New Trends in Macromolecular Crystallography at High Hydrostatic Pressure

R. Fourme[#], I. Ascone[+], R. Kahn[*], E. Girard[*], M. Mezouar [++], T. Lin[§], and J. E. Johnson[§]

[#] SYNCHROTRON SOLEIL, bât. 209H, Université Paris-Sud, 91898 Orsay cedex, France
[+]LURE, bât. 209D, Université Paris-Sud, 91898 Orsay cedex, France
[*]IBS, 41 rue Jules Horowitz, 38027 Grenoble cedex, France
[++]ESRF, BP220, 38027 Grenoble cedex, France
[§]Department of Molecular Biology, the Scripps Research Institute, 10550N, Torrey Pines Road, La Jolla, CA 92037, USA

roger.fourme@soleil.u-psud.fr

Abstract. Publications on structures of macromolecules under high pressure determined by X-ray crystallography were limited until recently to two small monomeric proteins, hen egg-white lysozyme at 1 kbar (Kundrot and Richards, 1987) and sperm whale myoglobin at 1.5 kbar (Urayama et al., 2002). These studies were performed with a polycrystalline beryllium cell. Technical limitations were the low pressure limit (2 kbar), the lack of optical survey and noisy diffraction patterns due to scattering of X-rays by beryllium. Using the high pressure beamline ID30 at the ESRF, we achieved a technical breakthrough by the combination of a diamond anvil cell, ultra-short wavelength X-rays from undulators and a large imaging plate. The field of high-pressure macromolecular crystallography was extended both for the accessible pressure range (increased by nearly one order of magnitude) and data quality. Results obtained on tetragonal hen egg-white lysozyme crystals (tHEWL) at 7.0 kbar demonstrate that high quality diffraction data can be obtained under high pressure. In the case of tHEWL, data at 1.6 Å resolution were measured on two crystals. The structure was redetermined at high pressure and refined. A detailed comparison between the 1.6 Å structures at normal and high pressure is given. Another protein is under study, (Cu,Zn) superoxide dismutase (SOD). Good orthorhombic crystals were preserved beyond 12 kbar, which is noteworthy as the functional unit of SOD is a dimer. Data collection at 9 kbar is in progress. The following step is a preliminary study of cowpea mosaic virus crystal (CPMV), the first example of crystallized macromolecular assembly studied at high pressure. Oscillation pictures of a cubic crystal of CPMV were recorded at several pressures up to 4.4 kbar. The initial crystal (space group P23 at 1 bar, preserved at 1.1 and 2.0 kbar) was disordered and diffracted to low resolution. At 3.3 kbar, a highly ordered I23 crystal was obtained, which diffracts to 2.6 Å resolution with high signal-to-noise ratio. At 4.4 kbar, the crystal no longer diffracted.

R. Winter (ed.), *Advances in High Pressure Bioscience and Biotechnology II*

1 Introduction

Crystallography is the physical method which can provide the most accurate global description of three dimensional macromolecular structures. As such, it is of particular interest for high pressure studies. Yet, the development of high pressure macromolecular crystallography (HPMX) has been slow. The main reasons are the following: the confinement of a single crystal in a high pressure cell adds constraints to normal data collection; the communities of macromolecular crystallographers and high pressure specialists are very weakly connected; and further and perhaps more importantly, the scientific interest of structural biologists for HPMX was modest. The pionneering study published in 1987 on the structure of tetragonal hen egg-white lysozyme (tHEWL) at 1 kbar [1] was performed using a beryllium cell with a pressure limit of about 2 kbar [2] and a rotating anode X-ray source. This device did not allow optical observation of the sample; it produced strong parasitic scattering and diffraction and had a low transmission at the X-ray wavelength used. Beryllium cells have been used in the last few years to study structures of two other small monomeric proteins, *staphyloccocal* nuclease [3] and sperm whale myoglobin [4,5]. In 1996, a diamond anvil cell (DAC) was coupled to a synchrotron radiation source to measure the compressibility of tHEWL crystals, but without follow-up data collection [6]. The next step forward came from our group in 2001, opening the field of HPMX at pressures beyond 2 kbar [7]. High quality diffraction pictures were obtained for protein crystals of monomeric (tHEWL) and dimeric CuZn superoxide dismutase (SOD) [7,8], then for crystals of a complex macromolecular assembly, cowpea mosaic virus (CPMV) [8]. Finally, acquisition of nearly complete data on tHEWL crystals compressed at 7 kbar was performed. The 7 kbar crystal structure was solved at 1.6 Å resolution and compared to the 1 bar structure at the same resolution [9]. A similar work is planned on CPMV.

This article presents an overview of the current state and future prospects of these developments.

1.1 Interest of Crystalline State and Structural Information Derived from HPMX

A macromolecular crystal - a well-defined sample which can be reproduced at will through given crystallisation procedure - has a structure and properties which are of great interest for high pressure studies. This particular state of matter is a periodic array of densely packed molecules in a solution. Its long range order may be extremely good, as shown by fwhm rocking curve widths of Bragg reflections in the range 0.01-0.001° [10,11]. This semi liquid/semi solid medium features significant plasticity and capacity to cure damages and to accommodate large changes in specific volume induced by pressure variations. The liquid phase within the crystal communicates with the external solvent through structural channels, hence the compression of the biopolymer remains hydrostatic, provided that pressure ramp is

sufficiently slow and continuous [7]. For these reasons, these crystals can withstand large variations of pressure while keeping excellent long range order. This has been observed consistently during all our experiments. In particular, for SOD crystals, pressure variations in excess of 10 kbar have been observed both by X-ray diffraction and IR spectroscopy [12]. Finally, as the unit cell is large, the number of Bragg reflections collected through a small oscillation of the crystal is so large that indexing a single diffraction picture is generally trivial. It is interesting to note that the fragile structure of a macromolecular crystal and the complexity of its components are indeed advantages for high-pressure crystallographic studies.

Information which can in principle be derived from HPMX is very rich:

(i) Information derived from a detailed analysis of the reciprocal space, including long and short range order, disorder and correlated motions, through mosaicity, resolution of diffraction data and diffuse scattering pattern respectively.

(ii) Global compressibility of crystal, through measurements of unit cell parameters.

(iii) Atomic coordinates and temperature factors of protein atoms and other ordered components (water molecules, ions), which give access to molecular architecture, inter- and intra-atomic distances, local compressibility of e.g. secondary structure motifs, mapping of cavities and solvent accessible surfaces.

Changes induced by pressure may be subtle, and their description and the characterization of their variations require accurate structural analysis. Hence, acquiring high quality diffraction data (in terms of accuracy, resolution and completeness) is a central goal for HPMX.

2 Instrumentation and Methods

Our experiments have been performed on ID30 at the ESRF (Grenoble), an undulator beamline which can be flexibly adapted to a variety of high pressure studies. Key instrumental features are a DAC, an undulator X-ray beam and a large area detector [7].

In the DAC, X-rays travel through two diamond anvils and the useful optical aperture is limited. This led us to use X-rays of ultra-short wavelength (<0.4 Å), with the following advantages: absorption and extinction by diamonds are reduced, X-rays diffracted by the crystal are confined within a narrow forward cone and long crystal-to–detector distances are required, which is beneficial for signal to noise. The counterpart is that the beam must be very intense (as the scattered intensity varies as $\sim\lambda^2$) and parallel (in order to get small spots on the distant detector). The solution is an undulator on a high energy storage ring without focusing of the monochromated beam. The single undulator currently installed on ID30 will be replaced by a pair of narrow gap in-vacuum undulators, increasing brightness by a factor ~8.

The detector should be large in order to be able to collect high resolution data in spite of the long crystal to detector distance, together with a good detective quantum efficiency (DQE). We have used a MAR image plate detector with a circular imaging plate of diameter 345mm. The wavelength which was chosen for this de-

tector is 0.3305 Å, near the Ba-K absorption edge. In effect, the sensitive layer of the imaging plate contains barium and the DQE of the detector is essentially governed by the stopping power of this element. At this wavelength, DQE is maximal at the energy of elastically scattered photons (Thomson scattering), but is lower for photons inelastically scattered by diamonds (Compton scattering) which have a lower energy, below the energy of the Ba-K absorption edge. Accordingly, useful signal is increased while background noise is decreased. As the energy change of Compton photons is resolution-dependent, it turns out that the improvement in the signal-to-background ratio is largest where most useful, i.e. in the high resolution range of the diffraction pattern where signals are weakest [7,8].

Two DAC of the cylinder/piston type [13], where thrust is generated by a toroidal stainless steel membrane [14], were used alternately during experiments. The procedure used to confine and compress the sample is standard [15]: the compression chamber is a cavity limited by the culets of the two diamonds and the wall of a cylindrical hole (diameter up to 400 μm) produced by spark erosion in an inconel gasket (thickness 180μm). A sample (maximum dimensions 400 x 400 x 150 μm^3) is selected from a batch of crystals grown in standard conditions; it is mounted in the cavity filled with either the mother liquor or a solution with a composition purposely adjusted to improve the stability of the crystal under high pressure. Squeezing the gasket seals the cavity. By gradually increasing the thrust applied to the DAC piston, the volume of the cavity is reduced with a concomitant increase of pressure within the enclosed liquid. Pressure is measured through the wavelength shift of a fluorescence line [16], excited by laser light, of a tiny ruby chip placed in the cavity. The sensitivity and precision of such measurement are respectively 20 and 100 bar. A commercial computer-controlled device is used both for optical observation of the crystal and for excitation and collection of ruby fluorescence. Once loaded, DAC takes place on a goniometer which is provided with three micrometric orthogonal translations and three degrees of rotation. A computer-controlled-procedure ensures the centring (accuracy 2 μm) of the crystal at the intersection of the vertical axis of rotation and of the X-ray beam axis.

Data collection proceeds by recording adjacent oscillation photographs about the vertical axis of the goniometer. In the normal filling mode of ESRF (intensity of the stored electron beam 180 mA), a typical one degree of oscillation exposure is 40-60 s for small proteins (tHEWL and SOD); for CPMV, exposure is 360 s for a 0.3° oscillation. As mentioned previously, a pair of narrow gap undulators will hopefully reduce exposures by a factor ~ 8.

There are two limitations in high pressure data collection. The first is the limited rotation range allowed by current DAC. Complete data sets from a single sample can be collected only from high symmetry space groups. The best case is obviously cubic space groups (this is the case for CPMV). For tetragonal HEWL, a nearly complete data set was obtained using only one pre-aligned crystal. For lower symmetry space groups, several crystals with different orientations are required. Samples with a very anisotropic shape tend to orient with the largest face parallel to diamond culets. This was the case for platelet-shaped SOD crystals for which the completeness of collected data is not yet sufficient. The second is that cryocooling the crystal is not possible, as hydrostatic compression must be preserved. To alleviate faster degradation under X-ray irradiation, the crystal is translated by incre-

mental steps (typically 20 μm x 20 μm) during data collection, taking advantage of the small cross-section of the X-ray beam to irradiate successively fresh portions of the sample. In the case of tHEWL and SOD crystals, this procedure proved quite effective; appropriate rotation ranges were 15-20° for each irradiated portion.

3 Results and Discussion

3.1 Studies on tHEWL

The first crystal structure of an enzyme determined by X-ray diffraction was that of lysozyme. This protein is probably the one that has been most extensively studied by various high pressure techniques; further, it is easily crystallized. We have used the tetragonal form (tHEWL) both as a development tool in our experiments and for the accurate description of the structure at unprecedented high pressure (7 kbar).

The compressibility of the unit cell was determined in the range 1 bar-8.2 kbar from 24 accurate measurements of unit cell parameters. In this pressure range, the average compressibility is ~0.01 $kbar^{-1}$. This compression is strongly anisotropic, as the length of the c axis of the tetragonal cell is nearly invariant [7].

Then, we studied the crystal behaviour in the range 8.2-9.2 kbar, using successive recordings of the same oscillation picture. In this pressure range, the evolution of the diffraction pattern and diffuse scattering shows a perturbation of the perfection of the crystal structure and finally a complete loss of three dimensional order [7]. These observations might be a consequence of partial or complete denaturation of lysozyme molecules induced by pressure; another hypothesis which is also considered after our analysis of molecular packing at 7 kbar is that, as pressure increases, packing becomes so tight that the crystal structure is destroyed. Clearly, further experiments are required.

Taking 8 kbar as upper limit, we found that between 1 bar and 8 kbar the crystal order remains extremely good (Fig.1) and that the crystal behaviour is reversible. Diffraction data sets were collected at several pressures in this range. Using improved experimental procedures, the best data were acquired at 7 kbar using two crystals [8]. Diffraction pictures were analysed without applying absorption correction. Structure factor amplitude data from the two samples were merged to get a single set at 1.6 Å resolution (HP). Starting from 1.33 Å resolution data on tHEWL at 1 bar obtained using high quality space grown crystal [17], we took as reference a subset of these data, with resolution limited to 1.6 Å (REF). The REF and HP structures were determined and refined in parallel. In order to make a comparison with minimal bias, the same programs, procedures and cut-off limits were used at for molecular replacement, automatic interpretation of electron density (wARP) and refinement (CNS). In table 1, preliminary results on the two structures are given. Fig.2 and 3 show respectively a portion of the electron density of the HP structure and superimposition of molecules in the REF and HP structures. A more

thorough analysis - including in particular the analysis of cavities, solvent accessible surfaces, intermolecular distances and hydration network - will be published elsewhere [9].

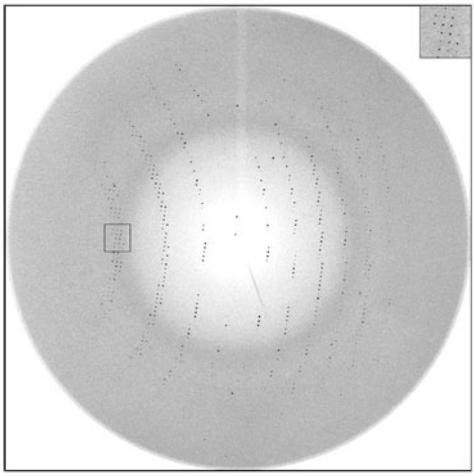

Fig. 1. tHEWL crystal at 7 kbar. Oscillation picture (0.75°). Wavelength 0.311 Å, exposure time 120 s at I = 70 mA

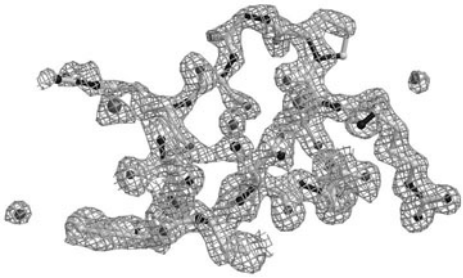

Fig. 2. Representative portion of the electron density of tHEWL at 7 kbar with the molecular model of the refined structure superimposed

Important conclusions from Table 1 are the following:

First, data quality of the HP set is unprecedented and close to usual standards. The slight difference between the two data sets comes essentially from absorption by diamonds and could be significantly reduced by calculated absorption correction. The resolution of the HP set is quite respectable, yet signal to noise and room temperature data collection would make difficult the measurement of data to the intrinsic resolution of the 1 bar set.

Second, the quality of the electron density in the HP structure is high (Fig. 2). Crystallographic results are accurate, according to crystallographic and free R factors and usual checks for testing the quality of structure determination.

Third, differences between the two structures are important but come primarily from the more compact packing of the HP structure. Changes to lysozyme molecules are generally limited and average temperature factors are only slightly smaller in the HP structure.

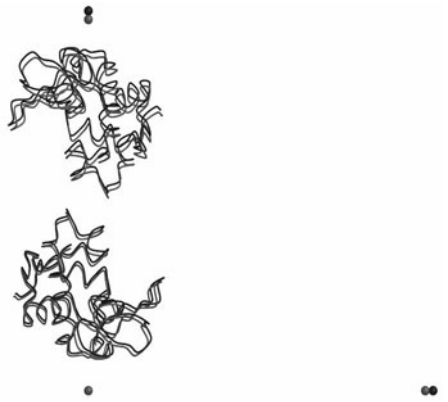

Fig. 3. Superimposition of tHEWL molecules at 1 bar and 7 kbar. Limits of a and b axis of tetragonal unit cells are represented by dots. (blue: 1 bar; red: 7 kbar)

3.2 Studies on CPMV

For the first time, crystals of a large macromolecular assembly, CPMV, have been studied by HPMX [8]. Yet preliminary, promising results have been obtained which are yet to be consolidated and extended to other systems.

CPMV is a member of the Comovirus family, a group of icosahedral plant viruses. Cubic and hexagonal crystals can be grown. When crystals of CPMV are grown in standard conditions, rhombic dodecahedral crystals are obtained. About 10% of these crystals conform strictly to the characteristics of space group I23 (non-extinction rule of the body centered space group : $h + k + l = 2n$). These crystals are good diffractors. The other crystals have also a cubic unit cell with the same parameter, but the diffraction pattern shows additional weak reflections with $h + k + l = 2n + 1$ (odd reflections). Intensities of odd reflections vary from one crystal to another. The space group P23 was tentatively assigned to these crystals, and the relationship between the I23 and P23 cell is interpreted as a first order transition of a special kind: with respect to the standard positions in the ordered I23 structure, virus particles are rotated randomly by about 7.6° [18].

Table 1. Summary of experimental parameters

	HP (7 kbar)	REF(1 bar)
Diffraction data		
Crystals	2, lab.-grown	1, space-grown
SR beamline	ID30, ESRF	W32, LURE
X-ray source	undulator	5 pole wiggler
Wavelength	0.3305 Å	0.90 Å
Detector	IP diam. 345 mm	IP diam. 180 mm
Resolution of collected data	1.6 Å	1.33 Å
Resolution used for refinement	1.6 Å	1.6 Å
Absorption correction	no	no
Completeness	93%	94%
Rsym (on I) for collected data	5.7%	4.2%
Redundancy	7.2	not specified
Refinement		
Rcryst	20.3%	17.5%
Rfree	24.9%	20.3%
Structural results		
Ordered water molecules	136	135
Na^+ ions	1	1
Cl^- ions	7	6
Double conformations	Ser 85, Ser 86	Lys1, Val 109
Aver. B, protein atoms	16.0 $Å^2$	16.9 $Å^2$
Aver. B, main chain	14.3 $Å^2$	15.1 $Å^2$
Aver. B, side chains	17.7 $Å^2$	18.9 $Å^2$
Aver. B, water molecules	30.8 $Å^2$	34.4 $Å^2$
RMS difference between atomic positions in RF and HP molecules:		
main chain	0.23 Å	
side chains	0.53 Å	

The structure at atmospheric pressure was solved and refined at 2.8Å resolution, using I23 crystals and synchrotron radiation [19]. The structure, assembly and molecular biology of CPMV were the subject of extensive fundamental studies leading to, among others, its use as a carrier of heterologous antigens allowing CPMV to function as a “dead virus” [20].

A DAC was loaded with a rhombic crystal in stabilizing liquor. This crystal was compressed by steps up to about 3.5 kbar and oscillation pictures (0.3°) were quickly recorded at each step. Diffraction was observed up to the maximal pressure. A second crystal was mounted and well-exposed oscillation pictures (0.3°) were collected at 1, 2, 3.3 and 4.4 kbar. At 1 and 2 kbar, pictures display odd reflections, diffuse scattering and the resolution is poor. At 3.3 kbar, only even reflections are seen and reflections are visible to 2.2 Å. At 4.4 kbar, diffraction was lost.

Conclusions are the following :

First, cubic CPMV crystals withstand high pressure to at least 3.5 kbar.

Second, pressure induces a disorder-to-order transition, by which a poorly diffracting P23 crystal is transformed into a well diffracting I23 crystal.

Third, the feasibility of quality HPMX has been demonstrated for a complex assembly with a unit cell parameter in excess of 300 Å.

We have now two targets for the short term. The first is the collection of a complete data set for the compressed I23 structure which will be used for the refinement of this structure, in order to get an accurate description of pressure-induced changes. The second is investigation of the effect of pressure on resolution for crystals of various assemblies to check whether, as expected from thermodynamic considerations, the ordering effect of pressure on crystals is more general than the very peculiar case of CPMV or not.

4 Conclusions

Many applications can be anticipated for HPMX [8]. If it turned out that pressure can be used as a routine tool for improving crystal quality, applications for macromolecular crystallography would be tremendous. Other most important applications might be (i) the use of pressure to modify Gibbs free energy in a large domain, for the exploration of phase transitions and protein substates (ii) the detailed analysis of interactions (between proteins within the crystal, monomers of an oligomeric protein or components of a complex assembly; water-water and water-molecules interaction) (iii) study of first steps of denaturation induced locally by pressure.

Such studies will require time on beam lines equipped for high pressure studies at third generation storage rings of intermediate or high energy. Presently, such beam lines are scarce and heavily overbooked. If HPMX is to be developed, decisions about the sharing of beam time between various fields on existing beam lines are urgently required. Further, new investments in SR beam lines should be planned.

References

[1] Kundrot, C.E. and Richards, F.M. (1987). J. Mol. Biol. *193*, 157-170.

[2] Kundrot, C.E. and Richards, F.M. (1986). J. Appl. Cryst. *19*, 208-213.

[3] Ekstrom, J.L., Ealick, S.E., Osterberg, F.H.O. and Gruner, S. (1995). Effect of pressure on protein dynamics: a crystallographic study. Am. Cryst. Assoc. Meet., Montreal, Canada Collected Abstracts A319.

[4] Urayama, P. (1999) High pressure crystallographic structure of myoglobin. IUCR meeting, Glasgow, UK. Collected Abstracts M11.0c.005.

[5] Urayama, P., Phillips, G.N., and Gruner S.M. (2002). Structure *10*, 51-60.

[6] Katrusiak, A. and Dauter, Z. (1996). Acta Cryst. D*52*, 607-608.
[7] Fourme, R., Kahn, R., Mezouar, M., Girard, E., Hoerentrup, C., Prangé, T. and Ascone, I (2001). J. Synchrotron Rad. *8*, 1149-1156.
[8] Fourme, R., Ascone, I., Kahn, R., Mezouar, M., Bouvier, P., Girard, E., Lin, T. and Johnson, J.E. (2002) Structure *10*, xx-xx.
[9] Girard, E., Kahn, R., Prangé, T., Ascone, I., Mezouar, M. & Fourme, R. (in preparation).
[10] Fourme, R., Ducruix, A., Ries-Kautt, M. and Capelle, B (1995) J. Synchrotron. Rad. *2*, 136-142.
[11] Fourme, R., Ducruix, A., Ries-Kautt, M. and Capelle, B (1999) J. Crystal Growth *196*, 535-545.
[12] Ascone, I, Cognigni, A. and Fourme, R. (in preparation).
[13] Chervin, J.C., Canny, B., Besson, J.M., and Pruzan, P. (1995). Rev. Sci. Instrum. *66*(3), 2595-2598.
[14] Le Toullec, R., Pinceaux, J.P. and Loubeyre, P. (1988). High Pressure Research *1*, 77-90.
[15] van Valkenburg, A. (1962). High Pressure Measurements. p.87 (Eds. A.A.Giardini and E.C. Lloyd). Butterworth: Washington.
[16] Mao, H.K., Bell, P.M., Shaner, J.W. and Steinberg, D.J. (1978). J. Appl. Phys. *49*, 3276.
[17] Vaney, M.C., Maignan, S., Ries-Kautt,M. and Ducruix, A. (1996). Acta Cryst. *D52*, 505-517.
[18] Schildkamp, W., Lin, T. and Johnson, J.E. (in preparation).
[19] Lin, T., Chen, Z., Usha, R., Stauffacher, C.V., Dai, J.B., Schmidt, T. and Johnson, J.E. (1999). Virology *265*(1) 20-34.
[20] Brennan, F.R., Jones, T.D. and Hamilton, W. D. (2001). Molecular Biotechnology *17*(1), 15-26.