horse of structural biology for at least the next decade. Most importantly, however, synchrotron radiation — by helping to ease the process of structure determination — has brought extra impetus to the expansion of X-ray crystallography into biology, allowing us to visualize many more structures of the molecules that govern chemical processes within the cell.

Acknowledgments
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