



CRYSTALLOGRAPHY WITH BIOLOGICAL MICRO-CRYSTALS

**H. BELRHALI¹, A. BRAM^{1,2},
S. CUSACK³ AND C. RIEKEL¹**

1 ESRF, EXPERIMENTS DIVISION

2 SIEMENS MICROELECTRONICS CENTER GMBH, GERMANY

3 EMBL GRENOBLE OUTSTATION c/o ILL, GRENOBLE



From left to right:
S. Cusack, C. Riekkel and H. Belrhali.

Crystals of biological macromolecules are produced from solution by decreasing the solubility of the macromolecules often by using a «precipitant». Crystallisation of biomacromolecules is still as much an art as a science and if the first hurdle is to get any crystal at all, the second hurdle is to get a crystal of sufficient quality and size for crystallographic data collection.

This is required because the intensity I_{hkl} of a diffracted ray by a crystal is proportional to the ratio of its diffracting volume V_{χ} to its unit cell volume V_c and also to the incident beam intensity I_0 :

$$I_{hkl} \propto (V_{\chi} / V_c) \times I_0$$

For macromolecules, V_c is generally large (typically 10^6 \AA^3) and the total diffracted intensity is split up into many hundreds of weak reflections. Crystals used for most biocrystallographic experiments on standard synchrotron sources therefore need to be typically a few hundred micrometers in linear dimensions. However it is often much easier to obtain micro-crystals up to a few tens of microns in size. The highly focussed intense undulator beams available on third generation synchrotron radiation sources now permit good quality data to be obtained on such previously unusable crystals.

In order to test this approach we have used the Microfocus beamline (ID13) with the aim of obtaining complete high resolution data set

collections on micro-crystals. The high flux density of the Microfocus beamline is particularly adequate for these studies but the associated problems of sample manipulation, visualisation and sample stability need also to be addressed.

As shown in Figure 1, the flux density at the sample position can be increased by focusing the beam. The focusing element on ID13 is an ellipsoidal mirror which produces a beam of approximately $30 \mu\text{m}$ with a gain factor of $\geq 7 \times 10^3$ as compared to a collimated beam. In the case of larger crystals, a microfocused beam could also be used as a probe to screen perfect sub-volumes or to irradiate fresh parts of crystals. The principle of the method is also shown in Figure 1.

INSTRUMENTATION

Experiments were performed with a monochromatic beam (Si-111) at a wavelength of 0.688 \AA . The beam

divergence at the sample position was $2.3(h) \times 0.44(v) \text{ mrad}^2$. The estimated flux in the $30 \mu\text{m}$ beam at the sample position was about 5×10^{10} photons/s when the synchrotron was operated in the 16-bunch mode. Under optimal conditions the flux can become $>10^{12}$ photons/sec.

The precise ID13 Nonius κ -goniometer was used for the experiments. A 30 cm MAR image-plate detector was installed and synchronised with the SPEC instrumental control system. Samples, held in tiny loops, were cooled to 100° K by an Oxford Cryostream[®] system. Crystals were optically centered on the goniometer by a QUESTAR[®] long-distance microscope. Fine tuning of the sample position in the beam was done in real time by observation of the diffraction pattern using an "X-ray eye" CCD camera. Translation of the whole goniometer, with micrometer precision, was also used to expose fresh volumes in the case of large crystals.

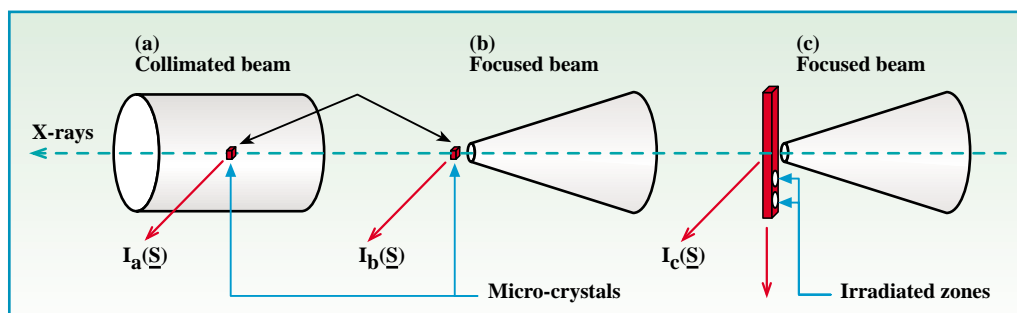


Fig. 1:
Intensity $I(S)$ diffracted from a micro-crystal in (a) a collimated and (b) a focused X-ray beam and (c) by a macro-crystal in a focused beam.



Macromolecules	Histidyl-tRNA synthetase + Histidine + AMP CPP + Mn ⁺⁺	Asparaginyl-tRNA synthetase	Prolyl-tRNA synthetase + tRNA ^{Pro}	T Protein:DNA	p52:DNA	Bacteriorhodopsin	HIV-1 p24:fab
Space group	P21212 (Orthorhombic)	P6422 (Hexagonal)	P4X212 (Tetragonal)	P212121 (Orthorhombic)	I212121 (Orthorhombic)	P63 (Hexagonal)	C2 (Monoclinic)
Cell parameters (Å, Å, Å // β*)	175, 215, 49	125, 125, 123 // 120	143, 143, 231	39, 113, 147	45, 129, 132	62, 62, 104 // 120	194, 48, 191 // 92.4
Crystal dimensions (mm ³)	40x40x100	30x30x50	100x100x100	100x100x80	500x80x50	30x50x5	100x100x20
Resolution limit (Å)	~2.9	~2.3	~3.5	≥ 2.00	~3.8	≥ 2.3	~4.0
Comments	92 % complete Rsym = 9.0 %	80 % complete Rsym = 15 % 3 translations	rapid decay 4 images	78 % complete Rsym = 8.4 % 2 translations	80 % complete Rsym = 8.0 %	91 % complete Rsym = 10.5 %	86 % complete Rsym = 12 %
Experimenters	H. Belrhali (ESRF) A. Yaremchuk (EMBL)	C. Berthet -Colominas L. Seignovert (EMBL)	S. Cusack, A. Yaremchuk (EMBL)	C. Müller (EMBL)	P. Cramer, C. Müller (EMBL)	E. Pebay-Peyroula (IBS), E. Landau and G. Rosenbusch (BIOZENTRUM)	C. Berthet- Colominas S. Cusack (EMBL)

Table 1: Macromolecule micro-crystals used on ID13 (beam size = 30 μm φ, λ = 0.6883 Å).

EXPERIMENTAL RESULTS

Using the experimental set-up described above, a number of interesting biological crystals have been examined (see Table 1). Most of the experiments, except the one with prolyl-tRNA:tRNA^{Pro} complex, led to usable data comparable in quality to data sets recorded on other beamlines, if not better. This is particularly true for the bacteriorhodopsin project, where for the first time ever three-dimensional crystals were measured to high resolution giving data from which a 2.5 Å crystal structure has been determined. Exposure times per image were of the order of 60 seconds. We note that patterns of comparable intensity require a 100 μm beam on ID2. This could be further improved by increasing the number of undulator segments on ID13.

In the case of the T protein: DNA complex project, the available crystals were mosaic. The use of the microfocused beam allowed the screening for more perfect sub-volumes of those crystals, allowing the collection of a complete high resolution data set. Similarly in the case of the HIV-1 p24/Fab complex, single crystal regions of a macroscopically twinned crystal could be picked out.

The price to pay for the high flux density is a faster radiation damage of

the crystals even at cryo-temperatures. Most of the samples showed a shorter lifetime in the beam compared to previous experiments performed on beamlines with less flux density. Larger crystals were systematically translated during the data collection in order to expose fresh volumes.

PERSPECTIVES

The results described above are very promising. However, before micro-diffraction on biological crystals becomes a routine technique, several points have to be addressed systematically.

Micro-crystal manipulation

It is indeed very difficult to “fish” micro-crystals using the classical stereo zoom microscopes. Higher resolution microscopes and alternative ways of fishing the crystals have to be explored, perhaps using micro-manipulators.

Sample observation

We encountered difficulties in the handling of very small crystals and in particular to observe them clearly while centering on the goniometer. In the frozen droplets, the crystals are extremely difficult to distinguish not only because of their size but also because of the frozen mother liquor droplet that surrounds each of them and produces light reflections.

We are presently exploring the use of crossed polarizers in order to enhance the visibility.

Sample lifetime

The lifetime of the crystals in the beam has to be increased. Lower temperatures and/or shorter wavelength could be explored. However it could be that the intrinsic limit due to primary radiation damage is being reached.

Detector

A large area CCD detector would increase the data collection rate, and therefore would permit a complete data set to be collected in a shorter time and allow at the same time φ-slicing to improve the signal-to-noise ratio. We aim to install a 2000 x 2000 pixel CCD detector with a read-out time of less than 10 seconds. In contrast the read-out time of the 30 cm diameter MAR image plate is about 200 seconds!

CONCLUSION

We have demonstrated the feasibility and utility of protein micro-crystal diffraction on ID13. For problems in which there are only micro-crystals (for example bacteriorhodopsin), ID13 now makes crystal structure determination possible. Development of appropriate tools for sample observation, manipulation and storing is required. ■

ACKNOWLEDGEMENTS

The hardware and software were set up by a combined EMBL-ESRF JSBG team. The authors would like to thank many people who have contributed to this project, i.e., C. Berthet-Colominas, J.-M. Bois, M. Chiu, L. Claustre, P. Cramer, P. Engstrom, S. Fiedler, F. Felisaz, E. Landau, F. Lapeyre, S. Monaco-Malbet, C. Müller, E. Pebay-Peyroula, D. Pognant, V. Rey Bakaikoa, G. Rummel, L. Seignovert, M. Tukalo, S. Wakatsuki, P. Wattecamp and A. Yaremchuk.