Macromolecular crystallography at synchrotron radiation sources: current status and future developments

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X-ray diffraction with synchrotron radiation (SR) has revealed the atomic structures of numerous biological macromolecules including proteins and protein complexes, nucleic acids and their protein complexes, viruses, membrane proteins and drug targets. The bright SR X-ray beam with its small divergence has made the study of weakly diffracting crystals of large biological molecules possible. The ability to tune the wavelength of the SR beam to the absorption edge of certain elements has allowed anomalous scattering to be exploited for phase determination. We review the developments at synchrotron sources and beamlines from the early days to the present time, and discuss the significance of the results in providing a deeper understanding of the biological function, the design of new therapeutic molecules and time-resolved studies of dynamic events using pump–probe techniques. Radiation damage, a problem with bright X-ray sources, has been partially alleviated by collecting data at low temperature (100 K) but work is ongoing. In the most recent development, free electron laser sources can offer a peak brightness of hard X-rays approximately $10^8$ times brighter than that achieved at SR sources. We describe briefly how early experiments at FLASH and Linear Coherent Light Source have shown exciting possibilities for the future.

Keywords: synchrotron radiation; macromolecular crystallography; beamline design; radiation damage; time-resolved studies; free electron lasers

1. Introduction—early history

In this article, we identify advances in modern-day macromolecular crystallography (MX) that have been made possible using synchrotron radiation (SR). There have been outstanding successes in the field providing insight into the relationship

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between structure and biological function and definitive data for therapeutic applications. SR gives a bright, highly collimated, tunable source of X-rays for experiments in the life and physical sciences with applications that range from engineering to cultural heritage. The brightness and collimation allow the study of large weakly diffracting macromolecules in the crystalline state, enabling smaller crystals and/or crystals with large unit cells to be used. The tunability of the radiation allows wavelengths to be precisely selected to exploit the anomalous scattering for phasing in structure determination. Methods in MX have been described in Blundell & Johnson (1976) and more recently by Rupp (2010). A recent review gives a splendid historical account of MX and SR (Dauter et al. 2010).

In a synchrotron, electrons are accelerated to speeds that are close to the speed of light and sent on a near circular or polygonic path by means of bending magnets. Electromagnetic radiation is emitted tangentially to the instantaneous path of the electrons, as predicted from theoretical calculations (Schwinger 1949), with wavelengths ranging from the infrared to the hard X-ray region. Relativistic effects confine the emission to a narrow cone in the forward direction with low angular divergence in the plane of the ring. As a consequence of radiation emission, electrons lose energy and the lost energy is restored by radiofrequency accelerator cavities placed around the ring.

The earliest synchrotrons were built as particle accelerators and colliders for elementary particle physics and the SR was considered an annoying by-product of the acceleration of the charged particles. In 1970, the first experimental use of SR in biology was made by Rosenbaum, Witz and Holmes at the Deutsches Elektronen Synchrotron (DESY), Hamburg, Germany, where it was shown that diffraction from a muscle fibre could be recorded with very much shorter exposure times than with a laboratory-based X-ray source (Rosenbaum et al. 1971). From that time, the special brightness of SR began to be appreciated for MX. Proof-of-principle experiments were reported from the Stanford Synchrotron Radiation Lightsource (SSRL) on the SPEAR storage ring (Phillips et al. 1976), while at the Laboratoire pour l'Utilisation du Rayonnement Electronique (LURE, Orsay, Paris, France) with the beamline set up by Roger Fourme, Max Perutz was one of the pioneering visitors with his haemoglobin crystals. Later, Enrico Stura, a graduate student of one of the authors (L.N.J.), in work carried out in 1979, showed the dramatic enhancement that could be obtained with crystals of glycogen phosphorylase (the largest single polypeptide chain (846 amino acids) to be studied by X-ray crystallography at that time; Wilson et al. 1983). Exposures that took 13h at the home source took only 6min at the LURE DCI storage ring and the diffraction pattern extended to a higher resolution (2Å with the SR compared with 3Å with the home source).

Progress was slow in those days (Dauter et al. 2010). Time at SR sources was limited. The early synchrotrons were inherently unstable. Collision experiments for elementary particle physics required stability only at the points where detectors sensitive to the position of the particles were located. Hence, there was a demand for stable storage rings dedicated to the production of X-rays and this led to the construction of second-generation sources. The synchrotron radiation source (SRS) at Daresbury, UK, commissioned in 1981, was the first such facility to be built with the MX beamline 7.2 (Helliwell et al. 1982), and
was soon delivering results. Other sources with dedicated MX beamlines followed, including the National Synchrotron Light Source (NSLS, Brookhaven, NY), the Photon Factory (PF, Tsukuba, Japan) and BESSY (Berlin, Germany). Other first-generation accelerators such as LURE, Cornell High Energy Synchrotron Source (CHESS, Cornell, NY), SSRL and DESY were upgraded for production of SR (reviewed by Helliwell 1984). Successful results and the demonstration of the possibility of Laue diffraction from microcrystals (Hedman et al. 1985) led to the demand for yet more brightness.

In the mid-1990s, third-generation sources with lower source sizes and lower emittance were built to meet this need. The first such sources with MX beamlines were the European Synchrotron Radiation Facility (ESRF, Grenoble, France; 1994), the Advanced Photon Source (APS, Argonne, IL; 1996) and Super Photon ring 8 (SPring8, Harima, Japan; 1997). SPring8, operating at a ring energy of 8 GeV and with a storage ring circumference of 1436 m, was until recently the largest storage ring dedicated to the production of SR in the world. (The largest is now PETRA III (circumference 2.3 km) in Hamburg, Germany.) The long straight sections in these storage rings can accommodate insertion devices, such as undulators or wigglers that consist of a periodic arrangement of many short dipole magnets of alternating polarity. The electrons move on a wave-like curve through the magnets with overall zero deflection of the beam and produce a synchrotron beam with higher peak brightness (undulators) or greater flux (wigglers) at higher energies than can be achieved with bending magnets. Other sources with radiation extending into the soft X-ray region were also built in 1994, such as the Advanced Light Source (ALS, Berkeley, CA) and Elettra (Trieste, Italy). Further third-generation sources now include the Swiss Light Source (SLS, Villigen, Switzerland), the Canadian Light Source (CLS, Saskatoon, Canada), Diamond Light Source (Chilton, UK), Soleil (Saint-Aubin, France), the Australian Synchrotron (AS, Melbourne, Australia) and the Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China) with more currently being constructed and commissioned.

Three synchrotron sources have been built in Third World countries as part of a successful programme to stimulate industry and the economy: the National Synchrotron Radiation Research Centre (NSRRC, Hsinchu, Taiwan; 1994), the Pohang Accelerator Laboratory (PAL/PLS, Pohang, Korea; 1994) and the Laboratory Nacional de Luz Sincrotron (LNLS, Campinas, Brazil; 1996). In 1997, on the decommissioning of the BESSY I synchrotron, the initiative of some visionary scientists led to an arrangement to ship components to the Middle East to found SESAME (Synchrotron-light for Experimental Science and Applications in the Middle East), a synchrotron being built by a consortium of seven countries (Bahrain, Egypt, Israel, Jordan, Pakistan, Palestine and Turkey with several others about to join), as a facility for science and a focus for peaceful cooperation.

2. MX beamlines

The synchrotron sources with MX beamlines are shown in table 1. In August 2010, the Protein Data Bank (PDB; www.rcsb.org) held 59700 biological macromolecular structures solved by X-rays, of which 43000 (72%) had been refracted.
Table 1. Past, present and near future Synchrotron Radiation Sources with MX beamlines (http://biosync.rcsb.org/BiosyncStat.html).

| Source | Energy (GeV) | Circumference (m) | Current (mA) | Number of active MX beamlines | Number of deposited structures (August 2010) | Year first structure deposited from 1995 |
|--------|--------------|-------------------|--------------|-------------------------------|--------------------------------------------|----------------------------------------|
| **Americas and Canada** | | | | | | |
| ALS (Advanced Light Source, Berkeley, CA) | 1.9 | 197 | 400 | 8 | 3033 | 1998 |
| APS (Advanced Photon Source, Argonne, IL) | 7 | 1104 | 100 | 22 | 7982 | 1997 |
| CAMD (Centre for Advanced Microstructures and Devices, Baton Rouge, LA) | 1.3/1.5 | 55 | 300/150 | 1 | 36 | 2003 |
| CHESS (Cornell High Energy Synchrotron Source, Ithaca, NY) | 4.7–5.5 | 768 | 250 | 4 | 1224 | 1995e |
| CLS (Canadian Light Source, Saskatoon, Canada) | 2.9 | 171 | 250 | 1 | 69 | 2006 |
| LNLS (Laboratorio Nacional de Luz Sincrotron, Campinas, Brazil) | 1.37 | 93 | 250 | 2 | 192 | 1999 |
| NSLS (National Synchrotron Light Source, Brookhaven, NY) | 2.8 | 170 | 300 | 13 | 4775 | 1995e |
| SSRL (Stanford Synchrotron Radiation Lightsource, Stanford, CA) | 3 | 234 | 200 | 5 | 2890 | 1995e |
| **Europe** | | | | | | |
| ALBA (Barcelona, Spain) | 3 | 269 | 400 | 1 | f | f |
| BESSY II (Berlin, Germany) | 1.7 | 240 | 200 | 2 | 414 | 2003 |
| Diamond Light Source (Didcot, UK) | 3 | 562 | 250 | 5 | 333 | 2007 |
| ELETTRA (Trieste, Italy) | 2 | 259 | 330 | 1 | 373 | 1997 |
| DORIS, DESY (Hamburg, Germany) | 4.45 | 289 | 140 | 7 | 2647 | 1995e |
| ESRF (European Synchrotron Radiation Facility, Grenoble, France) | 6 | 844 | 200 | 13 | 6662 | 1996 |
| Kurchatov SNC (Kurchatov Synchrotron Nano Centre, Moscow, Russia) | 2.5 | 124 | 300 | 1 | 4 | 2008 |
| LURE (Paris, France) | 1.8 | 91 | 250 | 3 | 261 | 1995e |
| Source                                      | Beamline | Max. Intensity | Int.   | No.  | Year |
|---------------------------------------------|----------|---------------|--------|------|------|
| MAXII (Lund, Sweden)                        | 1.5      | 90            | 280    | 4    | 552  | 1998 |
| PETRA III, DESY (Hamburg, Germany)          | 6        | 2304          | 100    | 2    | f    | f    |
| SLS (Swiss Light Source, Villigen, Switzerland) | 2.4      | 288           | 400    | 3    | 1450 | 2002 |
| Soleil (Saint Aubin, France)                | 2.75     | 354           | 500    | 1    | 39   | 2008 |
| SRS (Synchrotron Radiation Source, Daresbury, UK) | 2        | 98            | 200    | 5    | 1406 | 1995 |

**Asia and Oceania**

| Source                                      | Beamline | Max. Intensity | Int.   | No.  | Year |
|---------------------------------------------|----------|---------------|--------|------|------|
| AS (Australian Synchrotron, Melbourne, Australia) | 3        | 216           | 200    | 2    | 94   | 2007 |
| BSRF (Beijing Synchrotron Radiation Facility, Beijing, China) | 2.2      | 240           | 65     | 2    | 120  | 2003 |
| NSRRC (National Synchrotron Radiation Research Centre, Hsinchu, Taiwan) | 1.5      | 240           | 120    | 2    | 221  | 2001 |
| PAL/PLS (Pohang Light Source, Pohang, Korea)    | 2.5      | 280           | 180    | 3    | 345  | 2001 |
| Photon Factory (Tsukuba, Japan)               |          |               |        |      |      |      |
| SESAME (Synchrotron Light For Experimental Science in the Middle East, Allaan, Jordan) | 2.5      | 400           | 125    | 1    | f    | f    |
| SPring8 (Hyogo, Japan)                        | 8        | 100           | 1436   | 12   | 2693 | 1999 |
| SSRF (Shanghai Synchrotron Radiation Facility, Shanghai, China) | 3.5      | 432           | 200–300 | 1    | 25   | 2009 |

SR, storage ring; AR, accumulator ring.

*a* Operates parasitically in tandem with the high-energy physics research programme at Cornell.

*b* Includes X31, now closed.

*c* Closed 2003.

*d* Closed 2007.

*e* These sources were in operation before 1995 (when bioSync statistics started being collated) and hence the total number of structures over their operating period is larger than the figure given.

*f* These facilities are still to be commissioned.
solved by SR (http://biosync.rcsb.org/BiosyncStat.html). MX beamlines are in high demand from both academic and industrial users and are most productive. Over the past two decades, there have been many instrumentation and software developments and the MX technique has become accessible to non-experts (Wlodawer et al. 2008).

Most MX beamlines allow the X-ray wavelength to be tuned for the experiment. A wavelength of 0.98 Å is most commonly used with those between 0.7 and 2.0 Å usually accessible. Equipment to be found on an MX beamline end-station includes a fluorescence detector for precise wavelength selection; a goniometer for crystal mounting, alignment and rotation in the X-ray beam, and a cryo-cooling device to allow data collection at 100 K to mitigate the effects of radiation damage (discussed later).

Detector developments have been crucially important for MX. There has been a progression from X-ray film (1980s) to image plates (mid-1990s) to charge-coupled device (CCD) detectors (late-1990s, early-2000s) and most recently to pixel array detectors (PADs) (approx. 2009). Each stage has resulted in an increase in efficiency and precision. For example, the use of a 3 × 3 mosaic CCD detector in 1999 on the then new undulator beamline at APS enabled complete multi-wavelength anomalous diffraction (MAD) datasets at four different wavelengths to be collected in less than 30 min for a small 16 kDa protein (Walsh et al. 1999). At the time of writing, most beamlines use CCD detectors for collecting the diffracted photons. The latest development of PADs (Broennimann et al. 2006; Kraft et al. 2009) has provided improvements in precision and speed of data collection. Each pixel has its own signal processing and read-out electronics to enable pixels to be read out in parallel. As a result, PADs have negligible read-out time, and data may be collected continuously as the crystal is rotated so that there is no need for a shutter to delimit individual exposure times. Data collection for a small protein (105 amino acids, 1.42 Å resolution, approx. 110,000 reflections, 18,197 unique) was accomplished in less than 3 min on beamline I24 at Diamond Light Source with a PAD.

Automation is becoming routine for crystal mounting and is important for screening crystals. A number of commercial robots are now available. In the early days, experimenters would collect data on the first crystal that diffracted well after mounting by hand perhaps half a dozen crystals. With automation hardware, hundreds of crystals can be screened and software has been developed to automatically process the data and rank the results according to a set of predefined criteria in order to find the best diffracting crystal. A number of groups and consortia are exploiting genomic data to select protein targets for structure determination. They aim to determine a large number of new structures that do not have homologues in the protein database in order to expand structural knowledge. These structural genomics groups require great efficiency in structure determination in order to deliver their targets and automation is central to this goal (Joachimiak 2009).

The increased demand for high-quality experimental facilities placed pressure on the synchrotron beamline developers and operators to ensure equipment stability and reliability. Considerable ongoing effort was invested in motorizing and encoding all axes, updating equipment control systems and developing easy
to use graphical user interfaces for experimental control. This fundamental work provided the foundation for the automation of the MX experiment from sample mounting at the goniometer through to pipelining the data reduction process. Standard hardware for sample mounting was developed, particularly in Europe, where EU funding of several projects provided both resource and investment for this (Cipriani et al. 2006). The automation of the beamline hardware provided significant incentive to the software developers to include software automation (Beteva et al. 2006). The MX beamlines at SSRL were the first to offer remote access data collection where samples are mailed in, beamline scientists load the samples into the robotic crystal mounter and data collection is controlled remotely via a high-speed network link (Soltis et al. 2008). The notion of beamlines without resident users has become popular.

Several MX beamlines are targeting smaller crystals with increasing flexibility in both the actual beam size and also the position of the beam focus (Moukhametzianov et al. 2008). Over the years, as samples have become smaller, crystal viewing both prior to and during data collection has become important. Tiny crystals can be difficult to see at the best of times, but when they are embedded within a layer of vitreous ice, as needed for cryo-protection (discussed later), they can become almost invisible. A major step forward in sample viewing was the on-axis viewing systems, which allow the experimenter to have the same view of the crystal as that of the incoming X-ray (Perrakis et al. 1999). In later developments, the use of X-rays for alignment of the crystal has provided a more reliable method than visual optics for challenging problems (Song et al. 2007). Diffraction quality of a crystal may vary over different regions. At the microfocus beamlines (discussed later) or those with mini-beam collimation to provide a small beam (Cherezov et al. 2009; Fischetti et al. 2009), it is possible to scan small areas within larger crystals to detect the best portion of the crystal for diffraction (Aishima et al. 2010).

A dramatic example of work with microcrystals is the structural determination of the polyhedrin virus protein, which forms micron-sized crystals that encapsulate cypoviruses and baculoviruses. The structure of the trimeric polyhedrin protein from the Bombyx mori cytoplasmic polyhedrosis virus (CPV) was determined from crystals of 5–12 μm in size at beamline X06SA at SLS, the smallest crystals used at that time for de novo structure determination (Coulibaly et al. 2007). More recently, this work has been elaborated showing an ironic evolutionary twist. The polyhedrin protein from another insect virus, the baculovirus Autographia californica multiple nucleopolyhedrosis virus (AcMNPV), showed similar unit cell dimensions and space group to the CPV polyhedrin protein (cubic space group I23, unit cell $a = \sim 102\,\text{Å}$), and it was assumed that the folds of the two proteins would be similar. In fact, structure solution using crystals no greater than 5 μm at the Diamond beamline I24 showed that the AcMNPV polyhedrin protein fold was different and more closely resembled the canonical capsid protein structure of the picornavirus lineage of viruses (Ji et al. 2010). The similar crystalline arrangement adopted by the two polyhedrin proteins appears particularly well suited to the functional requirements of the polyhedra and has been either preserved or reselected during evolution.
3. The impact for biology of macromolecular crystallography

The impact of structural studies on biology and biological chemistry has been immense. The ultimate aim of structural biology is to understand the biological function in terms of three-dimensional structure. Myoglobin, the first protein crystal structure solved in 1959 (Kendrew et al. 1960), showed how the haem iron embedded in its special non-polar environment in the protein could take up and release oxygen without becoming oxidized. Without the protein, the iron is rapidly oxidized from the ferrous to the ferric state. The structure of lysozyme, the second protein and the first enzyme structure solved at atomic resolution (Blake et al. 1965), together with inhibitor-binding studies (Blake et al. 1967), showed the molecular basis for substrate recognition and catalysis. Lysozyme hydrolysates the linkage between sugars in the polysaccharide component of bacterial cell walls. Bacterial cell walls are composed of alternating sugars, N-acetylmuramic acid and N-acetylglucosamine, together with peptide cross-links that hold the polysaccharide chains together in a strong two-dimensional array. Lysozyme cleaves specifically between the N-acetylmuramic acid and N-acetylglucosamine residues. The structural studies showed the origin of the specificity for acetamido sugars, the selectivity for the bond cleaved, the involvement of two acidic residues in catalysis—one glutamic acid acting as a general acid and the other an aspartic acid residue acting as a nucleophile—with their chemical properties conferred by their environment in the protein. The studies led to a proposal for the mechanism of catalysis of the β(1,4) glycosidic linkage that used the recognition properties shown by the structure. The marvellous explanatory power of molecular recognition laid the basis for the use of MX in understanding the biological function and for modern-day structure-based drug design.

Lysozyme data collection with a laboratory source took approximately 14 days for a 2Å resolution dataset. The commissioning of the SRS in 1981 enhanced structural studies in the UK. Data-collection times were reduced and several large biological complexes were solved that could not have been addressed with laboratory-based home sources. These included functional studies on glycogen phosphorylase (Sprang et al. 1988), which led to an explanation for changes in phosphorylation, to foot and mouth disease virus (FMDV) (Acharya et al. 1989), which provided data for the design of safer and more effective vaccines, and the F1-ATPase (Abrahams et al. 1994), the membrane-bound protein that synthesizes ATP to provide energy for the cell. With the availability of more MX facilities at SR sources worldwide, many other large complexes have joined this gallery, of which only a small selection is illustrated in figure 1. Notable is the large fatty acid synthase complex of molecular mass 2.6 MDa. The experimental strategies in addressing such large complexes have been reviewed (Mueller et al. 2007). Each of the challenging problems solved with SR has contributed definite data for biological processes and a fundamental understanding of biology.

An important outcome of advances in MX through SR has been contributions to the design of new therapeutic agents. The structure of the protein defines the positions of its atoms in three dimensions. Once a protein has been identified as a target for therapy, a structural study on the binding of a lead putative drug molecule will show how the compound interacts with the protein. This knowledge can suggest how the compound may be modified to improve binding properties.
Figure 1. A gallery of selected important large biological structures that have been solved with data from SR sources. 

(a) F1-ATPase (351 kDa; PDB ID 1BMF). The three non-active $\alpha$ chains are in red, the three active $\beta$ chains in yellow and the $\gamma$ subunit in blue. 

(b) The nucleosome core particle (206 kDa; PDB ID 1AOI). The four histone proteins, H2A, H2B, H3 and H4, are in pink, yellow, blue and green, respectively and 146 bp of DNA in dark and light blue. 

(c) Cytochrome BC1 complex (480 kDa; PDB ID 1BE3). The transmembrane region is in the centre and the matrix space below. The bovine mitochondrial protein is a dimer and the 11 different subunits are shown in different colours. 

(d) RNA polymerase II in complex with DNA (dark and light blue spheres) and RNA (red spheres) (500 kDa; PDB ID 1Y1W). The 12 subunits are in different colours with the major subunit Rpb1 in grey. 

(e) The 70S ribosome in complex with tRNA (green spheres for the P site and dark red spheres for the A and E sites) (2500 kDa; PDB ID 2J00, 2J01). The 50S proteins and RNA are in light blue and cyan, respectively, and the 30S proteins and RNA in pale yellow and orange, respectively. 

(f) Fungal fatty acid synthase (2600 kDa; PDB ID 2UV9, 2UVA). The six $\alpha$ subunits are in cyan and form a central wheel. The six $\beta$ subunits are in green. The enzyme contains six different enzyme activities at different sites within the large (210 kDa and 230 kDa) polypeptide chains.
Table 2. Protein kinase inhibitors that have been approved for clinical use (adapted from Johnson (2009)).

| generic name | brand name | date approved | known protein kinase targets | disease |
|--------------|------------|---------------|-------------------------------|---------|
| fasudil      | Rho kinase (ROCK) | 1999 | cerebral vasospasm |
| imatinib     | Glivec    | 2001 | ABL, ARG, PDGFR, KIT | chronic myeloid leukaemia (CML), gastrointestinal stromal tumours |
| nilotinib    | Tasigna   | 2007 | ABL, ARG, KIT, PDGFR | CML with resistance to imatinib or intolerance |
| dasatinib    | Sprycel   | 2007 | ABL, ARG, KIT, PDGFR SRC and others | CML with resistance to imatinib or intolerance |
| gefitinib    | Iressa    | 2004 | EGFR | non-small cell lung cancers (especially adenocarcinomas) |
| erlotinib    | Tarceva   | 2004 | EGFR | non-small cell lung and pancreatic carcinomas (with gemcitabine) |
| lapatinib    | Tykerb    | 2007 | EGFR (ErbB-1, -2) | breast cancer—Her2-positive |
| sorafenib    | Nexavar   | 2006 | B-Raf, VEGFR, PDGFR, FLT3, c-KIT | renal cell carcinoma |
| sunitinib    | Sutent    | 2006 | VEGFR, PDGFR, FLT3, c-Kit | renal cell carcinoma, gastrointestinal stromal tumours |
| temsirolimus | Torisel   | 2007 | mTOR | renal cell carcinoma |
| pazopanib    | Votrient  | 2009 | VEGFR, c-Kit, PDGFR | renal cell carcinoma |

and where it should not be modified because that would prevent binding. Some of the successes from the early days of MX, where drug design was aided by structure, include captopril (Capoten) for the treatment of hypertension through inhibition of the angiotensin-converting enzyme, dorzolamide (Trusopt) for the treatment of glaucoma through inhibition of carbonic anhydrase, several of the drugs in use for the treatment of AIDS that inhibit the HIV protease and the anti-influenza drugs Relenza and Tamiflu that inhibit the flu virus surface protein neuraminidase.

Protein kinases are a class of enzyme that play key roles in the regulation of cell processes, including cell signalling, metabolism, growth and differentiation, and these enzymes have become prime targets for the treatment of cancer and also inflammatory diseases and diabetes, where the kinase, or the pathways that it regulates, may be defective. Eleven protein kinase inhibitors have been approved to date for clinical use (table 2). The most successful is imatinib (Glivec). This compound was approved in 2001 for the treatment of chronic myeloid leukaemia, a cancer that is triggered by a chromosomal translocation leading to the upregulation of the Abelson tyrosine kinase, Abl. This drug is nearly 100 per cent successful if the disease is diagnosed in the early stages. Structure contributed to the design of Glivec, but, as with all drug-design studies,
the production of a successful drug with the right pharmacological properties was dependent on the combination of clever chemistry, physiological insights and fast biological assays.

Pharmaceutical companies require a quick turn-around between an idea, organic synthesis and results. In the early days, crystallography was regarded as too slow to be part of a drug discovery programme but now SR combined with fully automated experimental MX stations can deliver a fast turn-around. A ligand-binding experiment may take less than 10 min from crystal data collection to structure solution. Most pharmaceutical companies now use SR as part of their drug discovery programmes and several companies have combined to build their own beamlines (e.g. at APS, SPring8 and SLS).

4. Physical aspects of the SR beamline design that have led to efficiency and precision

The optical layouts of MX beamlines over the years have changed little. The improvements to beamline quality have been brought about by source improvements and the manufacture of better quality optical components, such as focusing mirrors with improved surface finishes. This has been aided by the smaller source size produced by the latest synchrotrons, which reduces the length of mirror required, thus making manufacture easier. The early reports of beamlines for MX at the SRS, Daresbury, were concerned with the effects that the thermal distortion on optical surface would have on the beam focal properties and the large source size (14 mm initially and then 2 mm after installation of the high-brightness lattice in 1985)—the effect of these being significantly greater than the problems associated with the inability to manufacture X-ray optically smooth mirror surfaces (Helliwell et al. 1982, 1986; Brammer et al. 1988). Thermal instabilities in the optics have become less of a problem with modern sources despite greater peak heat loads owing to improvements in cooling techniques and with machine developments that allow ‘top up’ operation, where the circulating electron current is kept at a constant level for long periods (potentially weeks) rather than having daily beam dumps and refills. The first MX beamline on a third-generation source, the bending magnet beamline BM14 (initially identified as BM19 but renamed BM14 to indicate position around the ESRF ring; Deacon et al. 1995), demonstrated the advantages of a smaller source size with greater photon flux into a smaller focal spot size.

The size of the beam spot at the crystal is determined by a number of factors, principally the photon beam source size (which depends on the electron beam source size) and the quality of the focusing optical elements (usually the mirrors). Beam stability is key as there is no point in achieving a really small focus if variations in position of the source or instability of the components allow the spot position to vary. This required stability is becoming increasingly challenging as crystal sizes have reduced over the years. In the mid-1990s, beam spot sizes were around 200 × 200 μm, which governed the optimal size of crystals for the beamline (Brammer et al. 1988). Today, beam spot sizes of 5–10 μm can be achieved that allow data collection for very small crystals with improved signal to noise. Beam divergence is a limiting factor for very large unit cells, such as those encountered with virus crystals. With moderate crystal to detector distances, the diffraction
Spots may overlap, making integration of intensities problematic. With small beam sizes, undulator sources and stable focusing optics these problems have been alleviated.

At Diamond Light Source, the first three general purpose MX beamlines were built on undulator sources 2 m long with either 23 mm period (for two beamlines) or 21 mm period (for the third beamline). A standard optical layout was chosen to deliver the beam to the sample (Duke et al. 2010). The first element is a silicon double crystal monochromator with indirect liquid nitrogen cooling of the first crystal that is used for wavelength selection (range 5–25 keV; 2.5–0.5 Å). Independent horizontal and vertical focusing are achieved with two mirrors in a so-called ‘Kirkpatrick–Baez mirror pair’ arrangement. These mirrors are bimorph mirrors in which the optical surface is bent electrically via piezo-electric ceramics embedded down the length of the mirror rather than mechanically. At a wavelength of 0.979 Å, these beamlines deliver $10^{12} \text{ photons s}^{-1}$ into an 80 μm focal spot at the sample. At the microfocus beamline at Diamond, beamline I24, a second stage of focusing is used in the optical layout (figure 2). Here, the first pair of mirrors delivers a focal spot to a set of slits. This forms a secondary source for the second stage of focusing where another pair of mirrors provides a microfocus spot at the sample or detector position. The use of the secondary source means that the effects of long lever arms on instability are reduced and enables slits or a pinhole to be located at the secondary source and thus reduce the background. Currently, the average crystal size for samples brought to the MX beamlines at Diamond is 50–100 μm. At the microfocus beamline data from crystals as small as 5 μm have been recorded.

5. Radiation damage and cryo-crystallography

Radiation damage had been recognized in the early days of MX as a problem for precise data collection. A study on myoglobin crystals quantified the effects showing a systematic decrease in intensities on irradiation, whereby the unchanged fraction of the crystal decreased linearly with dose and a severely disordered part changed exponentially as a function of resolution (Blake & Phillips 1962). This led to procedures to monitor diffraction intensities as data collection proceeded and to change the crystal if intensities fell by more than a certain amount (typically 25%). The increased intensity of SR initially resulted in even more damage but ironically, at room temperature, a high dose for a short time was observed to be less deleterious than a low dose for a longer time, presumably because heating effects were less and free radicals formed from the radiolytic products of water had less time to diffuse throughout the crystal.

However, if SR was to be fully exploited, radiation damage required attention. In an important advance, it was shown that radiation damage could be mitigated by cryogenic vitrification of protein crystals (Hope 1988). At temperatures of 100 K, crystals become almost (but not quite) immortal (Garman & Schneider 1997; Teng & Moffat 2002; Meents et al. 2010). Control studies with small proteins showed that in general cryo-cooling changes neither the overall structure nor details of the functional sites but that the dynamic properties of a protein may be changed (Rasmussen et al. 1992). At 100 K, the thermal mobility of
Figure 2. Optical layout of the microfocus MX beamline I24 at Diamond Light Source. See http://www.diamond.ac.uk/Home/Beamlines/MX/MX/I24/specs.html.
atoms is reduced, resulting in higher resolution data. A further advantage has been the ability, by using careful selection of data-collection parameters, to collect a complete dataset from a single crystal and hence to avoid uncertainties in scaling data collected from many crystals. At cryo-temperatures, radiation damage appears to be linear with dose and less dependent on dose rate than at room temperature. There are several collaborative programmes that aim to understand the causes and consequences of radiation damage on the structures of biological macromolecules and their function (reviewed in Nave & Garman 2005; Ravelli & Garman 2006; Garman & McSweeney 2007). Most low-temperature studies are carried out by flash-freezing the crystal in the presence of cryo-protectants, but recent studies indicate that slow cooling may have advantages (Warkentin & Thorne 2010).

In the early days, crystals were mounted in thin-walled quartz capillaries and sealed with crystallization solution to keep the crystal hydrated. For data collection at 100 K, new procedures were developed in which the crystal is fished out of the crystallization well with a nylon loop or nylon mesh with dimensions similar to that of the crystal and rapidly frozen in a stream of nitrogen gas (Teng 1990). The method results in a reduction in the background scatter contribution from the capillary and the associated hydrating mother liquor. In order to avoid formation of ice crystals and associated mechanical stresses in cryo-freezing, cryo-protectants are added or may be part of the crystallization conditions (Garman & Mitchell 1996). Virus crystals are notoriously difficult to freeze and any loss in diffraction quality or increase in mosaic spread can make data collection impossible with crystals of large unit cells. Safety considerations with crystals of disease-causing viruses also lead to a necessity for crystal mounting in sealed tubes. With the blue tongue virus, cooling was not effective and data were collected at room temperature. For this massive approximately 50 MDa particle (crystals were $P2_12_12$, with cell dimensions $a = 796\,\text{Å}$, $b = 822\,\text{Å}$, $c = 753\,\text{Å}$), over 1000 crystals were examined at ID2, ESRF, in order to obtain data (with only one image per crystal) and to solve the structure (Grimes et al. 1999).

A dose limit of $2 \times 10^7\,\text{Gy}$ for a cryo-cooled biological specimen (defined as the dose that would cause the intensities of the diffracted spots to reduce by half) was estimated by extrapolation of data obtained from electron diffraction, assuming that the damage inflicted is directly proportional to the energy deposited, irrespective of whether by electrons or by X-rays (Henderson 1990) ($1\,\text{Gy} = 1\,\text{J}\,\text{kg}^{-1} = 6.24 \times 10^{12}\,\text{MeV}\,\text{kg}^{-1}$ deposited energy). Later, experimental studies gave a value $3 \times 10^7\,\text{Gy}$, a value that also includes other data quality indicators (the changes in the high-resolution diffraction limit, the agreement between equivalent intensities, the Wilson $B$-values and the number of damaged residues; Owen et al. 2006). Thus, doses to crystals should be kept lower than this in order to retain crystal and structural integrity. Programmes are available for calculation of the time taken to reach the dose limit (Murray et al. 2004; Paithankar et al. 2009), and for collection of the best data within particular experimental constraints (desired experimental outcome, crystal content, beamline flux and time available for data collection) (Bourenkov & Popov 2010).

Examination of protein structures for the chemical consequences of radiation damage has shown that the main damage results in the breakage of disulphide bonds and decarboxylation of aspartate and glutamate residues. The extent of damage is dependent on the environment and flexibility of the protein in

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the region (Burmeister 2000; Ravelli & McSweeney 2000; Weik et al. 2000). Metals in metallo-proteins have also been found to be susceptible to photo-reduction by radiation damage, making conclusions from X-ray structure on the role of the metals problematic (Carugo & Carugo 2005). A more welcome aspect of the structural effects of radiation damage was the demonstration that radiation damage changes could be used as a method of phasing the structure (radiation-induced phasing (RIP)) (Ravelli et al. 2003). A further imaginative use of radiation damage was to follow intermediates on the cytochrome P450-catalysed reaction of camphor hydroxylation (Schlichting et al. 2000). The enzyme uses a haem iron to catalyse the addition of molecular oxygen to hydrocarbons. The O₂ complex structure was determined from data recorded at 88 K with short (0.91 Å) wavelength to minimize damage. Guided by spectroscopic information, the complex in the crystal was irradiated with 1.5 Å radiation for 3 h at cryo-temperatures to produce a source of electrons generated by the radiolysis of water that resulted in an oxyferryl intermediate from the breakdown of the dioxygen molecule. Data for the resulting intermediate were again collected at low-temperature short wavelength. Finally, the product, 5-exo-hydroxy-camphor, was generated by warming the crystal to room temperature for 30 s to allow the reaction to proceed and then flash-freezing to 100 K before collecting the third dataset for the product complex.

6. Data analysis

X-rays are scattered by the electrons in the molecule. Structural determination is carried out by Fourier transform of the diffraction pattern resulting in an image of the electron density. From the electron density, the positions of the atoms can be determined, facilitated by the known geometry and stereochemistry of individual groups of atoms. However, the Fourier transform requires knowledge of both the amplitudes and the phases of the Bragg reflections. Amplitudes are derived from the intensities recorded on the detector, but all information of their phase is lost when the diffracted X-ray beams interact with the detector. The first step is the precise measurement of intensities. As Lawrence Bragg noted:

> It is a curious feature of X-ray analysis that there are two points in this course of investigation when it comes as it were to a focus. The first is the finale of experimental measurements, which are summed up in a list of values of \( F(hkl) \). All experimental effort and cunning has gone into making these values as accurate and as extensive as possible and they represent the raw material on which the whole analysis is based. The second is the list of atomic co-ordinates in which a set of numbers constitutes a full account of all that the investigator has succeeded in establishing.

(Bragg 1969, p. 1)

Integrated intensities are derived from the ‘raw’ diffraction images with a number of software options possible, each employing slightly different methods and algorithms and the choice is often down to personal or laboratory preference. The most frequently used are MOSFLM (Leslie 1999, 2006; Leslie & Powell 2001), DENZO/HKL (Minor et al. 2006), XDS (Kabsch 2010a–d) and d*TREK (Pflugrath 1997, 1999). For subsequent steps in the reduction of data, comprehensive program packages such as the CCP4 suite (CCP4 1994;
Winn (2003) and PHENIX (Adams et al. 2010) are available that integrate multiple programs into a coherent suite with standardized interfaces and file structures that greatly assist structure determination. The rapidity of diffraction data collection on third-generation sources coupled with the advances in computational speed have provided ample incentive both to speed up individual steps in structure determination and to automate the entire process including the connections between individual steps (Leslie et al. 2002). Expert systems to assist in the decision-making process have been produced (e.g. ELVES) (Holton & Alber 2004). Developments that enable almost real-time data processing and automated structure solution on beamlines equipped with rapid read-out CCD detectors include AUTORICKSHAW (Panjikar et al. 2005, 2009), BALBES (Long et al. 2008) and XIA2 (Winter 2010). The efficiency of the process is such that non-experts can readily learn crystal structure determination. A concern for graduate student supervisors is to make sure that their students understand the fundamentals of crystallography. There is a continuing need for workshops and tutorials.

7. Phasing

Phasing presents a special problem. For small molecules and some small proteins, where it is possible to record diffraction data to atomic resolution (better than 1.2Å resolution), structures are solved directly through direct methods (e.g. SHELX; Sheldrick 2008). The large number of small molecule structures (525 093 in September 2010) in the Cambridge Crystallographic Data Centre (CCDC) shows the success of these methods. Direct methods may be extended to proteins with data recorded at 2Å resolution, but this is not yet routinely used (Rodriguez et al. 2009). With the increasing numbers of protein structures in the data bank, many unknown structures can be solved using the known structure of a similar protein (approx. greater than 30% sequence identity) with the method of molecular replacement (MR) (Rossmann & Blow 1962). It has been estimated that around 75 per cent of structures deposited in the PDB could have been solved automatically without user intervention (Long et al. 2008). MR uses initial phases calculated from the related known structure suitably oriented and positioned in the unit cell of the unknown structure. Orientation and position space are explored to find the best fit and cycles of model building and refinement ensue until the final structure is defined (Evans & McCoy 2008). Strategies and verification procedures are in place to ensure that the starting model does not bias the final model.

However, for proteins for which no structure of another protein with sufficient sequence similarity exists, experimental methods are required. The method of multiple heavy atom isomorphous replacement (MIR), introduced by Max Perutz (Green et al. 1954), continues to be used with appropriate treatment of errors (Blow & Crick 1959). Heavy atom compounds (such as those of mercury, platinum, uranium or gold) are added to the crystal and act as reference markers for phase determination. For the method to work, the heavy atom compounds must not disturb the protein structure and more than one heavy atom derivative is required to provide a solution to the phase problem.
SR offers the unique advantage that the X-ray wavelength can be tuned to the appropriate absorption edge in order to optimize anomalous scattering. Anomalous scattering arises from resonance between beams of X-ray waves and electronic transitions from bound atomic orbitals. From the early days, it was shown that anomalous scattering data could provide additional phase information and improve precision (Blake et al. 1965). The atomic scattering factor \( f \) is described by three components—the normal scattering term \( f^o \), which is dependent on the Bragg angle and is independent of wavelength, and two anomalous scattering factors, one real \( f' \) and one imaginary \( f'' \), which are wavelength \( \lambda \) dependent \( f = f^o + f'(\lambda) + f''(\lambda) \). The anomalous scattering effects become significant when the wavelength of the incident X-ray beam approaches the wavelength of the absorption edge (figure 3). The real part of the anomalous component of the scattering factor, the dispersive term \( f' \), is 180° out of phase with the normal scattering factor \( f \) and hence reduces it slightly. The imaginary absorptive term \( f'' \), which is proportional to the atomic absorption coefficient, is advanced in phase by 90°. This results in a breakdown of Friedel’s Law, the law that states the diffraction pattern is always centrosymmetric even though the structure lacks a centre of symmetry, i.e. the structure factor amplitude for reflection \( F(hkl) \) is equal to that for reflection \( F(-h - k - l) \). In the presence of an anomalous scatterer in a protein

![Graph showing anomalous scattering factors for Se from seleno-methionine thioredoxin.](image)
molecule, this law breaks down \((F(hkl) \neq F(-h-k-l))\). The resulting difference is termed the Bijvoet difference. From measurements of this small difference, the sine of the phase angle can be determined. If the anomalous scatterer is an integral part of the protein (say a metal ion), then there is an ambiguity in the phase represented by the determination of the sine of the phase and not the phase itself. If the anomalous scattering atom is the heavy atom of an isomorphous replacement structure, the ambiguity can be resolved from a combination of the isomorphous information, which gives the cosine of the phase, and the anomalous information, which give the sine of the phase (Blow & Rossmann 1961; North 1965). For MX beamlines, the K-edges of the elements between Cr and Zr and the L-edges between La and U are commonly accessible.

The ability to tune and select wavelength at SR sources led to the development of the multi-wavelength anomalous dispersion (MAD) technique (Hendrickson 1991), in which measurements on the same crystal are made at three wavelengths—one chosen to maximize the absorption \(f''\) effect \((E_{\text{peak}}\text{ in figure } 3)\), one chosen to maximize the dispersive \(f'\) effects \((E_{\text{edge}}\text{ in figure } 3)\) and one remote from the absorption edge whose value is chosen based on the knowledge of the crystal chemical composition. These measurements can be used to solve the phase problem unambiguously. Because the measurements are made with the same crystal, a drawback of the lack of isomorphism between native and derivative crystals in the MIR method is removed. Although anomalous phasing had been demonstrated for a number of biological molecules of moderate complexity, it required beamline developments (such as those at NSLS, SSRL, PF and LURE) to allow the method to become productive. Developments included new crystal monochromators (such as the double-crystal monochromator with two crystals of parallel glancing surfaces or the single crystal channel cut monochromator) for rapid tuning without the need to re-align the beam and precise tuning of the X-ray wavelength (the different energies for \(f'\) and \(f''\) can be as small as 0.0002 Å (2.5 eV)). Other improvements included cryo-methods to preserve the crystal, and the introduction of image plate or CCD detectors to measure the small anomalous differences precisely (Hendrickson 1999). Almost all MX beamlines now have this capability. MAD requires careful measurements at three wavelengths, which can be demanding. As shown by Helliwell (1984) and further developments by Gonzalez et al. (1999), under certain optimized conditions two wavelengths can be sufficient. However, single wavelength anomalous dispersion (SAD) (Wang 1985; Rice et al. 2000; Dauter et al. 2002) is now more generally used. Here, measurements are made at a single wavelength selected to exploit the maximum anomalous scattering \((f'')\) and the phase ambiguity is resolved by other methods that use maximum-likelihood density modification, such as SOLVE/RESOLVE (Terwilliger 2000). SAD has the advantage of speed and less likelihood of radiation damage.

For anomalous scattering experiments, wavelengths have to be carefully tuned to the optimal wavelength (figure 3). An anomalously scattering atom in a protein environment may have slightly different properties from those calculated for the isolated atom. The intensity values for \(f'\) and \(f''\) result from the transition of electrons to the unoccupied orbitals and therefore, along with the precise position of the resonant frequency itself, are dependent on the oxidation state and stereochemistry of the atom. These transitions give rise to the so-called ‘white
line’ from early observations on photographic film. The effects are more apparent for L-edges; they are also observed for K-edges. A fluorescence detector is used to select the optimal wavelength for data collection.

Metallo-proteins containing Cu, Zn, Mn, Fe have a natural anomalous scatterer at wavelengths that are accessible on most synchrotron MX beamlines, but few other proteins do. Dauter et al. (2000) have shown that alkali halides soaked into a crystal (e.g. as bromide or iodide salts) can be effective. The use of bromo-substituted uridine has been useful for nucleic acid structures. The advance that really made anomalous scattering more widely applicable was the method based on the substitution of selenium for sulphur in the naturally occurring amino acid methionine (Se-Met) through recombinant DNA techniques (Hendrickson et al. 1990). Selenium ($f^0 = 34$ electrons; $f'' = 5$ electrons; $f' = -9$ electrons) has a K-absorption edge of 0.9795Å, which is accessible and convenient for the collection of MX crystallographic data. The method was demonstrated in ab initio structure determination with the biologically important protein RNase H, part of the HIV reverse-transcriptase machinery (Yang et al. 1990). The Se-Met method has become routine for proteins that can be expressed in Escherichia coli where bacterial strains exist that are methionine auxotrophs (i.e. they cannot make their own methionine) and it has been extended to other hosts (Walden 2010). Not all proteins or protein complexes can be expressed in heterogeneous systems, and human proteins pose particular challenges.

Some of the most interesting macromolecular complexes have to be isolated from tissue. Almost all of these will contain sulphur either as cysteine and/or as methionine residues. The sulphur absorption edge is 5.015Å. However, even at wavelengths of approximately 1.5Å, the sulphur anomalous signal can be used for phasing, provided that sufficient precision is obtained in the measurements, as shown by Hendrickson & Teeter (1981) with a home source and reviewed in Cianci et al. (2008). A number of small- to moderate-sized proteins have been solved by this method (e.g. the growth factor VEGF at 1.69Å wavelength; Wagner et al. 2006). A recent development at MX SR beamlines is the construction of purpose-built long-wavelength beamlines that will allow exploitation of anomalous scattering close to the absorption edges of sulphur (or phosphorus edge 5.78Å) for more challenging problems. These beamlines will give due consideration to the problems faced in working with long wavelengths, namely air absorption, detector sensitivity and goniometer design, such as the mini-kappa diffractometer to allow collection of Friedel pairs close together in time. When these beamlines are operating, structure determination for almost all biological protein molecules should become routine.

SR is plane-polarized in the plane of the ring. This phenomenon has not been exploited although it was known that anomalous scattering exhibits anisotropic behaviour near the absorption edge. The anomalous anisotropy is dependent on the chemical arrangement of bonds around the anomalous scatterer with respect to the plane of polarization. Schiltz & Bricogne (2008) have shown how the anisotropy of anomalous scattering can be used to assist phase calculations. However, on most MX beamlines, the rotation axis of the crystal is horizontal and so is exactly along the direction of polarization of the beam. Anisotropic anomalous scattering is dependent on the orientation of the chemical groups attached to the anomalous scatterer with the plane of polarization and for certain
orientations anomalous scattering can be diminished. For the method to be exploited, data-collection strategies are needed that allow rotation of the crystal about different axes, such as with the mini-kappa goniometer.

8. Time-resolved studies

Fast data-collection times at SR sources opened up the possibility of time-resolved studies on dynamic events within crystals. In particular, Laue diffraction that uses a broad wavelength polychromatic beam of radiation allowed times for data collection to be reduced substantially (Moffat 2001). Laue diffraction is not widely used. Firstly, because the crystal must remain intact during the experiment and not show any lattice distortion that frequently occurs when conformational changes take place in the crystal. Secondly, there are problems in initiating the reaction homogeneously throughout the crystal. This is difficult to do by diffusion of substrate into an enzyme crystal, which may take a few seconds to several minutes depending on the size of the substrate and the channels through which it must diffuse (Geremia et al. 2006), but light-activated reactions have provided some most interesting results. Experiments of the pump–probe type have been used to follow events in haem-containing proteins and the photo-cycle of photoactive yellow protein. The reaction is initiated by an ultra-short laser pulse, which is followed by a short brilliant X-ray pulse to probe the structure. Experiments require specialized instrumentation at the beamlines to synchronize the pump and the probe.

An early successful example was the study on the photo-induced dissociation of carbon monoxide from myoglobin on a nanosecond time scale (Srajer et al. 1996). Later, data from a mutant myoglobin were recorded at ESRF on the 100 ps time scale (Schotte et al. 2003). The synchrotron was operated in single bunch mode and a chopper was used to isolate a single intense X-ray pulse (approx. 100 ps) with wavelength spread of 0.72–1.2 Å. The carbonmonoxy–myoglobin structure was followed frame by frame at 1.8 Å resolution after photolysis using cumulative images from 32 X-ray pulses with a variable pump–probe time delay. Time-resolved mid-infrared spectroscopy on the flash-photolysed mutant myoglobin had indicated a short-lived CO intermediate with a 140 ps lifetime, shorter than that shown by wild-type myoglobin. With a pump–probe time delay of 100 ps, the structures revealed transient conformational changes far more dramatic than the structural differences between the initial carbonmonoxy and final states and correlated side-chain motions responsible for rapidly sweeping CO away from its primary docking site at the haem to adjacent pockets. In carbonmonoxy–myoglobin, the release path for CO to the outside is blocked by protein. The observations showing how the protein could change and allow release were therefore of considerable interest.

At the BioCARS 14ID B beamline (APS), a single exposure with a 153 ps X-ray pulse from the synchrotron operated in the hybrid mode was used in a Laue diffraction experiment with the photoactive yellow protein (Schmidt et al. 2010). Twenty-four Laue patterns were required for a complete dataset, with the crystal at different orientations between each exposure. Data were collected for a series of time delays from 3 to 256 ns. At each point, a Laue diffraction pattern was
obtained using eight repeated 153 ps pulses (in order to improve precision) with a 2 s time delay between to allow recovery with each exposure preceded by a laser pulse. In comparison with the structure in the dark, the intermediate of the light-activated state after 256 ns showed a change in isomerization of the chromophore from a cis to a trans conformation and changes in protein conformation. With this beamline observations can also be carried out at different temperatures adding a fifth dimension to structural studies.

A third recent example is the time-resolved Laue diffraction studies with the photosynthetic reaction centre from the purple bacterium Blastochloris viridis (Wohri et al. 2010). The purple bacterium membrane protein converts light energy to a reduced quinone as part of the pathway of photosynthesis. In the first steps, the absorbed photon is transferred to a special pair of chlorophyll molecules and a high-energy electron is then transferred from one pigment molecule to another, creating a stable charge separation. Conformational changes are thought to accompany the intermediates on the pathway but they have been difficult to quantify. With 3 ms time-resolved Laue diffraction studies using 86 μs pulses at beamline ID09 at ESRF, light-induced conformational changes in the bacterial photoreaction centre were observed that mainly involved the side chain of a tyrosine residue (shifts of 1.3 Å). The tyrosine lies adjacent to the special pair of bacteriochlorophyll molecules that are photooxidized in the primary light conversion event. This movement accompanies deprotonation of the tyrosine residue and provides a mechanism for stabilizing the primary charge separation reactions of photosynthesis. In this and several other studies for specialized systems, detailed insights into the structural basis of transient events have been obtained.

9. Free electron lasers (FELs): ultra-bright sources for the future

FELs were considered in the early 1970s and 1980s for the production of even more intense X-rays than synchrotron storage rings. A workshop to discuss the science case was held in 1994 that resulted in decisions to construct FELs for scientific research. FELs are based on the principle of self-amplified spontaneous emission (SASE). The first SASE FEL lasing in the vacuum ultraviolet (VUV) region (wavelength 109 nm) was achieved in 2000 at DESY, Hamburg (Andruszkow et al. 2000). Following this, an FEL source in the soft X-ray region was built (2005) at FLASH, Hamburg (initially with wavelength 13 nm, then 7.0 nm and currently 4.5 nm; figure 4a). Most recently, a hard X-ray FEL source has been commissioned for users, the Linear Coherent Light Source (LCLS), Stanford (operational in 2009 initially with wavelength 6.5 Å and currently 1.5 Å). Further, hard X-ray FEL sources are under construction: the SPring8 Compact SASE Source (SCSS X-FEL), Riken Harima Institute Hyogo, Japan (operational 2011), the European X-ray FEL (XFEL), Hamburg (commissioning in 2014) and a number of other facilities are in their planning or development phase.

On MX beamlines at third-generation synchrotron sources, X-radiation is most commonly produced by undulator magnets in which each electron radiates independently, hence the total flux produced by a bunch of N electrons is just N times the flux of one electron. The technical revolution behind FELs lies in
the use of very long undulators arranged in a straight path so the X-rays are emitted in phase, as in a laser light beam. As the electrons travel on their slalom path through the undulator, they emit X-ray photons. The photons travelling in a straight line at the speed of light overtake the electron bunches and the electromagnetic field of the photons interacts with the electrons. Electrons that are in-phase with the photons get decelerated, while those out-of-phase are accelerated. Over the length of the long undulator, this process packs the electrons into micro-bunches that are overtaken by exactly one photon wavelength for each of their wave period. As a result, all the electrons of the micro-bunches radiate in synchrony, producing an extremely short, coherent and intense pulse of X-rays. At LCLS, the peak brightness at 8 keV is estimated to be greater than $10^{32}$ photons s$^{-1}$ mm$^{-2}$ mrad$^{-2}$ per 0.1 per cent bandwidth. This is $10^8$
times greater than the radiation emitted by undulators on a third-generation synchrotron source. The pulse length is extremely short—it can be varied from 10 to 80 fs ($1\text{fs} = 10^{-15}\text{s}$) at 8 keV and X-ray flux is approximately $10^5$ X-ray photons per Å$^2$. The pulses are delivered at 30 Hz, a parameter that will be increased to 60 Hz and possibly 120 Hz. At LCLS, the 14.2 GeV FEL is placed in the last kilometre of a 3 km long tunnel (that was originally built to house a longer linear accelerator and which goes under Interstate Highway 280). The undulator length is 100 m (figure 4b). X-ray lasing was found to take place within a length of 60 m. The undulator length was overestimated to be on the safe side.

With such a brilliant source of X-rays what can be done with biological material? A single pulse of such high peak-power X-rays will blow up the sample. In an important paper, Hajdu and colleagues (Neutze et al. 2000) provided computer simulations of the predicted damage associated with photo-absorption and Auger electron emission and showed that coherently scattered X-rays from a single molecular complex could be collected before the sample explodes from Coulomb forces, provided that the pulse was short enough (in the range of femtoseconds). Later, it was calculated that some electronic damage (removal or rearrangement of electrons) may also take place on the femtosecond time scale (Hau-Riege et al. 2007). In simple terms, scattering takes place on the femtosecond time scale, while most damage takes place on the picosecond time scale. By recording scattering patterns from many protein complexes injected individually into the X-ray beam, orienting these patterns and averaging in order to enhance the signal from a single shot, a three-dimensional pattern can be obtained that can then be phased to produce a three-dimensional image of the scattering object. These calculations opened up the possibility of detecting scattered radiation from a single macromolecular complex and determining structure without the need for crystallization. Crystallization is the major rate-limiting step in any structural biology problem and there is the apprehension that some complexes, such as some of those embedded in the membrane or some very large assemblies, might never be amenable to crystallization. The implications for the use of FELs in structural biology are therefore enormous.

Direct phasing of the scattering pattern is possible because the sample is isolated and of known finite dimensions against a blank background. As first noted by David Sayre, phases can be retrieved by iterative phase-retrieval methods from the over-sampled scattering pattern (Fienup 1982; Miao et al. 2000, 2004; Marchesini 2007). Methods for three-dimensional reconstruction assume that the particles are reproducible for each shot. Sample variability will reduce the resolution obtainable, but these effects may be alleviated by grouping structures into distinct structural sub-states, as is done in single particle imaging by electron microscopy (Maia et al. 2009).

Spectacular proof-of-principle of the flash imaging procedure with very short exposures and computation of the reconstructed image came from work at FLASH, where a faithful image of a trial object composed of two cowboys (approx. 1 µm in size) etched onto a silicon nitride substrate was successfully achieved (Chapman et al. 2006). A year later (Chapman et al. 2007) in an ingenious time-delay holography experiment, the dynamics of polystyrene spheres in the intense FEL pulses was monitored with 32.5 nm radiation and 25 fs pulses each delivering $1.6 \times 10^{12}$ photons. An explosion occurred well after the
initial pulse (greater than 1 ps) and there were no observable changes up to 350 fs delay after the first pulse. In the first results for imaging a biological sample, Hajdu and colleagues (M. Seibert 2010, personal communication) report imaging of the mimivirus, a large virus with a viral capsid 0.45 μm in diameter presenting a pseudo-icosahedral appearance and which is covered by long fibrils. From images collected at LCLS with wavelength 6.9 Å, pulse length 70 fs and $1.6 \times 10^{10}$ photons $\mu m^{-2}$ delivered to the sample, a two-dimensional reconstructed image of 16 nm resolution was obtained showing the viral shell of protein and phospholipids and some internal structure.

With a FEL transverse coherence width that exceeds the beam diameter and a beam focus of a few micrometres diameter, it has become possible to record coherent hard X-ray diffraction patterns from individual micron-sized crystals (nano-crystals) comprising just a few (10–20) unit cells (Kirian et al. 2010). A nanocrystal with $10 \times 10 \times 10$ unit cells each of a 100 Å would give a crystal size of $0.1 \times 0.1 \times 0.1 \mu m$. With crystals, the Bragg scattering is proportional to the square of the number of molecules and hence the scattering will be greater than that for a single molecule and also stronger at higher resolution. Although this application of FEL radiation requires crystallization, it will be applicable to those macromolecular complexes that produce only nano-sized crystals, and with clever delivery systems it will allow pump–probe time-resolved experiments. The method was successfully demonstrated at LCLS for nano-crystals of photosystem I, a large complex that carries out the second step in the capture of light for photosynthesis in plants and cyanobacteria. Indexing of the diffraction patterns was possible with the existing MX software, but estimates of intensities from multiple still images required new software (Kirian et al. in press). Calculations suggest that images from approximately 10 000 nanocrystals can give a full three-dimensional dataset at 9 Å resolution. With the present 30 Hz repetition rate, the data could be collected in less than 6 min. However, the throughput is lower owing to the need to target a hit rate of about 20 per cent, so that patterns with multiple crystals are minimized. With 1.5 Å data, atomic resolution structures will be possible but this will require many more images.

Crucial to these experiments is the delivery of sample into the beam. Two devices have been developed for use at FELs that deliver a stream of hydrated proteins or nano-crystals into the beam with hit rates that vary from one per second to many per second (Bogan et al. 2008; DePonte et al. 2009). Hence, realistic data-collection times are more likely to be a few hours. New fast read-out area detectors have been developed that can match these data-collection rates (Denes et al. 2009; Strüder et al. 2010). For coherent diffraction imaging, the phase-retrieval methods demand a nearly complete dataset and hence the design of the backstop is crucial to avoid losing the low-resolution terms. Data-handling problems are immense. The field is in its infancy but shows exciting promise. The exploration of matter with hard X-ray FELs has shown some unexpected results. With neon as the sample target, it was observed that, during the course of a single approximately 20 fs pulse of hard X-rays at LCLS, rapid photo-ejection of inner shell electrons occurs that produces ‘hollow’ atoms and an intensity-induced X-ray transparency (Young et al. 2010). Under these conditions, the ratio of elastic to inelastic scattering increases. It is suggested that, for biomolecular imaging, an X-ray pulse shorter than the hollow atom lifetime should result in less radiation damage.
The principles of coherent diffraction imaging with short pulse FEL radiation also apply to micron-sized objects such as viruses, micro-organisms and human cells (Bergh et al. 2008). For imaging macromolecules at atomic resolution, radiation with wavelength of approximately 1–1.5 Å is needed, but for imaging cells at 1 nm resolution (say), softer X-rays in the ‘water window’ (between 2.3 and 4.4 nm) are preferable in order to provide contrast of the carbonaceous material against a water background. The application of soft X-ray coherent diffraction imaging has the potential to image cells at a higher resolution than can be achieved with an optical microscope and offers the opportunity to work with thicker specimens than possible in the electron microscope. Three-dimensional tomography will be necessary in order to provide detailed biological information (for example to distinguish the arrangements of subcellular organelles and protein complexes) but, with FEL radiation, only one shot is possible before the sample is destroyed. Several ways of providing three-dimensional imaging for non-reproducible objects have been proposed (Schmidt et al. 2008) but await experimental verification.

10. Concluding remarks

MX at SR sources is in a vibrant phase with many new biological problems to be addressed that technological advances have now made possible. Among such are membrane proteins that constitute approximately 30 per cent of the proteins encoded in the human genome but which have been difficult to express and crystallize. Their crystals are often small and fragile and benefit from the advances in crystal handling and the optical designs of modern SR experimental stations. Demand and supply of beam time are now more favourably matched than in the past, allowing experimenters more time to focus on the quality of data and experimental design. A significant trend has been the study of large assemblies and the correlation of X-ray diffraction results with those from small angle scattering (Mertens & Svergun 2010), for information on shape and size and response in solution, and with cryo-electron microscopy for the study of the whole assembly, where in some cases near atomic-resolution can be achieved (Zhang et al. 2008). X-rays and the electron microscope provide different but complementary ways of imaging in space and time (Zewail & Thomas 2010). The transformations in the past 30 years have been immense. The present results have given a breath-taking demonstration of the physical and chemical principles of protein topologies, protein assemblies and their biological function, but there is some way to go before we have a more complete picture of the architectural principles and dynamic properties that govern macromolecular complexes and assemblies within the cell (Harrison 2004).

The amazing achievements of synchrotron-based MX could not have been realized without the international openness and cooperation in the field where those acting behind the scenes often get little acknowledgement of their contribution. We thank all, past and present, near and far, for their contributions and discussions over the years, which have brought us to this point today and we look forward to those waiting in the wings to take the field into an undoubtedly exciting future.
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