# LTP MX421
# Report on cumulated activity from 2005-II to 2007-II
**(prepared in view of continuation of the LTP for 18 months, from 2008-II to 2009-II)**

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| ESRF | **LTP title:**<br>Application of ultra-short wavelengths for high-pressure and conventional macromolecular crystallography<br>Long Term Project (LTP) from 2005 II to 2008 I | **Experiment number**:<br>MX421 |
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## Collaboration on diamond anvil cells

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## 0. Introduction

Biophysics under high pressure is a developing field. On the one hand, the fundamental and specific interests of high-pressure perturbation are better appreciated. On the other hand, the adaptation of several biophysical techniques - including two major structural biology methods, NMR and X-ray crystallography - to high-pressure studies has contributed to enlarge the scope of applications.

The main goal of LTP MX421 was the development of high-pressure macromolecular crystallography (HPMX), completed by the investigation of the possible impact of HP instrumentation and methodology to the broader field of conventional MX. In 2000, HPMX was still in its infancy. After the pioneering experiments of Kundrot and Richards (*J. Mol. Biol,193, 170-187, 1987*) on hen egg-white lysozyme (HEWL) at 0.1 GPa (1000 atm.) using a Be cell and a laboratory X-ray source, nothing new emerged until 2000 except the use by Katrusiak and Dauter (*Acta Cryst. D52, 607-608, 1996*) of a diamond anvil cell (DAC) with synchrotron radiation to measure the compressibility of two crystalline forms of HEWL (up to 1 GPa for one of the forms) without further data collection for structural analysis. The starting point of the HPMX project at ESRF was an experiment performed in 2000 II at ID30 (proposers R. Fourme, I. Ascone and M. Mezouar), which demonstrated that the combination of DAC, high energy undulator radiation, and a large area detector was a milestone for HPMX. The technique was developed during the period 2001-2004 in the context of standard ESRF proposals on ID30 and ID27. Then, proposed by a core of six scientists, LTP MX421 created from 2005 II the frame appropriate for a coordinated development of instrumentation and investigation of new science based on projects both from the core group and from external collaborations. All LTP experiments were performed on ID27 which incorporates now, in addition to its outstanding capabilities for microdiffraction in extreme (P,T) conditions, a unique experimental set-up specialized for HPMX.

The report includes the cumulated activity performed in the LTP from 2005 II to 2007 II (i.e. 5/6 of the total allocated beamtime) but also, for clarity, the trajectory of the whole HPMX programme from the beginning. This document is intended to be the support for a continuation of the LTP during 18 months (2008 II to 2009 II).

## I. Instrumental and methodological developments

**High energy MX optimization**

ID27 is a microdiffraction high energy beamline with a Si 111 channel-cut monochromator and KB mirrors. The beam can be focused to a spot size down to a few microns in the range 18-50 keV. The pair of U23 undulators deliver an extremely intense X-ray beam in the energy domain of interest. Hardware, software and control have been improved in order to get reliable MX data by the rotation method even at very high acquisition rates (exposure times per frame down to the subsecond range). Centring of crystals is performed with micron accuracy. In conclusion, this beamline has been completed to feature main characteristics of dedicated MX beamlines, but it is unique since it is optimised for high energy.

**High-pressure cell design and sample loading**

DAC are of course an essential component for HPMX. Basic needs of HPMX include a large useful aperture in order to get high-completeness data and a pressure domain from ambient up to at least 2 GPa. We have used three types of DAC, all designed with a piston-cylinder design with thrust on the piston generated by a toroidal membrane inflated by helium. DAC that were initially available had been developed for very high-pressure research in geophysics and materials science with a modest useful aperture (42°). Special cells were built with an aperture of 62°. Finally, we have designed, constructed and successfully used in 2007 a new DAC based on conical diamonds (Boehler mount), which provide a large useful aperture (82°) up to about 2.5 GPa. (11). Different metals for gaskets have also been tested and selected for the particular conditions of our experiments. In order to get rid of preferential orientation of plate-shaped crystals in the DAC cavity, we have used diamond splinters introduced in the compression cavity (9, 11). These various improvements have extended considerably the possibility of collecting full data sets even in the case of crystals with low-symmetry space group and/or anisotropic habit (11). High-completeness data have been collected for cubic, hexagonal, tetragonal and orthorhombic crystals.

**Detectors**

HPMX require detectors with a reasonable detective quantum efficiency (DQE) for high-energy photons. We have used three types of detectors. The first one was a MAR345 imaging plate (1). We have shown that the optimal wavelength in terms of DQE and signal-to-noise ratio is close to the Ba K-edge on the high-energy side, because the stopping power for elastic scattering is then maximum whereas Compton inelastic scattering is attenuated. High-quality data were obtained, but the data flow was slowed down by the long readout time (about 60

sec), which is larger than the typical exposure time of a frame (30-45 sec). The interest to use a detector with a much faster readout was an incentive for the installation of a MAR165 CCD as a component of the beamline equipment. This detector allowed us to improve considerably the data flow for HPMX and other experiments on ID27 (10). The drawback is a DQE lower than the MAR345 one, as the 40-micron layer of $Gd_2O_2S$ is optimised for 12 keV energy experiments. We have also tested for a couple of hours a MAR555 flat panel detector and found that the good stopping power at short wavelengths as well as the small point spread function of this detector would be well adapted to HPMX. Unfortunately, this detector is not currently really operational. A detector with better DQE between 18 and 50 keV remains one of the main technical problems to be solved for HPMX.

**HPMX data collection and analysis**

Procedures for accurate centring of the sample, determination of crystal-to-detector distance and wavelength are available (1, 8, 11), so that the crystal cell compressibility as a function of pressure can be accurately determined. Indexation and integration of Bragg reflections in connection with e.g. small spot size are routinely performed with DENZO and XDS. The quality of HPMX data are generally similar to MX conventional data (4, 8).

**Radiation damage and choice of photon energy**

As for HPMX compression must be truly hydrostatic, diffraction data should be collected at room temperature (or at moderately low temperature, but not on cryocooled samples). In that case, degradations due to the interactions of photons with the sample are not limited to the primary ionisation processes. How to derive as much diffraction information as possible from a given sample in these physical conditions is thus a central question. We defined the data collection efficiency (DCE) as the information content, with a pre-assigned signal-to-noise threshold, that can be collected per unit volume of sample (4). Higher DCE can be achieved in three ways

- Improving the signal-to-noise of Bragg reflections: why the HPMX data collection mode provides high DCE was analysed and confirmed by experimental results on CpMV virus crystals (4).
- Translating the sample across the finely collimated beams, in order to irradiate successively fresh zones of sample (1).
- Selecting the photon energy. During session 2007 II, we have performed metrology at two photons energies: 18 keV (the lowest energy safely achieved with the ID27 optics)

and 33 keV (at the Iodine-K edge, the energy used most frequently up to now for HPMX experiments). First, the absolute flux density of photons was determined at the two energies (in collaboration with Kadda Medjoubi, from the SOLEIL Detector Group). Second, we have determined the DCE and the useful sample lifetime at 18 and 33 keV of HEWL crystals mounted in capillaries at room temperature or cryocooled in loops. On cryocooled samples, a large number of high-resolution data sets could be acquired from a single sample at both photon energies. The increase of the average B value per data set is somewhat lower at 33 keV, in spite of the poor efficiency of the CCD at this energy which needs to be compensated by useless irradiation of the crystal. Due to the fairly large size of HEWL crystals and lack of beamtime, we could not accumulate enough data sets to reach the Henderson's limit ($2x10^7$ Gy absorbed by the sample), so that these preliminary observations need to be consolidated. At room temperature, DCE is much better at 18keV; on the one hand, the intrinsic DCE seems somewhat higher at 18 keV (intrinsic means the physical behaviour of the crystal irradiated at room temperature under the combined effects of primary, secondary and tertiary processes); and, on the other hand, the low efficiency of the CCD detector at 33 keV is the main penalty. These results may lead us to use softer radiation for future HPMX experiments at room temperature, at least in cases where larger aperture of the diffraction cone is not a limitation. With softer radiation, the absorption of X-rays by diamonds is larger and cannot be neglected. This led us to incorporate in the data analysis process a systematic absorption correction of structure factor amplitudes based on the calculation of beam paths through diamonds.

**Impact on other communities**

General tools used by protein crystallographers (for example software for data analysis) and the instrumentation and technical procedures developed for HPMX (large aperture DAC, high level area detectors, use of diamond splinters to reorient crystal in the compression cavity, choice of photon energy, beam metrology) have been transferred to non-biological HP single crystal crystallography, thus contributing to keep ESRF at the forefront in this field.

## 2. Results

Questions that have been addressed so far using HPMX and associated know-how fall in five categories

**Effects of pressure on interactions and structure in the case of elastic compression**

When increasing pressure is applied to a macromolecular crystal, the first step is elastic compression of the initial (fundamental) state. From the refined structures of various biomolecules (3, 7, 9, 10, 16, 17), we obtained consistent data on the reduction of H-bond lengths, decrease of cavity volumes, evolution of buried surfaces, bound water molecules and atomic thermal parameters. With respect to temperature and denaturing agents, this brings additional information on the physical chemistry of biomolecules, and may shed some light on the primary stages of unfolding.

**First steps of pressure-induced protein denaturation or dissociation of multimeric proteins**

Denaturation of a monomeric proteins under high pressure was observed on early experiments on hen egg-white lysozyme (1). Beyond 0.82 GPa, the compressibility of the crystal is abnormal and diffraction is gradually converted into diffuse scattering at around 0.9 GPa. Data have been collected in 2007 I at various pressures, and a careful analysis is under way to describe small variations induced by pressure and possibly suggest a path for the onset of unfolding.

The stability of multimeric proteins is a classical problem that has been extensively investigated by various spectroscopic methods. The dimeric protein bovine Cu, Zn superoxide dismutase (SOD) has a particular behaviour: this functional dimer can withstand in solution pressure in excess of 1 GPa without dissociation, as shown by XAS and IR. Crystals can also withstand at least 1 GPa. As a first step towards higher pressure, diffraction data were acquired at 0.57 GPa (due to DAC limitations at the time of data collection) and the corresponding 3D structure was refined (16). In contrast, the tetrameric protein urate oxidase dissociates, as generally observed, at low pressure. We have captured the onset of tetramer dissociation at modest pressure (0.14 GPa). Indeed, main interfaces of the tetramer are weakened by pressure as evidenced by the abnormal behaviour of H-bond lengths, buried surfaces and thermal parameters in these regions (9). These results have been completed in 2007 using HPMX, HP optical fluorescence, HP-SAXS on ESRF ID02 and activity measurements. Complementary results suggest a detailed path for the pressure-driven dissociation. Further, we have found that HP reveals higher energy states which are involved during the catalytic function at ambient pressure (swelling of the active site region, flexibility of regions around the access channel by which the substrate gets into the protein and the product is released etc…(15).

**Adaptation of life to high pressure and prebiotic chemistry.**

The molecular basis of the adaptation of life to high pressure is a classical and important problem with fundamental and biotechnological issues. We have undertaken the study of proteins from psychro-piezophilic bacteria. These proteins are adapted both to low temperature (around 3°C) and high pressure (0.1-100 MPa) encountered in cold deep sea.

We have studied the catalytic domain of a cellulase from *Pseudoalteromonas haloplanctis,* for which the structure has been recently determined at 1.4 Å resolution by the IBCP team at Lyon. Two high-quality 1.8 Å resolution data sets were recorded at ambient pressure and 175 MPa respectively. Refinements led in both cases to low residual R-factors (R and Rfree of about 16% and 20 % respectively) (17). Subtle differences between the two structures have been evidenced and their significance with respect to high-pressure adaptation is being investigated. We have collected during the recent 2007 II session a third data set of cellulase at a higher pressure (630 MPa) which may be useful by enhancing relevant structural differences between ambient and low pressure structures. We plan to study several other proteins in collaboration with the same team.

Prebiotic chemistry is a fascinating and yet broadly open problem. It is commonly admitted that the seminal world was a RNA world. RNA molecules played a crucial role because they were able both to store / propagate genetic information and to act as catalysts, thus breaking the "egg and hen" problem. In many scenarios, such molecules appear in a reactive context under extreme conditions. For this reason, we have studied the molecular adaptation of base-paired double-helix architecture. This was done on crystals of the octanucleotide d(GGTATACC), with molecules in the A-DNA form. Such crystals can withstand pressure up to about 1.9 GPa. From four high resolution crystal structures at pressures from ambient to 1.39 GPa, we found that the axial compression is very large with a denser stacking of base-pairs, but the diameter of the helix and the base-pair geometry remain nearly invariant. In fact, these octanucleotide crystals provide information also on the B-DNA form. Indeed, B-DNA molecules reminiscent of the A-B equilibrium in solution are trapped in channels of the A-form crystal structure and are revealed by a characteristic fibre diffraction pattern. The B-DNA form is stable up to at least 2 GPa and the variation of the average base-pair stacking distance as a function of pressure was determined. The general conclusion is that the double-helix base-paired architecture is a molecular spring which can withstand very high pressure while keeping the geometry of base-pairs. This property may have played an important role in the emergence of such molecules among seminal components of life. This work gave the first

HPMX results on nucleic acids, and the pressure range explored in this project is currently the largest of any HPMX study (10, 13).

**Using pressure perturbation as a way to explore energy landscape from fully folded to unfolded states.**

A protein in solution flips rapidly between various conformations (conformers) with slightly different Gibbs free energy and specific volumes. Some of these conformers correspond to states occupied by the protein during function. As a rule, the free energy, the disorder and the specific volumes of the various conformers are correlated (as one moves from bottom to top of the "energy funnel", disorder increases while the specific volume decreases; the fundamental state is at the bottom, and unfolded state(s) at the top of the funnel). By applying pressure, the proportion of molecules in the fundamental state tends to decrease at the benefit of higher energy conformers that have smaller specific volume. This selective promotion has been evidenced and quantified by HP NMR on proteins such as ubiquitin and dihydrofolate reductase (DHFR) (*Akasaka, Biochemistry 42, 10875-85, 2003*). We have postulated that this selective promotion may be even more pronounced in the crystal (9). Accordingly, HPMX would allow to trap states of high biological relevance without recourse to fast freeze or kinetic experiments. Exploring the energy landscape of a macromolecule might be the most important application to biology of high-pressure perturbation (8). In view of the importance of this field and of the amount of beamtime required to make complete experiments on higher energy conformers, additional proposals have been submitted. The first proposal was from a japanese team (K. Akasaka et al.) and a german team (H. Kalbitzer et al.). No beamtime was allocated to this proposal. The second was from A. Houdusse et al. (Institut Curie, Paris) on the myosin motor. Beamtime was allocated to this proposal and experiments were performed in early 2007. The experiment was not successful as samples grown in Paris turned out to be degraded after travelling to ESRF. We also allocated some beamtime from the 2007 II session for at least commissioning this difficult project. Crystals of myosin II and VI grown in Grenoble diffract rather weakly and have a relatively short lifetime when irradiated. The use of softer radiation providing a much better DCE seems the obvious way to extract more information from these crystals during future experiments.

**Interest of HP and HPMX data collection procedures for conventional MX.**

First, pressure tends to reduce entropy of a system at constant temperature. In our studies involving compression in the elastic regime, the effect of pressure is an increase of crystal

order. We have observed in a particular case, cowpea mosaic virus (CpMV) particles in the cubic form, a spectacular effect of pressure on the crystalline order. At ambient pressure, most CpMV crystals are disordered; the disorder comes from small misorientations of weakly interconnected capsids with respect to the perfect tri-periodic arrangement. At high pressure, capsides are packed with a perfect long-range order due to stronger packing interactions mediated by water molecules. Resolution improves from 5 Å to 2.6 Å (7). Although this is a special case (packing transition of quasi-spherical particles), it would be quite interesting to investigate more systematically the effect of pressure on poor-quality crystals of important macromolecules.

Second, the data collection mode used for HPMX data collection does not follow the current paradigm. We have shown that using a parallel, very collimated beam of ultra-short wavelength and a distant detector improved quite significantly data collection efficiency (DCE). In the first publication on this subject (4), the respective contribution of photon energy and other factors in this improvement was not unraveled. A quantitative analysis of the effect of energy at 18 and 33 keV has been undertaken during 2007 II (i) measurement of absolute flux density and (ii) monitoring of crystal degradation by acquiring repeatedly data sets on crystals at room temperature and cryocooled (see section "Radiation damage and choice of photon energy" in paragraph 1. of this report).

**3. Conclusion**

HPMX is now a mature technique that can be applied to a variety of biomolecules (8, 11). Quality standards of conventional MX data collection can be achieved under high pressure. Pressure opens new and relatively simple routes to investigate high-energy states of bio-molecules: we have fully realized the potential of these pressure-induced high-energy conformers during the course of the LTP, and some very preliminary work in this field has been included in the LTP.

The core team of the LTP has now extended collaborations with other groups in France, in Europe and beyond. HPMX has been evaluated as a highlight of high-pressure research at the ESRF by the last ID27 beamline review committee, and HPMX was selected for the ESRF Highlights 2002 and 2007. An invited review article on HPMX for Annual Review of Biophysics 2009 (the review journal with the highest impact factor in biophysics) is in preparation.

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***Articles produced during the HPMX programme at ESRF***

***1*** - "High-pressure protein crystallography (HPPX): instrumentation, methodology and results on lysozyme crystals"
R. Fourme, R. Kahn, M. Mezouar, E. Girard, C. Höerentrup, Prangé, T. & I. Ascone. (2001) J. Synchrotron Rad., **8**, 1149-1156.

***2*** - "Opening the high-pressure domain beyond 2kbar to protein and virus crystallography"
R. Fourme, I. Ascone, R. Kahn, M. Mezouar, P. Bouvier, E. Girard, J. E. Johnson & T. Lin. (2002) Structure, **10**, 1409-1414.

***3*** - "New trends in macromolecular crystallography at high hydrostatic pressure"
R. Fourme, I. Ascone, R. Kahn, E. Girard, M. Mezouar, T. Lin & J. E. Johnson. (2003) Advances in high-pressure bioscience and biotechnology II: Proceedings of the 2nd International Conference on High-pressure Bioscience and Biotechnolog. September 16-19, 2002, Dortmund. Editor R. Winter, Springer Verlag, pp 161-170.

***4*** - "Using a quasi-parallel X-ray beam of ultrashort wavelength for high-pressure virus crystallography: implications for standard macromolecular crystallography"
R. Fourme, E. Girard, R. Kahn, I. Ascone, M. Mezouar, A.-C. Dhaussy, T. Lin. & J. E. Johnson (2003) Acta Cryst., D**59**, 1767-1772.

***5*** - "State of the art and prospects of macromolecular X-ray crystallography at high hydrostatic pressure "
R. Fourme, E. Girard, R. Kahn, I. Ascone, M. Mezouar, T. Lin. & J. E. Johnson. (2004) 'High-pressure Crystallography', Editors A. Katrusiak & M. F. Mac Millan, Kluwer, Dordrecht, pp 527-542.

***6*** - "A new dimension in structural biology: high-pressure macromolecular crystallography"
E. Girard, R. Kahn, I. Ascone, M. Mezouar, A.-C. Dhaussy, T. Lin, J. E. Johnson & R. Fourme. (2004) High Pressure Research, **24**, 173-182.

***7*** - "The First Crystal Structure of a Macromolecular Assembly under High Pressure: CpMV at 330 MPa"
E. Girard, R. Kahn, M. Mezouar, A-C. Dhaussy, T. Lin, J. E. Johnson & R. Fourme (2005) Biophysical Journal, **88**, 3562-3571.

***8*** - "High-Pressure Macromolecular Crystallography (HPMX): Status and prospects"
R. Fourme, E. Girard, R. Kahn, A.-C. Dhaussy, M. Mezouar, N. Colloc'h & I. Ascone (2006) Biochim. Biophys. Acta **1764**, 384-390.

***9***- "High-pressure macromolecular crystallography: The 140-MPa crystal structure at 2.3 Å resolution of urate oxidase, a 135-kDa tetrameric assembly"
N. Colloc'h, E. Girard, A.-C. Dhaussy, R. Kahn, I. Ascone, M. Mezouar & R. Fourme (2006) Biochim. Biophys. Acta **1764**, 391-397

***10*** - "Adaptation of base-paired double-helix to extreme hydrostatic pressure"
E. Girard, T. Prangé, A-C Dhaussy, E. Migianu, M. Lecouvey, M. Mezouar, R. Kahn & R. Fourme (2007) Nucl. Acids Res. **35**(14), 4800-4808

***11*** – "Toward full-fledged high-pressure macromolecular crystallography (HPMX) "
E. Girard, A.-C. Dhaussy, B. Couzinet, J.-C. Chervin, M. Mezouar, R. Kahn, I. Ascone & R. Fourme *J. Appl. Cryst.* 40, 912-918

***Invited articles***

***12*** - "Protein and virus crystallography under High Hydrostatic Pressure"
R. Fourme, I. Ascone, R. Kahn, M. Mezouar, P. Bouvier, E. Girard, J. E. Johnson & T. Lin. *ESRF Highlights 2002*

***13*** - "Molecular springs at the onset of life"
E. Girard, T. Prangé, A-C Dhaussy, E. Migianu, M. Lecouvey, M. Mezouar, R. Kahn & R. Fourme
*ESRF Highlights 2007*

***14*** - "High-pressure macromolecular crystallography"
R. Fourme et al. (in preparation for the 2009 issue of Annual Review of Biophysics)

***Articles in preparation***

***15*** - "Destabilization and inactivation of tetrameric urate oxidase by high pressure perturbation. Implications for catalytic mechanism."
E. Girard et al.

***16*** - "The crystal and molecular structure of bovine superoxide dismutase at 570 MPa"
I. Ascone et al.

***17*** - "Pressure adaptation of the catalytic domain of a cellulase from psychropiezophilic bacteria"
N. Aghajari et al.

***Proceedings***

Ninth International Conference on Synchrotron Radiation Instrumentation (SRI), Pohang, Korea (2006). Conf. Proc. 879 American Institute of Physics, Synchrotron Radiation Instrumentation.
X-ray crystallography at high pressure to probe conformational fluctuations in biological macromolecules
E. Girard, R. Kahn, A-C Dhaussy, I. Ascone, M. Mezouar & R. Fourme

9th International Symposium on Protein Structure and Function, Karachi, Pakistan (11-14 Jan 2007). Conf. proc. (in press)
High-pressure perturbation in molecular biophysics: Why and how? The status of high-pressure macromolecular crystallography.
R. Fourme, E. Girard, R. Kahn, A.-C. Dhaussy, M. Mezouar & I. Ascone

***Invited presentations (from 2005)***

IUCR Congress, Florence, 23-31 aug 2005
When Macromolecular Crystallography Meets High-pressure Techniques...
E. Girard et al.

ERL Workshop. Chess, Cornell University, June 5-6 2006
High-pressure and conventional macromolecular crystallography using ultra-short wavelengths. The case of the Cornell ERL source.
R. Fourme et al.

10 years of BM14 at ESRF SOLEIL progress and challenges ahead Grenoble, 23 Jan 2007
High-pressure Macromolecular Crystallography
R. Fourme et al.

Science at synchrotrons Feb 2007 Cape Town
Macromolecular crystallography and its combination with pressure perturbation (HPMX)
R. Fourme et al.

9$^{th}$ International Conference on Biology and Synchrotron Radiation
13-17 August 2007, Manchester, England
Progress in instruments and methods towards fully-fledged high-pressure macromolecular crystallography (HPMX). Recent structural results on nucleic acids and proteins
E. Girard et al.

GTBIO, meeting of the AFC (French branch of IUC) 8-11 oct 2007, Lille, France
Study of the destabilization of the active site of urate oxidase by high-pressure crystallography, SAXS and fluorescence.
E. Girard et al.

Pressure, Protein Hydration and Structural Dynamics (JSPS Core to Core Program Project 17009, international seminar)

Meeting #1. Okinawa, Bankoku Shinryoukan, Japan Jan 15-19 2006
X-ray crystallography at high pressure to probe conformational fluctuations in virus and monomeric and multimeric proteins
R. Fourme et al.

Meeting #2 Montpellier Aug 27-30 2006
High-pressure studies of bovine superoxide dismutase
I. Ascone et al.

Meeting #3 Santa Fe Jan 21-25 2008
High pressure macromolecular crystallography studies of A- and B-DNA up to 2 GPa.
R. Fourme et al.