Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Structural Biology

High-pressure macromolecular crystallography and NMR: status, achievements and prospects

Roger Fourme[1], Eric Girard[2] and Kazuyuki Akasaka[3]

Biomacromolecules are thermodynamic entities that exist in general as an equilibrium mixture of the basic folded state and various higher-energy substates including all functionally relevant ones. Under physiological conditions, however, the higher-energy substates are usually undetectable on spectroscopy, as their equilibrium populations are extremely low. Hydrostatic pressure gives a general solution to this problem. As proteins generally have smaller partial molar volumes in higher-energy states than in the basic folded state, pressure can shift the equilibrium toward the former substantially, and allows their direct detection and analysis with X-ray crystallography or NMR spectroscopy at elevated pressures. These techniques are now mature, and their status and selected applications are presented with future prospects.

Addresses
[1] Synchrotron Soleil, BP48 Saint Aubin, 91192 Gif sur Yvette, France
[2] IBS, UMR 5075 CEA CNRS UJF, 41 rue Jules Horowitz, 38027 Grenoble Cedex, France
[3] High Pressure Protein Research Center, Institute of Advanced Technology, Kinki University, 930 Nishimitani, Kinokawa 649-6493, Japan

Corresponding authors: Fourme, Roger (roger.fourme@synchrotron-soleil.fr), Akasaka, Kazuyuki (akasaka@waka.kindai.ac.jp)

Current Opinion in Structural Biology 2012, **22**:636–642

This review comes from a themed issue on **Biophysical methods**

Edited by **S Samar Hasnain** and **Soichi Wakatsuki**

For a complete overview see the Issue and the Editorial

Available online 4th September 2012

0959-440X/$ – see front matter,

http://dx.doi.org/10.1016/j.sbi.2012.07.007

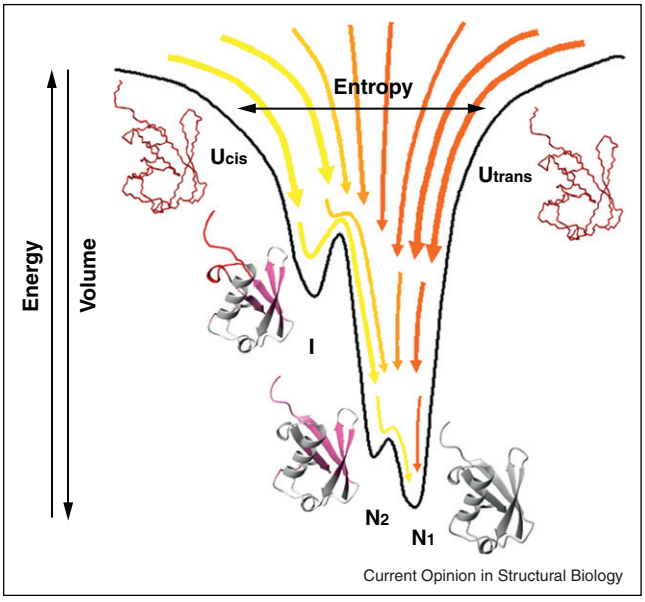
Introduction

Pressure is increasingly used for molecular biophysics studies. We believe that this evolution builds on two basic reasons: pressure perturbation has a uniqueness that can exploit hidden but crucial characteristic features of proteins, and technical and methodological difficulties related to sample environment under pressure have been overcome to a greater extent for a large number of biophysical methods.

Like temperature, pressure is a fundamental thermodynamic variable for defining protein conformational states. While entropy is the conjugate variable for temperature, volume is the conjugate variable for pressure, and volume is more intimately related with structure of biomacromolecules than entropy. In a modern view, a protein in solution equilibrates, in general, among multiple conformational states, with all functional substates forming part of the energy landscape under physiological conditions [1•,2••]. Although most proteins exist dominantly in their basic folded state (the so-called 'native' state), they may be visiting alternate substates that exist at a few to a few tens of kJ/mol higher in Gibbs free energy.

Importantly, these substates differ substantially in their partial molar volume (PMV), the effective volume of a protein in solution, the difference (ΔV) amounting to a few tens to ~a few hundred mL/mol of protein [3]. In most actual proteins having various alternate substates, we postulate that the PMV decreases ($\Delta V < 0$) in parallel with the loss of conformational order (namely the extent of unfolding) (the volume rule) [2••]. This volume rule (previously described and termed the volume theorem) is an essential characteristic of the volume behavior of proteins, which predicts the conformational variation of a protein with pressure. The validity of this rule has been tested successfully by a number of proteins so far investigated by high-pressure NMR (HPNMR). A general background of this rule would be that successive filling of internal cavities of a globular protein with water causes a loss of volume in parallel with a loss of conformational order. It must be kept in mind, however, that at high temperatures where the contribution of 'thermal volume' [4] (the temperature-sensitive space between the protein and water) to PMV becomes dominant, it is possible that this rule may no longer hold. Thus, by carrying out experiments around physiological temperatures, or even at lower temperatures by taking advantage of the fact that water freezes only at -21 °C at 200 MPa (1 pascal (Pa) = 1 N m^{-2}; 1 MPa = 10^6 Pa = 10 bar = 0.01 kbar), we can use pressure to shift the conformational equilibrium toward substates with smaller PMV according to Le Chatelier's principle. Typically at a pressure of 1 kbar or 100 MPa, a higher-energy substate with a volume lower by $\Delta V = -100$ mL/mol would gain a surplus of stability by -10 kJ/mol as $P\Delta V$, which would increase its population by a factor of $\exp(-P\Delta V/RT) \sim 50$. This might be sufficient to invert the population distribution between the substate and the basic folded state and make the spectroscopic or crystallographic detection of the substate possible [2••]. By applying higher and higher pressure, one can populate conformers of proteins with higher and higher energy but with smaller and smaller PMV. In favourable cases, one may scan the entire energy landscape, from the basic folded state in the bottom of the funnel, through intermediately folded states, perhaps up to

Figure 1



Schematic representation of the energy landscape of folding for ubiquitin, a small globular protein, based on the results of variable-pressure NMR and a kinetic folding experiment (adapted from Figure 15 in [2••]). The horizontal axis represents the conformational entropy of the polypeptide chain. The vertical axis represents the conformational order and is determined by the solvent-averaged energy. According to the volume rule of protein, the partial molar volume (PMV) decreases in parallel with the loss of conformational order. Variable-pressure NMR detects two major folded conformers N1 and N2, one locally folded conformer I and a fully unfolded conformer U. They are arranged in the decreasing conformational order: N1 > N2 > I > U.
Representation: Ribbon model (gray) for the folded part; ribbon model (purple) for the distorted part; wire model (brown) for the unfolded part. Arrows indicate folding pathways: one directly from Utrans to N1 (brown) and the other from Ucis to N1 (yellow) via cis-proline trapped intermediate, closely identical to I.

the fully unfolded state(s) close to the top of the funnel, with pressure as sole variable (Figure 1).

To the experimentalist, pressure is more friendly than temperature. Firstly, hydrostatic pressure can be varied in a smooth and practically continuous manner, and the same pressure can be attained instantly at all sample points. Secondly and importantly, in dilute solutions most proteins behave reversibly under pressure with little problems from irreversible aggregation, allowing thermodynamic and structure analyses simultaneously on higher-energy substates.

Given the unique potential of pressure perturbation as stated above, investigating details of the protein conformational substates at (or near) atomic resolution under pressure was a tantalizing prospect. Accurate mapping of pressure-dependent changes in protein conformation opens new prospects to explore the conformational space available to protein function, adaptation, evolution and aggregation, giving opportunities to test the precise mutants for protein engineering purposes as well. Two high-resolution methods, macromolecular crystallography (MX) and solution NMR, have been adapted so far for this purpose. Their present status, complementarities and future prospects are surveyed in this article with some examples giving insight into the dynamic nature of protein conformation.

High pressure MX

Materials and methods

In this review, high pressure macromolecular crystallography (HPMX) means that the sample, during data collection, is in equilibrium under pressure, that is, in a well-defined thermodynamic state, contrary to the pressure cryocooling method by which a crystal is quenched at liquid nitrogen temperature while pressurized in a column of helium gas before MX data collection at atmospheric pressure [5]. This different method has been applied in particular to study the alteration of citrine structure by pressure [6] and is further discussed in a review article [7•].

The first pressure cell used for HPMX was made of beryllium. The design prevented sample observation and limited both the diffraction resolution and the pressure range available (100 MPa). It was used to solve in 1987 the structure of hen egg-white lysozyme at 100 MPa [8•], a pioneering work that discussed several important issues, in particular suggested that the most significant motions in the molecule were of large structural motifs. A variant of this cell, reaching 200 MPa, was used to study sperm whale myoglobin [9•] and the T4 lysozyme [10]. The renewal of HPMX was associated to another device, the diamond-anvil cell (DAC), first introduced in 1996 for compressibility measurements of lysozyme crystals without data collection [11], then extensively used for HPMX studies since 2001 [12•]. Cells have been purposely designed for HPMX [13], in which the thrust applied to diamonds is generated by a built-in pneumatic system ensuring a linear and reversible response. They cover the required pressure and temperature ranges (0–2.5 GPa and 20–125 °C) and their useful apertures for incoming and diffracted X-ray beams are large (~85°) thanks to diamonds with a conical seat [14]. Procedures for loading, pre-orienting one or several crystals in the DAC and collecting a large amount of data from each sample were developed [13]. Pressure is monitored by the wavelength shift of laser-excited ruby luminescence, which is the usual method to determine the actual pressure of a sample compressed in a DAC [15]. Hard X-rays (photon energy 33–37 keV) are optimal for HPMX, as used on the ID27 beamline at the ESRF (Grenoble, France) [12•] albeit lower-energy photons have also been used, for example, 26 and 30 keV at beamline CRISTAL of SOLEIL, France (Eric Girard and Roger Fourme, private communication) and 18 keV at beamlines BM30A of