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Review

High-Pressure Macromolecular Crystallography (HPMX): Status and prospects

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Abstract

Recent technical developments, achievements and prospects of high-pressure (HP) macromolecular crystallography (MX) are reviewed. Technical difficulties associated with this technique have been essentially solved by combining synchrotron radiation of ultra-short wavelength, large-aperture diamond anvil cells and new sample-mounting techniques. The quality of diffraction data collected at HP can now meet standards of conventional MX. The exploitation of the potential of the combination of X-ray diffraction and high-pressure perturbation is progressing well. The ability of pressure to shift the population distribution of conformers in solution, which is exploited in particular by NMR, can also be used in the crystalline state with specific advantages. HPMX has indeed bright prospects, in particular to elucidate the structure of higher-energy conformers that are often of high biological significance. Furthermore, HPMX may be of interest for conventional crystallographic studies, as pressure is a fairly general tool to improve order in pre-existing crystals with minimal perturbation of the native structure.

Keywords: Hydrostatic pressure; Protein crystallography

1. Introduction

Nearly two decades after the pioneering results of Kundrot and Richards on the crystal structure of hen egg-white lysozyme at 100 MPa [1], structural information obtained by macromolecular crystallography under high pressure (HPMX) remains surprisingly scarce. Several reasons can be invoked to explain this situation: (i) lack of interactions between the macromolecular crystallography and the high-pressure communities; (ii) doubts about the interest of high pressure in the solution of biological questions; (iii) technical difficulties in combining macromolecular crystallography and high pressure; (iv) lack of synchrotron radiation beamline dedicated to HPMX, and severe competition to have access to multipurpose high-pressure beamlines. The exploitation of the potential of the combination of X-ray diffraction and high-pressure perturbation is still in its infancy. This article aims to demonstrate that this situation is changing since the last few years, that most technical difficulties of HPMX are now solved and that coupling MX with pressure perturbation has a considerable potential to give new insights in fundamental biological questions. HPMX is also of interest for the whole biocrystallography community by giving ways to improve, at least in some cases, the crystal order of pre-existing crystals.

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2. Fluctuations and conformers in biological macromolecules

A protein molecule in solution, at given temperature and pressure, exists as a dynamic equilibrium mixture of subensembles of conformers [2] differing, in general, in their topology of folding, in partial molar volume and in thermodynamic stability. The concept of conformational substrates has been described [3]. Spectroscopic studies of proteins, particularly vibrational and NMR spectroscopy, have provided a wealth of data about internal dynamics and conformational substates [3–6]. The dynamic nature of proteins has been proposed to have a function in catalysis. Recent results on the prolyl *cis–trans* isomerase cyclophilin A in the substrate-free state and during catalysis show pre-sampling of conformational substates before catalysis that are harvested for catalytic turnover [7]. This is directly analogous to findings of the role of pre-existing equilibrium for allosteric signaling and ligand binding [5,8]. A picture is therefore emerging in which conformational events that occur during protein function are already present before ligand binds. Proteins have evolved to sample multiple defined conformations that are critical for function.

The most powerful way to elucidate relations between protein function and conformational fluctuations is the determination of three-dimensional structures of relevant conformers. The prerequisite to perform such a programme is the preparation of a sample that is sufficiently homogeneous, i.e., in which the majority of macromolecules belong to the same subensemble. This condition must be preserved throughout data collection by one of the few methods of structural biology able to supply 3D-information.

Detection and analysis of structures of higher-energy conformers are limited, particularly under physiological conditions, either because their equilibrium populations are small or because they exist only transiently in the folding process. Increasing the equilibrium population of conformers can be obtained, in some cases, by varying the pH or concentration of a denaturant [9], but it is not generally assured that conformers under such extreme conditions are intrinsic conformers. Pressure is the appropriate perturbation to modify the population of intrinsic conformers. Le Chatelier's principle states that if a system at equilibrium is disturbed by a change in temperature, in pressure, or in the concentration of one of the components, the system shifts its equilibrium state so as to counteract the effect of the disturbance. Consequently, an increase in pressure favors reduction of the volume of a system. Let us consider the simple case of a protein in solution existing as an equilibrium mixture of the native subensemble N and another subensemble I, which differs in partial molar volume ($\Delta V_0 = V_I(p_0) - V_N(p_0)$) and in free energy ($\Delta G_0 = G_I(p_0) - G_N(p_0)$). If the temperature of the system is considered as a constant, the variation of the Gibbs free energy due to application of pressure p and the equilibrium constant between and I are described respectively by:

$$\Delta G = G(I) - G(N) = \Delta G_0 + \Delta V_0(p - p_0) + 1/2\Delta\beta(p - p_0)^2$$

and

$$K = [I]/[N] = \exp(-\Delta G/\mathrm{RT})$$

where ΔG and ΔG_0 are the Gibbs free energy changes from N to I at pressure p and p_0, respectively, ΔV_0 is the partial molar volume change, $\Delta\beta$ the change in compressibility coefficient defined as $\Delta\beta = (\partial\Delta V/\partial p)_T$, R is the gas constant, T the temperature and K the equilibrium constant [10,11].

Accordingly, considering the first order term in the development of ΔG only, when a solution consists at atmospheric pressure of a series of subensembles differing in volumes, higher and higher pressure will populate ensembles of smaller and smaller volume, each of which could be a target for structural analysis [12].

Under pressure, a protein may shift to a smaller partial molar volume through (schematically) two kinds of fluctuations:

In the first case, fluctuations are within the same subensemble of conformers (which is the case when the higher-energy conformers are well-separated in energy from the lower-energy subensemble). The stable state of the system is mildly affected by pressure in such a way that the protein and the surrounding solvent occupy a smaller volume [13]. At the microscopic scale, the volume compression is attained by a shift of the population among microscopic states in favor of lower-volume microscopic states within the subensemble. In this regime, the structural response of the protein–solvent system can be called "elastic" since it does not involve a transition from native to non-native state (there is no denaturation, even partial, of the protein). Elastic relaxation is structurally characterized by a moderate change in interatomic distances, reversible when the pressure is released, but not necessarily homogeneous throughout the protein structure.

In the second case, fluctuations are among several subensembles of conformers having different average free energies. In many proteins, peculiar intermediate conformers, often with distinct local conformational changes, are found. Their structures may be depicted qualitatively with some local structural transition or unfolding of the known folded structure. Specific structures suggest that these conformers are designed for function and are closely related to kinetic intermediates. A few representative examples are: changes at the active site of the enzyme dihydrofolate reductase enzyme during catalysis [14], barrel opening fluctuations allowing for small hydrophobic ligands to enter β-lactoglobulin [15], and locally disordered conformer of prion protein [16]. Results suggest that, in solution, relatively mild pressure range of a few hundred of MPa allows to detect higher-energy conformers, from the basic folded conformer to the fully unfolded, thus covering most of the conformational space allowed for the protein.

The major effect of pressure is the increase of the population of higher-energy conformers. By choosing an appropriate pressure, an intermediate conformer can be stably trapped in local free energy minimum and submitted to structural analysis. The combination of pressure perturbation with shape-sensitive structural methods extends considerably possibilities to study most of the biologically relevant conformational space of a

protein [12], which is crucial for advancing our understanding of specificity of ligand binding, signal transduction, molecular recognition and conformational diseases.

3. Structural methods with pressure perturbation

3.1. Nuclear magnetic resonance (NMR)

In multidimensional NMR spectroscopy, NOE distances and torsion angles are used to create average atomic coordinates. Although NMR may be considered in detecting signals from all fluctuating conformers in solution, in reality it reports on a single structure [17] neglecting coexisting minor conformers. Moreover, even within the subensemble of the major conformer, any fluctuation with a frequency of $>10^3$ s^{-1} is averaged out during the relatively long acquisition time (ms to s). Direct structural characterization of higher-energy conformers is possible by increasing sufficiently their equilibrium population through the application of pressure. The combination of NMR with pressure has gained much attention in recent years [18–22]. Structural changes can be monitored at essentially all individual amino acid residues on ^{15}N- or ^{13}C-labeled protein samples (MW < 30 kDa) under variable pressure up to about 400 MPa [12]. In recent developments, NMR "snapshots" of a fluctuating protein structure under variable pressure were obtained at atomic resolution [23].

3.2. Small angle X-ray scattering (SAXS)

SAXS is a technique in which the X-ray intensity scattered by a solution of (ideally) randomly oriented identical macromolecules is recorded as a function of scattering angle. Modern data interpretation tools, primarily developed by the group led by Dmitri Svergun (EMBL-Hamburg), have considerably extended the usefulness of SAXS for the determination of low resolution structures in solution. On the one hand, scattering curves can be accurately predicted from known 3D-structures. On the other hand, several methods have been developed for ab initio determination of molecular shape. They include (i) spherical harmonics, (ii) the use of a large number of small uniform-density spheres and simulated annealing optimization under looseness and disconnection penalties and (iii) the description of a protein shape as a chain of dummy residues whose distribution simulate typical neighboring characteristics in polypeptides (for a review, see [24]). Main applications of SAXS are shape determination at low resolution, detection of quaternary changes and the determination of complex structure from two components of known structures by adjusting their relative positions by automated or interactive rigid-body refinement. Such applications can be performed at high pressure, which is used to increase the population of particular conformers [25,26].

3.3. Macromolecular crystallography (MX)

Imaging a single macromolecule by X-ray diffraction is extremely difficult due mainly to the weakness of scattering and competition between scattering and radiation-induced degradation. Although there are some prospects for single- or few-molecule imaging in connection with the advent of fourth generation synchrotron radiation sources emitting extremely intense and short bursts of X-rays, the current solution to these basic problems relies on virtues of cooperativity between a very large numbers of scattering units. This is realized by exploiting a special kind of sample, a crystal that is (ideally) a 3D-periodic array of a very large number of identical molecules. The word "diffraction" is associated with interference effects in scattered radiation resulting from order in the crystal. Diffracted beams produce so-called Bragg reflections, which are detected on an area detector. The image finally obtained by X-ray crystallography is an electron density map calculated via a Fourier transform based on amplitudes and phases of diffracted beams. This electron density map is then interpreted in terms of a molecular model. By essence, the electron density obtained by X-ray diffraction is time- and space-averaged over myriads of molecules. The sharpness of the electron density map, which is quantified by the so-called "resolution", is related essentially to the quality of the periodic arrangement in the crystal (which depends on the deviations with respect to ideal periodic array) and on the amplitude of static or dynamic fluctuations of a given atom in the various copies of the molecule. Disorder (in the broad sense) in the crystal reduces diffracted intensities, especially of higher-angle reflections that encode higher-resolution information. In practice, resolution is estimated from the extent of experimental diffraction data with an acceptable signal to noise ratio. In cases of highly ordered crystals, each atom may appear as a separate entity and atomic resolution is reached.

X-ray crystallography can provide the three-dimensional structure of unlabeled macromolecules of arbitrary complexity at near-atomic and sometimes atomic resolution. As previously described for NMR and SAXS, pressure can be used to shift the population of substrates and trap a particular higher-energy subensemble of conformers. Nevertheless, effects of crystalline medium with respect to solution deserve a thorough discussion.

A macromolecular crystal is a particular state of matter in which molecules, arranged in a periodic array and connected by weak forces, coexist with a liquid phase (typically, 30–80% of the total crystal volume). At room temperature, it is often possible to grow crystals of extremely high quality [27]. Indeed, the semi-liquid/semi-solid state of such crystals is both sufficiently rigid for 3D-periodicity over macroscopic distances, and sufficiently plastic to accommodate local rearrangements leading to near-perfect long-range order, a state associated with an energy well. Under pressure, the liquid surrounding the crystal and the liquid phase within the crystal communicate through channels in the crystal structure, ensuring hydrostatic compression of the polypeptide chain. Plasticity allows accommodating fairly large pressure-induced volume variations or fluctuations. For these reasons, macromolecular crystals generally withstand pressure quite well (of course below the denaturing pressure). When a crystal initially obtained at atmospheric pressure is submitted to a gradual increase of pressure, the first step is, in general,

fluctuations within a single (native) ensemble of conformers. The structure accommodates small changes due to compression, although remaining essentially the same for both 3D-structure of the protein and crystal packing. At higher pressure, the conformational equilibrium may be shifted beyond the native ensemble. We suggest that the crystalline state may act to some extent as a "conformation filter" favoring the most populated conformation because monodispersity allows better long range order, which is beneficial in terms of free energy minimization. In short, the crystalline state might provide better monodispersity than solution, a key point for the accurate structural characterization of higher-energy (non-native) conformers, provided that conformational transitions are not so large to be incompatible with crystal packing. Furthermore, we have found that the crystalline state may allow compressing protein molecules beyond the denaturation pressure in solution. For instance, tetragonal crystals of hen egg-white lysozyme must be compressed beyond 820 MPa to observe the emergence of crystal degradation through the appearance of diffuse scattering between Bragg reflections and finally a complete loss of diffraction [28]. In solution, changes are detected at lower pressures by fluorescence intensity [29] and rotational relaxation time [30] of lysozyme.

Another question, which is of high interest for conventional crystallography, is related to the possible role of pressure as a tool to improve order in a pre-existing crystal and accordingly to improve resolution. Following the principle of microscopic ordering, an increase of pressure at constant temperature leads to an ordering of molecules and a decrease in the entropy of the system. Compression of a crystal at moderate pressure should improve order. This has been verified for the crystal structures of Cowpea Mosaic Virus (CpMV) in the I23 space group that have been carefully refined and compared at 0.1 and 330 MPa [31]. The structures of capsid proteins are essentially the same, but the high-pressure structure is better ordered. This is a case of compression within the native subensemble of ordered proteins, and similar results can be expected for other systems. When structures of the same protein are determined both by NMR in solution and by X-ray diffraction, the current observation is that some regions that are disordered or undergo motions of large amplitudes in solution are stabilized by packing constraints in the crystal. Along the same line, tighter environmental constraints resulting from the application of high pressure might stabilize regions that are disordered in the crystal structure at atmospheric pressure. In the case of CpMV crystals, we have observed a pressure-induced disorder-to-order transition in cubic crystals from P23 to I23 space group with spectacular improvement of resolution [32]. A mechanism has been proposed for this transition, based on increased packing constraints and stronger inter-capsid bonding at high pressure that prevent orientational disorder of capsids observed at atmospheric pressure [31]. Such a mechanism is probably rather specific (e.g., packing of near-spherical objects). The ordering effect of pressure would deserve more systematic studies, which have been yet limited by lack of synchrotron radiation beam time.

4. Steps in HPMX

In HPMX data collection, several problems have to be addressed:

(i) The crystal should remain visible within the pressure cell, and the applied pressure should be monitored during data collection.
(ii) In order to preserve long range order, the crystal must be submitted to hydrostatic compression. The surrounding medium must be a fluid and, in particular, crycooling is excluded.
(iii) Obtaining accurate electron density requires data that are highly complete and isotropically distributed in reciprocal space. These conditions are difficult to fulfil when the sample is confined in a cell. Furthermore, in such conditions, data should be corrected for absorption, unless absorption is very small.
(iv) As the crystal is small and enclosed in a cavity, diffraction is weak and there are multiple sources of parasitic elastic and inelastic scattering. Accordingly, optimizing the ratio of diffraction signal to background is crucial.

The first device used to compress protein crystals was a polycrystalline beryllium cell [33] that has a pressure limit of about 200 MPa. Moreover, this device is opaque to visible light and produces substantial parasitic scattering. Published structures obtained with such Be cells are hen egg-white lysozyme (HEWL) [1] and more recently sperm whale myoglobin [34]. The diamond anvil cell (DAC) with the metal gasket technique [35] for encapsulation of crystal bathed in the mother liquor, was first used for compressibility measurements on HEWL crystals up to 1 GPa [36]. With DAC, the pressure range is extended, the sample is visible and parasitic scattering is reduced.

Over the last few years, fully-fledged HPMX has been developed on both protein and virus crystals compressed in a DAC, using the ESRF high-pressure beamline ID30, recently rebuilt and upgraded as ID27. This project matured at LURE, a synchrotron radiation facility at Orsay, France, where research under high pressure by diffraction, X-ray absorption and IR spectroscopy was very active. R. Fourme and R. Kahn had experience with crystallography at extreme conditions, and long-established motivation for high-pressure protein crystallography. The success of protein crystallography experiments at ultra-short wavelengths [37] was an incentive to use such exotic radiation for HPMX. In this context, the general HPMX project outline was proposed by R. Fourme and I. Ascone with the primary motivation to investigate the structure of bovine Cu,Zn superoxide dismutase (SOD) in order to complete results obtained on this protein by pioneering high-pressure X-ray absorption spectroscopy measurements [38]. Collaboration was established between scientists at LURE, IBS and ESRF. During the first shifts at ESRF in 2000, commissioning of the HPMX experiment was performed using orthorhombic SOD crystals and tetragonal HEWL crystals. Compressibility curves were derived and several diffraction data sets, albeit of insufficient

completeness, were collected and processed [28]. These results led us to focus our first HPMX studies on crystals with high symmetry (tetragonal or cubic) lattices. The work on HEWL, a monomeric protein, was pursued with an account of the 3D structure in space group $P4_32_12$ at 700 MPa [39]. The second project was on a virus. Collaboration was established with J. Johnson's group at the Scripps Research Center at San Diego, USA, who supplied cubic crystals of the CpMV capsid. A pressure-induced disorder-to-order transition was evidenced [32] and the refined structure of the capsid at 330 MPa and its evolution with respect to the structure at atmospheric pressure were completed [31]. With the improvement of diamond anvil cells and experimental procedures, the study of crystals with cells of lower symmetry such as SOD or urate oxidase became possible. New collaborations have been established on other protein and nucleic acid crystals. The recently reported study of urate oxidase [40] is a result from such collaboration.

5. Equipment for collection of HPMX diffraction data

For our experiments, we use two diamond anvil cells of the cylinder-piston type [41] where the thrust applied to the piston is generated by a toroidal membrane [42] inflated by helium. Two co-axial diamond anvils are mounted on metal supports: the first one is part of the cell body and the second one is attached to the piston. The actual pressure in the sample cavity is determined using the wavelength shift of the fluorescence emission of a ruby chip at 694.2 nm [43]. The optical system that measures the fluorescence is placed on the X-ray path between the DAC and the detector and can be remotely removed when the pressure measurement is completed. The same device, coupled to a CCD camera, is also used to image the sample on TV monitors.

The beamline incorporates a two-circle goniometer installed on a stacking of three orthogonal translation tables.

The X-ray optics used for HPMX has a single component, a monolithic two-reflection Si (111) monochromator. The divergence of the unfocused monochromatic beam is very small. We use ultra-short wavelengths, about three times shorter than in conventional protein crystallography. An imaging plate detector was used to collect the diffraction data. The wavelength (λ=0.331 Å, on the high energy side of barium *K* absorption edge) was selected in order to maximize the detective quantum efficiency of the imaging plate detector for elastic scattering and detect less efficiently Compton scattering [28]. Such beam characteristics are crucial, as they reduce systematic errors in integrated intensities of Bragg reflections and they increase the signal to background ratio (see [44] for a detailed discussion). In the rotation geometry, the approximate λ^2 dependence of the integrated intensity of a diffraction spot induces a reduction by nearly one order of magnitude compared to conventional protein crystallography data collection ($\lambda \approx 1$ Å). This reduction is compensated by the very high brilliance of undulators. The effect of wavelength on the sample lifetime is an important issue, especially as data collection proceeds at room temperature. The effect of ultra-short wavelength X-rays on the lifetime of biological samples remains poorly documented. When the transmission through the crystal is high, which is the case here, the absorbed energy is practically wavelength-independent, but its relationship with radiation damage in the crystal is not clear. Further experiments are required to get a more definitive conclusion.

The detector was a MAR345 imaging plate system. The size of this detector is suitable for large crystal-to-detector distances. On ID27 [45], exposure times are, with respect to ID30, reduced by a factor about eight with the two in-vacuum undulators switched on. In this latter case, exposure times for typical protein crystals are in the range 30–60 s for an oscillation of one degree. Accordingly, the BRUKER CCD detector available on ID27, which has a much faster readout (a few seconds), will be evaluated for future HPMX experiments.

6. HPMX methods

Our experiments have been performed using general purpose DACs in which anvils and their supports were modified in order to increase the useful apertures for incoming and diffracted X-ray beams. Initial apertures (42°, full cone opening) were increased to 62° by using thinner diamonds (thickness 1 mm) and modified supports.

Gaskets are fabricated by standard procedures. We use Cu gaskets up to 600 MPa. Cu-Be or Inconel gaskets are used for higher pressures. The gasket is indented by applying thrust to the piston diamond and a cavity is machined by spark-erosion at the centre of the indentation. The final diameter and thickness of the cavity are typically 400 and 180 μm, respectively. The pressurized volume is thus sufficient to host a crystal that may be relatively large.

After ultrasonic cleaning, the gasket is placed again on the top of the fixed anvil, exactly as it was during indentation. A ruby chip is deposited at the centre of the piston anvil. A crystal is fished in the crystallization drop using a nylon loop then quickly transferred to a small drop (3–5 μl) of mother liquor covering the cavity. The drop must be put just before the deposition of the crystal in order to reduce evaporation of its most volatile components. The crystal is then gently pushed into the cavity. Finally, the piston is remounted and the cell is closed by screwing the cap.

The DAC is then centered on the goniometer. The centre of the cavity must be accurately located on both the goniometer rotation axis and the X-ray beam path [28].

Diffraction data analysis software, like DENZO [46] and XDS [47], were successfully used for image processing and data integration. After centering the crystal, a few images (frames) are recorded. From these images, information necessary to undertake data collection (lattice type, cell parameters and orientation matrix) is derived. Data collection is performed by the rotation method with the high-pressure cell rotating about the vertical axis. The beam is collimated by slits to typically 40–60 μm in both directions, which defines the irradiated zone on the sample. Successive frames are displayed on the computer monitor and data integration proceeds on-line. Irradiating successively different regions of the sample during data collection is an efficient way to alleviate the relatively fast

degradation of crystals exposed to X-rays at room temperature. When there is evidence for significant degradation of the exposed zone, the crystal is translated in order to irradiate a fresh portion of the sample. After selection of useful frames, data are scaled and merged together using SCALA from the CCP4 suite [48]. Finally, a set of structure factor amplitudes suitable for structural analysis and refinement is obtained.

7. HPMX results

We have compressed numerous protein crystals and virus crystals. The most difficult step appears in general to be sample loading, including crystal fishing, deposition and sealing of the cavity. The evaporation of the most volatile components of the mother liquor during loading may change its composition and destabilize the crystal before pressure ramping. This may require some adjustment of the composition of the mother liquor. Pressure can then be ramped. We observed that macromolecular crystals withstand high pressure very well, for reasons that were given in Section 3. The stability domain of crystals with pressure is specific to each study and depends on a variety of factors (wild or mutant protein, complexation with an inhibitor, composition of mother liquor, etc.). Loss of diffraction (revealing a loss of long-range order in the crystal) has been observed near 400 MPa for CpMV [31,32] and 820 MPa for HEWL [28]. SOD crystals could be compressed without loss of diffraction beyond 1 GPa [28]. Among all samples that we have compressed up to now, the most sensitive to pressure were urate oxidase crystals, for which the critical pressure is about 180 MPa [40].

We have presently completed the refinement of three high-pressure crystal structures: CpMV [31], HEWL [39] and urate oxidase [40] and compared the refined structures at atmospheric pressure (AP) and at high pressure (HP). It turns out that the quality of data recorded at high pressure as evaluated by usual indicators (mosaicity, R_{sym}, completeness) meets, or is very close to, usual standards [44]. The conclusion is similar for the quality of crystallographic results (R factor, R_{free}, Ramachandran plot, errors on bond lengths and bond distances) [31,39,40]. Accordingly, HPMX is now a mature technique that can provide quantitative and accurate structural information on a variety of macromolecular systems. In particular, information on the evolution of isotropic temperature factors, ordered water molecules, hydrogen bond lengths, contact distances, cavities and buried surfaces was derived from these first studies. The last restrictions with respect to standard crystallography are being solved. In particular, collecting data with high completeness from few crystals was one of the most challenging technical problems of HPMX. As mentioned previously, initial studies were performed on crystals in cubic or tetragonal lattices, so that highly complete data sets could be collected in spite of the modest apertures of our first DAC (about 42°). Progress was achieved by increasing the cell apertures up to the current value of 62°. Furthermore, anisotropic crystals (e.g., plate-shaped) were forced to adopt various orientations in the cavity by inserting a diamond splinter selected from broken diamonds. Merging data from crystals with different orientations allowed us to study less symmetric crystals, e.g., in orthorhombic systems, which is the case of urate oxidase [40]. New large aperture cells have been designed (in collaboration with J.C Chervin and B. Couzinet at the IMPMC, Paris) and are being tested. With this equipment, supplemented by crystal mounting procedures using splinters, the completeness problem should become marginal in most cases.

8. Conclusions

The concept of conformational substates and exploration of the conformational landscape was put forward three decades ago [6]. Combined studies of pressure-perturbed macromolecules in the crystalline state (in particular by HPMX, a newcomer which has a bright future) and in solution (in particular by vibrational and NMR spectroscopies) will shed new lights on the connections between structure, energy landscapes, dynamics and function.

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