High Pressure Protein Cryo-crystallography: Studying Pressure Effects without the Pressure

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Introduction: Pressure is a basic and important physical parameter for biological systems, with more than 70% of the Earth’s surface covered by oceans at an average pressure of 400 bar (1 bar = 10^5 Pa = 0.987 atm) and with deep ocean pressures up to 1200 bar [1]. Pressures found in the biosphere, though small compared with pressures used in other areas of condensed matter research (>10^5 bar), have large effects on enzyme specificity and activity, molecular associations, protein folding, viral infectivity, and cellular morphology [2, 3]. The observation of large effects with moderate physical perturbations hints at the complexity of biological systems, ranging from the myriad of interacting biochemical pathways in a cell to allosteric interactions transmitted through the folds of a protein.

Kilobar pressures also have potential applications in biotechnology. High pressure was initially applied in the areas of agriculture and food sciences with commercial products appearing in the early 1990’s [1]. Pressure inactivation of microorganisms resulted in preservation methods that, unlike pasteurization, had fewer detrimental effects on food flavor, texture, and nutrition. The importance of pressure in pharmaceuticals, medicine, and biomaterials sciences is beginning to be appreciated [1, 4, 5]. Enzyme reactions under high pressure or in supercritical fluids may be promising in the synthesis of pharmaceuticals by operating under solvent conditions that increase the thermobarostability of industrial enzymes. In medicine, pathogens such as herpes, immunodeficiency viruses, and certain prion proteins are inactivated by pressure, which may be useful in the ex vivo treatment of blood. Even physiologically generated pressures, such as during colon peristalsis, have biological effects, for example, on the adhesion properties of epithelial cells in colon cancer [6].

High pressure methods: A technique was developed for cooling a protein crystal while under pressure with the goal of “freezing-in” pressure-induced structural changes by going below a glass transition temperature. Once cooled, high pressure structures could be studied at ambient pressure so long as temperatures were kept within a metastability region. Data acquisition was similar to ambient pressure cryo-crystallography, eliminating limitations due to bulky pressure cells on the beam line. The technique was based on a method by Thomanek et al. [7], who, in searching for ways of cooling crystals for protection against X-ray radiation damage, observed that myoglobin crystals slow cooled at 2.5 kbar still diffracted well.

In order to evaluate low temperature metastability (the “freezing-in” of structural changes), we also used a room temperature method in which the protein crystal was actively pressurized on the beam line using a pressure cell modified from Kundrot and Richards [8]. The crystal resided in the central bore of a beryllium pressure cell and was in contact with the mother liquor, which was used as the pressurizing medium.

Results: Room temperature (RT) and low temperature (LT) structures of sperm whale myoglobin were solved at ambient (low) pressure (LP) and at high pressure (HP; 1.5 to 2 kbar), with the LT-HP structure solved from crystals prepared using the high pressure cooling protocol. Metastability was evaluated by comparing pressure-induced structural changes at RT and LT.
Because data for the LT-HP crystal was taken at ambient pressure, anisotropic changes could be frozen-in even though compressive changes had relaxed. Therefore, structural comparisons were separated into isotropic (compressive) and anisotropic (conformational) effects, with emphasis on identifying collective reorientations of the protein backbone. Same temperature LP and HP structures were isotropically scaled to the radius of gyration, then least squares superposed. Regions showing large displacement were identified by plotting differences in the backbone position as a function of residue number (Figure 1). An estimated detection limit of 0.1 Å on backbone displacements was based on root-mean-squared differences (rmsd) observed between nominally identical structures, and on effects of refinement model errors and simulated annealing temperatures [9].

The largest displacements occurred in the A helix, AB loop, CD loop, F helix, GH loop, and H helix (Figure 2). Similar pressure-induced changes at RT and LT were seen in the AB loop, CD loop, F helix, and the H helix. Poorer agreement was observed for the A helix and GH loop regions, though the GH loop region seemed to be a naturally variable region as seen in the controls. Good agreement in the largest displacements between pressure comparisons at RT and LT suggested the ability to “freeze-in” pressure induced structural changes.

Fig. 1 Differences in backbone position upon pressurization. Differences were determined after isotropic scaling and superpositioning of refined structures. Differences are plotted for room temperature (RT) and low temperature (LT) pressure comparisons and controls. Controls were between nominally identical structures. For details on the analysis, see reference [9].

Fig. 2 Comparison of pressure-induced changes at room (center) and low (right) temperatures. Yellow ribbon = room pressure positions; green ribbon = high pressure positions, amplified by a factor of 15 for clarity. The leftmost structure indicates regions where differences at the two temperatures were most similar (bold, with arrows indicating the general displacement direction) and least similar (italics). Similarity between the largest displacement regions (F helix, AB loop, CD loop, H helix) suggests low temperature metastability.
Future directions: Recent near-atomic resolution structures [10] and high pressure Raman studies [11] on myoglobin suggested the presence of rigid body movements, especially in the F-helix region of myoglobin as observed here. Myoglobin is a compact globular protein, and so may have represented a particularly challenging protein for initial pressure studies. Nonetheless, results have demonstrated the ability to detect collective motions upon pressurization using x-ray crystallography, and there is every reason to believe the technique can be extended to reveal pressure-induced functional changes in other proteins.

An intriguing possibility is the use of pressure as a cryoprotectant for protein crystal cooling. High pressure cooled crystals used here had low mosaicities (~0.4°) and diffracted to ~1.5 Å resolution despite being cooled to liquid nitrogen temperatures without chemical cryoprotectants. Cooling rate was -1.7 K/s, 30-60 times slower than achieved during flash cooling. Pressure may slow ice formation rates, and, in fact, high pressure flash cooling is a common method for preventing ice damage in biological samples for electron microscopy [12]. Investigators interested in using pressure during cooling should be aware of possible pressure-induced structural rearrangements; however, we observed larger rmsd’s upon flash cooling than upon pressurization, so the ability of high pressure cooling to lock-in reorientations is not expected to be a limiting concern. A high pressure protocol for flash cooling directly onto a cryo-loop is under development in Professor Sol Gruner’s lab at Cornell.

Atomic resolution structural information in proteins is vital when extending and applying biophysics and biotechnologies to high pressures, for example, in elucidating the structural basis for pressure effects on ligand binding, in understanding the compressibility of proteins and its relation to collective motions, and in studying protein stability and pressure-induced protein unfolding. High pressure protein crystallography is a field in its infancy with less than 10 of the greater than 18,900 structures deposited in the Protein Data Bank determined under kilobar pressures. The “freezing-in” method highlighted here provides new possibilities in exploring pressure effects using structural biology.

Acknowledgments

I thank Cayce Butler, Marcus Collins, Adam Finnefrock, Raphaël Kapfer, Emil Lobkovsky, Martin Novak, Matt Renzi, Mark Tate, Gil Toombes, and Weiru Wang for assistance in data collection; Richard Gillilan for help in data visualization; the CHESS and MacCHESS staff; Prof. George Phillips for generously providing protein crystals; Apostolis Gittis and Prof. Eaton Lattman for assistance in structural refinement; Profs. Hans Frauenfelder and Robert Austin for useful discussions; and Prof. Sol Gruner for his kind and invaluable guidance.

References