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The Long-wavelength Macromolecular Crystallography I23 at Diamond Light Source

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Long-wavelength macromolecular crystallography (MX) has been proposed for a long time as a tool for phasing novel macromolecular crystal structures without additional heavy atom labelling. Making use of anomalous diffraction from atoms natively present in the crystal, such as sulphur and phosphorus, has become increasingly popular over the past years. Nevertheless, the full potential of this technique, has not been fully exploited due to lack of dedicated experimental setups able to easily access wavelengths longer than 2 Å. Since the wavelengths for the absorption edges of sulphur and phosphorus are significantly longer, standard beamline setups are not suitable to provide high-quality, high-resolution data, as the experiments are limited by the increasing absorption effects and the diffraction angles for longer wavelengths. Currently only two synchrotron beamlines offer access to optimised sample environments using wavelengths longer than 2.7 Å: BL1A at Photon Factory, Japan and I23 at Diamond Light Source, UK. Here, we describe the challenges and solutions implemented at the in-vacuum long-wavelength MX beamline I23 and present first results.

1. Introduction

Macromolecular crystallography is the most successful technique in structural biology, contributing to date more than 130,000 structures to the total 145,000 structures in the protein data bank. The success of this technique is owed to the significant investment in automation of all aspects of the structure determination pipelines, from cloning, protein expression and purification, crystallisation, automated sample changing and data collections, all the way through to automated processing and phasing. Recently, Grimes *et al.*¹⁾ reviewed these developments at Diamond Light Source with an outlook towards the future of MX.

The majority of MX experiments are nowadays performed at synchrotron beamlines, typically at wavelengths around 1 Å. The wavelength is an important parameter to select in an MX experiment and synchrotron beamlines typically cover a range from 0.7 to 2.0 Å. For crystal screening and

high resolution data collections, $\lambda = 1$ Å is a good compromise between diffraction and detector efficiency of Si based pixel-array detectors. Tuning the wavelength also allows exploiting anomalous dispersion, a resonance effect observed close to the characteristic absorption edges from elements present in the crystal structure. Three main applications exist to exploit the anomalous contrast in MX when tuning the wavelength close and across absorption edges: element identification, location of sulphur positions to assist model building and experimental phasing.

To identify particular anomalously scattering elements in the electron density, typically two data sets are needed, one above the absorption edge (higher energy or shorter wavelength side) and a second one below (lower energy or longer-wavelength side). Atoms from the element under investigation will show peaks in anomalous difference Fourier maps calculated from the data above the absorption edge, which are not present below the edge (or significantly

Table 1 Wavelengths and energies for elements of biological relevance in the tender X-ray range and their anomalous contribution f'' to the scattering factor for selected wavelengths.

	P	S	Cl	K	Ca
λ [Å]	5.7788	5.0155	4.3929	3.4369	3.0704
E [keV]	2.1455	2.4720	2.8224	3.6074	4.0381
f'' (1 Å)	0.17	0.24	0.31	0.48	0.51
f'' (2 Å)	0.71	0.90	1.12	1.68	2.01
f'' (3 Å)	1.45	1.82	2.24	3.26	3.89
f'' (4 Å)	2.34	2.90	3.55	5.55	6.68
f'' (5 Å)	3.41	4.09	5.00	8.81	10.01

reduced in the case of L or M absorption edges). This technique has recently been reviewed in Handing *et al.*²⁾

At low resolution, when side chains are not clearly resolved, it can be very difficult to build the amino acid sequence into the electron density. Anomalous difference Fourier maps from a long-wavelength dataset can facilitate model building by revealing the position of the sulphur atoms from methionine and cysteine residues, providing anchor points to fit the sequence into the density.

Experimental phasing by MAD (multi-wavelength anomalous diffraction) and SAD (single-wavelength anomalous diffraction) methods is a very powerful technique to overcome the crystallographic phase problem for structure determinations of novel structures, particularly when no homology model for molecular replacement exists. Anomalous scatterers can be introduced by soaking, co-crystallisation or by replacing the amino acid methionine by seleno-methionine.³⁾ The selenium K absorption edge is at $\lambda = 0.98$ Å making this technique easily applicable at synchrotron beamlines, for those cases where protein production and crystallisation are not adversely affected by seleno-methionine incorporation. Ideally, the intrinsic anomalous signal from sulphur should be sufficient for native phasing, however, with the sulphur K absorption edge at $\lambda = 5.02$ Å, this signal is weak within the wavelength range of standard beamlines.

While a first native phasing experiment from sulphur (S-SAD) was already described in 1981,⁴⁾ it was only in 2000 when a first novel structure was published.⁵⁾ However, it took another 10 years until more challenging crystal structures were reported⁶⁻⁸⁾ using standard MX beamline setups at wavelengths between 1.8 and 2.0 Å.

The absorption edges of the elements present in the naturally occurring amino acids are outside the typical wavelength range of synchrotron beamlines (Table 1). While C, N and O are in the soft X-ray range, the edges from phosphorus are in the so-called “tender X-ray regime”, still accessible with silicon based technology for both monochromators and detectors. However, the complexity is

increasing significantly when performing experiments with tender X-rays, or with wavelengths longer than 2 Å. This is due to two physical effects. Firstly, the X-ray absorption cross section increases approximately with the cube of the wavelength. Therefore, standard sample environments in air are no longer adequate, windows in the beam path have to be avoided and, at wavelengths close to the sulphur K absorption edge, even the sample will absorb most of the incoming photons, causing radiation damage. In addition, absorption corrections need to be seriously taken into account, to correct for the different absorption lengths of individual reflections, as function of sample size and morphology. Figure 1a shows the transmission through different thicknesses for lysozyme crystals as a function of wavelength, highlighting the effect of absorption.

The second aspect to consider at long wavelengths is the increase of diffraction angle. Bragg's law describes the relation between wavelength and diffraction angle 2θ for a given reflection (constant d-spacing) as $\lambda \sim \sin \theta$. Hence, diffraction angles increase significantly to longer wavelengths and standard flat detectors are no longer the most effective way to collect complete diffraction data (Fig.1b).

H. Stuhmann performed first pioneering experiments in the 1990s at wavelengths around the sulphur and phosphorus absorption edges, however, neither the sample environment nor the detectors available at this time allowed to perform a successful structure determination.⁹⁾ Synchrotron technology, in particular detectors, had to develop further until concepts for dedicated instruments optimised for long-wavelength MX, providing easy access to wavelengths $\lambda > 3$ Å could be developed and implemented. Currently, two such beamlines exist, BL1A at Photon Factory, Tsukuba, Japan¹⁰⁾ and I23 at Diamond Light Source, Didcot, United Kingdom.¹¹⁾

The long-wavelength MX beamline I23 is one of seven beamlines for macromolecular crystallography at Diamond Light Source. Beamlines I03, I04 and I04-1 mainly aim for highest throughput applications, with I03 also offering access to containment level 3 experiments. Beamline I24 was the first dedicated tunable microfocus beamline for MX,

Fig.1 a) Transmission for different path lengths through lysozyme crystals. b) Resolution as function of diffraction angle (2θ) for different wavelengths.

while VMXi was designed for in-situ screening experiments and VMXm will open new opportunities for diffraction experiments from sub-micron sized crystals. I23 is a beamline which is radically novel in its design, operating in vacuum, with a custom-made detector, allowing experiments to wavelengths as long as 5.9 Å. In the following this unique facility will be introduced.

2. The in-vacuum long-wavelength Beamline I23

The motivation to extend the accessible wavelength range beyond $\lambda = 2$ Å is to get access to the absorption edges of calcium, potassium, chlorine, sulphur and phosphorus, elements of high relevance in biology. In Table 1 the energies and wavelengths for these absorption edges are given and their anomalous contributions to the scattering factor f'' are presented. For sulphur f'' increases by about a factor of two when increasing the wavelength from $\lambda = 2$ Å to $\lambda = 3$ Å and even a factor three to $\lambda = 4$ Å. This significant increase of the anomalous signal can open new opportunities for native phasing experiments, towards structure solutions at lower resolutions or with lower sulphur content.

However, such experiments are not trivial as two main challenges have to be taken into consideration, the increasing diffraction angles and absorption effects (Fig.1).

2.1 In-vacuum crystallography

Performing experiments in vacuum addresses two important aspects, air absorption and air scattering. Diffracted X-rays are not attenuated between the crystal and the detector surface, making the experiments efficient in terms of radiation damage. Similarly, elimination of air scattering allows high signal-to-noise ratios by reduction of the noise.

The long-wavelength MX beamline is the first beamline for

macromolecular crystallography operated entirely in vacuum. The complete sample environment, including sample hotel for storage, goniometer and detector share the same vacuum space. The entire beamline is windowless, directly connected to the storage ring vacuum. The pressure difference from high vacuum (2×10^{-8} mbar) inside the I23 end station to the ultra-high vacuum of the storage ring is realised by differential pumping.

2.2 Sample holders & transfer

Sample cooling in vacuum has to be realised by thermal conduction. Heat is conducted from the sample through the sample mount, holder and goniometer to a pulse tube cooler (PTC) which is mounted on top of the vacuum vessel. This cooling path consists mainly of copper, with the flexible parts of the goniometer being realised by stacks of copper sheets. The temperature of the second stage of the PTC is 14 K under full thermal load, rising to around 40 K in the goniometer head. A good thermal contact between the goniometer head and the sample holders is realised by magnets providing a contact force of around 10 N.¹²⁾ Various materials were tested to support the crystals. A good compromise had to be found between thermal conductance, low X-ray absorption and scattering and mechanical properties for crystal harvesting. Litholoops (Molecular Dimensions) and laser cut mounts / loops from black kapton (DuPont) have been successfully used for experiments on I23. Both materials can be considered as thermal insulators, but due to the short distance (< 1 mm) from the tip of the copper pins to the crystal on the sample holders, cooling is sufficient to keep samples below the glass transition temperature during the experiments.¹³⁾ Figure 2 a and b shows details of the sample holders.

Samples are transferred into the vacuum end station

Fig.2 a) Image of the a litholoop as attached to the I23 sample holder. b) I23 sample holder with adaptor. c) Sample transfer block with 4 sample holders. d) I23 sample holders on adaptors mounted on a unipuck base for fast screening.

using a modified version of the Leica VCT100 system. Four samples can be transferred together per sample transfer block (Fig.2c). The transfer block is pulled from a liquid nitrogen bath into the transfer shuttle. A radiation shield inside the shuttle is kept cold by liquid nitrogen after pumping the shuttle. An air-lock system allows the insertion of transfer blocks into the sample hotel inside the vacuum end station using the shuttle while keeping the system under high vacuum conditions. 5 blocks with in total 20 samples can be kept in the sample hotel. To mount crystals onto the goniometer, the sample hotel can be rotated into the pick-up position. A linear mechanism with a gripper is used to mount and unmount individual samples from the transfer blocks onto the goniometer.

Sample transfer into the vacuum end station and inside, from the sample hotel to the goniometer, is slow, hence special adaptors have been designed which are compatible with the BART robots¹⁴⁾ available at the high-throughput MX beamlines I03, I04 and I04-1. This allows very fast and efficient crystal pre-screening. The best crystals will then be re-sorted from the unipuck bases (Fig.2d) on I23 transfer blocks (Fig.2c).

2.3 Detector

As illustrated above, large diffraction angles have to be accessible at longer wavelengths. The Pilatus 12M detector covers an angular range for diffraction experiments from $2\theta = -100^\circ$ to $+100^\circ$. Initial long-wavelength and vacuum tests for the Pilatus technology¹⁵⁾ were performed at the Diamond beamlines I18 and B16,¹⁶⁾ followed by a full characterisation of the technology at BESSY2.¹⁷⁾ The detector, designed

and built by Dectris (Baden, Switzerland), consists of 120 Pilatus2 modules arranged in a cylindrical geometry of 24 banks of 5 modules. The sensor modules are located inside the vacuum end station and are water-cooled to 12°C . 156 individual electrical feedthroughs connect the sensor modules with the detector readout electronics in a box outside vacuum attached to the back of the end station vacuum vessel. The detector can be moved 250 mm along the beam, with the sample typically being placed on the cylinder axis.

2.4 Goniometer

As for the detector, at the start of the project there was no commercial system available for the I23 goniometer. After discussions with several companies and engineering groups, the I23 in-vacuum goniometer was commissioned to the Astronomy Technology Centre (ATC), Edinburgh, UK. Their long-standing expertise in designing and building cryogenic positioning systems was key to this project. The goniometer is built in inverse kappa geometry¹⁸⁾ with an alpha angle of $\alpha = 50^\circ$. To facilitate the thermal design with the flexible copper cooling links, the overall rotation ranges of the goniometer had to be restricted for omega to $\omega \pm 270^\circ$, kappa to $\kappa - 10$ to $+180^\circ$ and phi to $\phi \pm 180^\circ$.

2.5 Software

Data acquisition at Diamond MX beamlines is achieved using the Generic Data Acquisition (GDA) system.¹⁹⁾ The GDA system user interface is shared across all MX beamlines and allows users to collect their datasets in a simple and hardware-safe fashion. More complex data collections such as the inverse beam or using a multi-axis goniometer are also easily utilisable. The latter type of data collections are both exploited by the strategy software ASTRA, developed by Global Phasing Ltd., in order to maximise the chances of collecting the best dataset for anomalous phasing.

Because the speed and the number of data collections have tremendously increased over the years, it is essential to be able to process datasets efficiently and quickly. Diamond has implemented a set of data processing pipelines using Xia2²⁰⁾ and autoPROC,²¹⁾ utilising Dials²²⁾ and XDS²³⁾ for exhaustive processing, but also fast-DP a near-real-time data processing software for quick assessment of the data. A second series of pipelines (post-processing) enable automatic molecular replacement against the collected data if a model is provided (DIMPLE),²⁴⁾ or structure solution by experimental phasing if some anomalous signal is detected (fast-EP and big-EP).²⁵⁾

Finally this overabundance of data needs to be easily accessible to users, hence a database ISPYB²⁶⁾ and its interface 'synchweb'²⁷⁾ have been developed, to guide users through their data collection and processing results. I23

data is presented in the same way as for all the other MX beamlines, with the special detector geometry, correction for detector shadowing automatically taken into account by the processing pipelines.

3. Results

Several novel structures have been solved based on the long wavelength data collected at I23 over the past two years.²⁸⁻³³⁾ The first published structure was that of ThcOx, an oxidase protein from the cyanobactin pathway.³⁴⁾ Crystals were highly non-isomorphous and varied in diffraction quality. Molecular replacement failed, labelling with SeMet was consistently unsuccessful and, despite various attempts to bind heavy atoms, it was not possible to find a derivative to allow phasing. The structure could be solved from data collected at a wavelength of $\lambda = 3.096$ Å from a crystal diffracting to 3.15 Å, with anomalous data extending to only 4.2 Å. While for native phasing experiments at wavelengths around $\lambda = 2$ Å typically high multiplicity data is needed (~ 100), for ThcOx 400° of diffraction data, resulting in a multiplicity of 26, was sufficient to solve the structure. Figure 3a shows the ThcOx structure with the anomalous difference Fourier map (5σ).

The salmonella-secreted effector SseK3 (PMID: 29449376) was also solved by SAD,³⁰⁾ but in this case the data showed some pathologies, such as pseudo-translation and anisotropy, potentially explaining the difficulty of solving the structure on standard beamlines. 1440° of data were collected with 0.1 s exposure and 0.1° oscillation from a single crystal containing seleno-methionine labelled proteins, at a wavelength of 2.755 Å. The position of the selenium and sulphur atoms could be found in SHELXD and confirmed the presence of four SseK3 in the asymmetric unit. At the experiment wavelength, the Se anomalous signal is still stronger than the S one, hence the highest anomalous peaks correspond to Se. After phase improvement, most of the structure could be built automatically. The anomalous difference Fourier map (5σ) is displayed in Fig.3b.

A third example, demonstrating the capabilities of the beamline towards high-resolution data collections, is the structure of the plastic degrading enzyme PETase.²⁹⁾ Phases could be obtained from a first crystal based on data collected at a wavelength of 2.455 Å and data to 1.7 Å resolution. 3600 images collected with 0.1 s exposures per 0.1° per image were of sufficiently high quality to solve the structure. A second crystal with different morphology turned out to be a different polymorph, with the space group being $P2_12_12_1$, rather than $C222_1$, the crystal used for phasing had belonged

Fig.3 a) anomalous difference Fourier map (5σ) for ThcOx. b) anomalous difference Fourier map (5σ) for SseK3. Electron density ($2F_o - F_c$, 1σ) around Trp257 of PETase.

to. As the crystal diffracted to the edge of the detector at longer wavelengths, a wavelength of 1.240 Å was chosen and diffraction data to a resolution of 0.92 Å were collected from 1800 images at $\kappa, \phi = 0^\circ$, followed by 800 additional images collected opening the kappa arm of the goniometer to $\kappa = -50^\circ$ ($\phi = 70^\circ$) for complete diffraction data. The combination of low-background in-vacuum crystallography, the kappa goniometer and the curved detector allowed to collect very high quality data without any signs of radiation damage from this important enzyme. Part of the electron density ($2F_o - F_c$, 1σ) is shown in Fig.3c.

4. Summary & Outlook

The long-wavelength MX beamline I23 at Diamond Light Source has been a very challenging project. The novel approach to establish in-vacuum crystallography required several bespoke solutions which have been discussed above.

The next generation cryogenic vacuum transfer system, including a new transfer shuttle and air-lock is currently being built and will soon replace the existing Leica VCT100 system. While the original system was designed to transfer grids for cryo-electron microscopy, the new system will be optimised to transfer crystals on pins.

While the beamline can access wavelengths up to 5.9 Å, for native phasing based on the anomalous signal from sulphur, a wavelength of 2.75 Å has been found to be a good compromise between the increase of the anomalous signal towards the sulphur edge and the increased sample absorption effects, compromising the data quality at longer wavelengths. So far, only standard empirical absorption corrections have been applied, as implemented in XDS/XSCALE or Aimless. To apply analytical absorption corrections based on the knowledge of the individual path lengths through diffracting and non-diffracting material for every reflection, the shape and volume of the sample need to be known. X-ray tomography can provide this information. Hence, an X-ray tomography camera has been incorporated into the I23 vacuum end station. After the diffraction experiment, an additional tomography experiment can be performed, followed by reconstruction and segmentation. As both experiments are performed with the same goniometer, the orientation of the crystal will be preserved.^{35,36)} A second approach towards longer wavelengths is to use laser shaping to remove excess non-diffracting material and to cut crystals into known shapes, ideally spheres.³⁷⁾ This technology will make absorption corrections trivial and open new opportunities for macromolecular crystallography, such as native phasing from increasingly difficult projects at lower resolution close to the sulphur and phosphorus edges and potentially allow measurement of anomalous signals from both sodium and magnesium.

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