High-Pressure Protein Crystallography and NMR to Explore Protein Conformations

Marcus D. Collins,[1] Chae Un Kim,[2] and Sol M. Gruner[2,3]

[1]Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195-7290

[2]Cornell High Energy Synchrotron Source, [3]Department of Physics, Cornell University, Ithaca, New York 14853; email: smg26@cornell.edu

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Abstract

High-pressure methods for solving protein structures by X-ray crystallography and NMR are maturing. These techniques are beginning to impact our understanding of thermodynamic and structural features that define not only the protein's native conformation, but also the higher free energy conformations. The ability of high-pressure methods to visualize these mostly unexplored conformations provides new insight into protein function and dynamics. In this review, we begin with a historical discussion of high-pressure structural studies, with an eye toward early results that paved the way to mapping the multiple conformations of proteins. This is followed by an examination of several recent studies that emphasize different strengths and uses of high-pressure structural studies, ranging from basic thermodynamics to the suggestion of high-pressure structural methods as a tool for protein engineering.

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Energy landscape: a map of the internal (or sometimes free) energy of a protein as a function of its atomic coordinates

Microstructural: refers to the detailed atomic coordinates of a protein structure

INTRODUCTION

In 1914, Percy Bridgman (5) reported that egg white coagulates under pressure, thereby launching the field of high-pressure biology. High-pressure biology is not esoteric: Most of Earth's biosphere exists under pressures greater than hundreds of atmospheres (43, 50). Further, pressure effects on biomolecular function are both numerous and often of significant magnitude (7, 8, 14, 17, 23, 40, 41, 49, 50, 54).

Many experimental methods have been developed since Bridgman's time to explore high-pressure biophysics. We review some of the more recent of these, namely, the use of pressure as a tool for detailed studies of proteins via X-ray scattering, crystallography, and NMR. A recent review (16) focused largely on methodological improvements in crystallography; hence, the present review focuses on the structural biology findings in sum. The goal is to convince the reader that this structural data provides new ways to use pressure to explore the conformational space available to proteins, to test hypotheses of protein function, and to engineer proteins.

Consider the variables conjugate in free energy that the experimenter can directly control. For temperature, the conjugate variable is entropy, a vitally important but less intuitive quantity that in a protein energy landscape picture (6, 18, 58) can be thought of as the accessible area near an equilibrium structure—that is, the fluctuations about the equilibrium. Similarly, the presence of salts or denaturing chemicals perturbs the structure of a protein in complex, indirect ways that may be difficult to visualize. Pressure has the advantage that its conjugate is volume, something readily and directly related to structure and to the conformational changes needed for protein function.

Pressure has remarkable effects on proteins, including a tendency to unfold them. At lower pressures at which the protein still maintains a functional form, many proteins undergo dramatic shifts in activity (7, 8, 14, 17, 40, 41, 49, 50, 54). These effects can be related directly to measurable structural deformations of the protein. The perspective adopted in this review is that these deformations guide a mechanistic understanding of the molecule.

We first introduce the reader to thermodynamic versus microstructural viewpoints of protein energy landscapes in the context of high-pressure experiments. Next, we review the historical development of high-pressure structural studies of macromolecules and provide recent examples of insights gained from crystallography and NMR. Finally, we show that pressure allows the exploration of

enzyme functional pathways and a means of modulating those pathways.

THERMODYNAMIC AND MICROSTRUCTURAL PERSPECTIVES OF PROTEIN ENERGY LANDSCAPES

Proteins are now frequently described using the concept of a multidimensional free energy landscape (6, 18). This landscape is a function of all conformational variables of a protein system such as the protein molecule, solvent, ions, and ligands.

The landscape may be viewed from either thermodynamic or statistical mechanical perspectives. In the thermodynamic perspective we think about states of the protein system that have macroscopic properties such as temperature, volume, and compressibility. In general, these states are not tied to any one exact conformation of the protein, but rather include very many such conformations (6). Until recently, most protein pressure studies measured only macroscopic thermodynamic variables, for instance, a determination of protein compressibility by measuring the fractional volume change of a protein system upon application of a unit pressure. Ultimately, one wishes to understand how the system free energy changes as a function of such variables. An extension of this approach is to consider the shape of the multidimensional free energy surface (i.e., the energy landscape) versus a set of conformational coordinates that can represent any of many degrees of freedom of the system. **Figure 1** shows a simplified energy landscape using citrine as an example.

Thermodynamics, by definition, provides averaged information about the behavior of large numbers of molecules. While this information is useful, it is difficult to gain insight from it about detailed changes around specific residues when proteins are pressurized. Today the experimenter has exquisite control of microscopic details of proteins. Crystallography allows visualization of the internal organization of a protein and genetic engineering allows synthesis of precise mutants. More direct questions are needed about how the energy landscape varies with detailed perturbations. For example, consider an enzyme whose activity is pressure dependent. What is the effect of pressure on the structure of the active site? If the protein is mutated by substituting one amino acid for another at an active site, does the pressure dependency of enzymatic activity increase or decrease? If so, can we devise models of the energy landscape that allow us to explain or, more ideally, to predict the sign of the change? Can we make the same kinds of predictions about distant, allosterically coupled amino acids? This type of information would directly assist engineering protein activity.

High-pressure studies may also help us understand the basis of both allostery and the conformational substates (CS) that represent well-defined minima in the free energy landscape. For example, hemoglobin exists in various distinct CS, each readily identified spectroscopically and with known, distinct structures (27). Spectroscopic pressure studies have shown that the equilibrium between relaxed (R) and tensed (T) hemoglobin in solution shifts toward R at high pressure (52). In a crystallographic study of primarily T hemoglobin, would applied high pressure shift the hemoglobin atomic coordinates to a more R-like structure? That is, is there a progressive decrease in the root-mean-square (RMS) difference in atomic coordinates of the high-pressure T-state hemoglobin and the known R-state structure? Although this experiment has not yet been performed on hemoglobin, it posits well-defined, experimentally accessible, quantitative questions about the relative atomic coordinates in the molecule that directly elucidate the interconversion between CS. A similar experiment has been performed on myoglobin (see below) (53).

HISTORICAL DEVELOPMENT OF HIGH-PRESSURE STRUCTURAL STUDIES OF PROTEINS

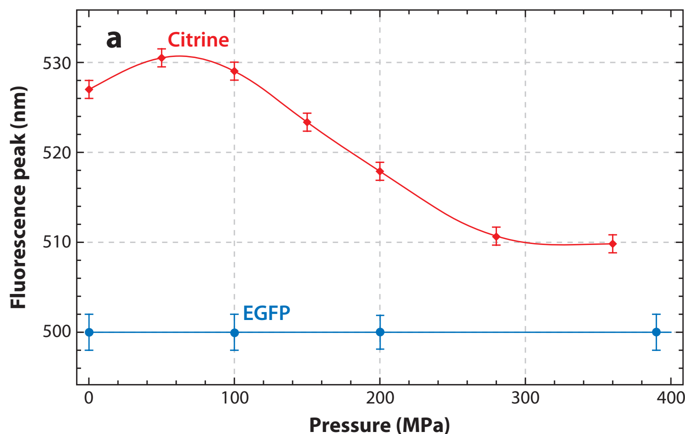
This section reviews the use of high-pressure crystallography and NMR to explore protein

Conformational coordinate: an averaged coordinate, such as the center of mass of a protein subdomain, or the angle between two helices

Allostery: the tendency of two or more conformational changes in a protein to favor each other

CS: conformational substate(s)

RMS: root-mean-square (usually in reference to atomic displacements)



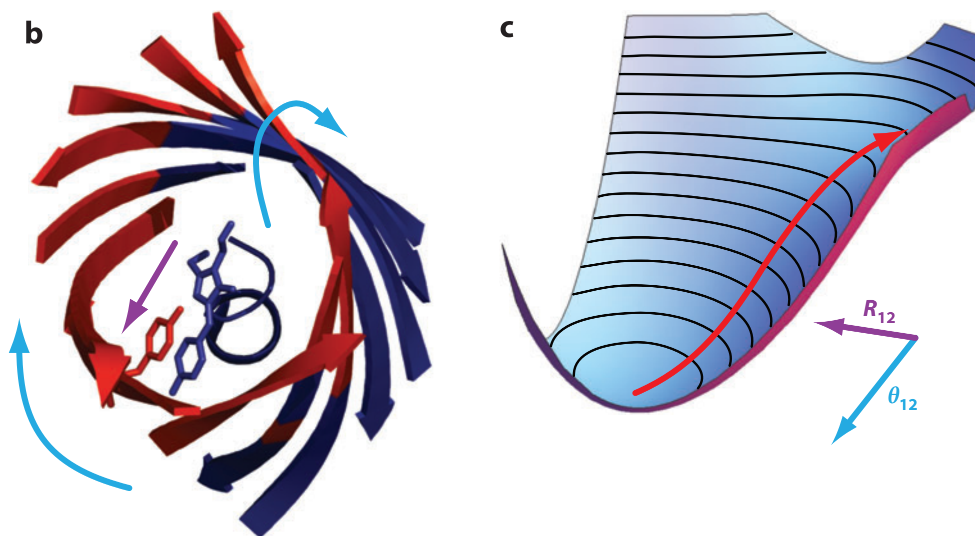


Figure 1

High-pressure deformations reveal the free energy landscape of citrine. (*a*) Peak fluorescence wavelength of citrine (*red diamonds*) and enhanced green fluorescent protein (EGFP) (*blue circles*) as a function of pressure. Curves are meant only to guide the eye. Reproduced with permission from Reference 3. (*b*) The citrine structure. Cluster 1 (see text) is shown in red, Cluster 2 in blue. Light blue arrows indicate rotations of Cluster 1 relative to Cluster 2 as pressure increases, while the purple arrow indicates the pressure-induced displacement of Cluster 1's center of mass relative to that of Cluster 2. Adapted from Reference 4. (*c*) A hypothetical (internal) energy landscape with two conformational coordinates: R_{12}, the distance between the clusters' centers of mass, and θ_{12}, the angle between principal axes of the two clusters. As pressure increases, work is done and the protein has higher internal energy, as indicated by the red arrow, which travels up a shallow valley in the energy landscape.

landscapes. The primary focus is on cases for which detailed atomic structures are known and can be connected to the effects of pressure at the macroscopic scale. Generally, one looks for locations in a given protein that display deformation under pressure. Proteins are globally linked objects. Thus, if one region of a protein compresses more than other regions, relative spatial readjustments to accommodate that heterogeneous compressibility may

propagate pressure effects on other parts of the protein.

First Steps

In 1973, Thomanek et al. (51) reported X-ray crystallographic data collected from a crystal of sperm whale myoglobin pressurized to 250 MPa in liquid isopentane and frozen in liquid nitrogen. Isopentane was chosen as a pressurization medium because it is hydrophobic, so it hardly penetrated the crystal but could be readily flaked off the crystal when frozen. The extracted crystal, always kept near liquid nitrogen temperatures, was mounted on an X-ray diffraction stage. The first critical observation was that the crystal survived pressurization and diffracted well. A number of Bragg reflections had intensities that differed from crystals at ambient conditions. The authors speculated that pressure-induced structural changes persisted at low temperature, even though the pressure had been released; i.e., structural effects due to pressurization were somehow locked-in as long as the crystal was kept near liquid nitrogen temperatures. The crystal did not compress uniformly; rather the compressibility was small along one crystallographic axis (b^*) and larger along the other axis (a^*). No structure was solved, as the focus of the paper was on freezing crystals to prevent radiation damage and to improve Mössbauer spectroscopy data collection.

In the mid-1980s, Kundrot & Richards (37, 38) developed a beryllium specimen chamber specifically designed for X-ray crystallography and used it to solve the structure of hen egg white lysozyme at up to 100 MPa. The chamber is a rod into which a crystal could be inserted and then pressurized in mother liquor. The beryllium rod was strong enough to operate at 100 MPa, yet sufficiently small and X-ray transparent that it was readily mounted on a standard X-ray goniometer. The motivation was, in part, to identify the most compressible regions in a protein that might be important to function or solvent accessibility. At the time, acoustic spectroscopy was the best means to measure protein compressibility; Kundrot & Richards set out to make a more direct measurement.

They discussed several important themes. The lysozyme domains had measurably different compressibilities—in fact one of the two is incompressible. These domains are not contiguous in the amino acid sequence but are purely structurally defined—in this case by a hypothesized hinge around an active site. However, no net rotation about the suggested hinge was detected. It has since become possible to analyze pairs or sequences of structures for such motions in a better defined way, as discussed below for the case of citrine.

The Debye-Waller temperature factors (or *B*-factors) decreased on average, but no coherent trends appeared and many *B*-factors even increased. This proves to be generally true of pressurized proteins. Kundrot & Richards observed that, despite different compressibilities, both domains moved toward the center of mass of the molecule, perhaps suggesting a different nature for the hinge motion inferred by earlier researchers. Side chain reorientations were the main source of compression, and buried side chains reoriented much less than surface side chains, demonstrating the well-packed nature of the protein.

Most importantly, the high-pressure lysozyme structure suggested new ways that one could identify structurally defined subdomains for further analysis. The ability to reduce the number of structural coordinates needed to describe an energy landscape is fundamental to the power of high pressure in studying protein structure and dynamics. This study strongly suggested the most significant motions were of large structural motifs, not an atomic free-for-all. This leads us to speculate whether the distinctly compressible domains seen in lysozyme are coincident with the structural sectors identified by Halabi et al. (20).

The Kundrot & Richards work had several unintended consequences that discouraged high-pressure crystallography. First, the absolute magnitudes of the deformations were generally sub-angstrom, leading others to draw

BPTI: bovine pancreatic trypsin inhibitor protein

HPr: histidine-containing protein

the erroneous conclusion that they were not biologically important. In fact, sub-angstrom motions around active sites can greatly alter enzyme activity. Difficulties with crystal cracking led others to conclude that crystal pressurization is difficult. Instead, recent experiences with dozens of different proteins have shown that crystals are typically robust against cracking while pressurized.

Crystal cracking during pressurization involves some perhaps nonintuitive subtleties. Protein crystals usually contain more water than protein. Consider a protein 50 Å across as a sphere scaled to 30 cm, about the size of a basketball. When stacked into a simple cubic lattice, the basketballs occupy only 52% of the crystal volume; the remaining volume is filled with water molecules (~1 cm across on this scale). Imagine all the 1-cm marbles that can fill the volume between the spheres in the basketball crystal. Although some of the water is bound to the protein surface, most is effectively bulk water several centimeters removed from the protein. Thus, pressurization of a protein crystal is qualitatively different from pressurization of a typical solid crystal: There is no strain gradient across the faces of the crystal because the interstitial water transmits pressure uniformly throughout the crystal to each protein molecule. So why would a crystal crack under pressurization? It must be because pressure is affecting the shape of each protein molecule or the nature of the soft forces that hold the crystal together. Whereas in most solids crystal cracking is a result of macroscopic strain, with proteins crystal cracking is a molecular manifestation of pressure.

NMR Leads the Way

NMR spectroscopists began to study small proteins such as bovine pancreatic trypsin inhibitor (BPTI; the first high-pressure NMR structure we are aware of) (56), histidine-containing protein (HPr) (28), ubiquitin (35) and the GTPase Rap1 (26), among others (25, 39, 42, 48). It remains challenging to solve full structures by NMR, so although it was clear that pressure was inducing alternative protein conformations, it was not always clear exactly what those conformations were.

Histidine-Containing Protein

These studies are well characterized by the work on HPr (28), which is involved in a phosphorylation pathway ultimately responsible for carbohydrate transport. From biochemical studies it is known that active HPr is itself phosphorylated at Ser46, which affects HPr's substrate binding surface. To account for the allosteric effect of phosphorylation, the authors presumed that fluctuations must be elevated at the binding surface, including the His15 active site, and that Ser46 phosphorylation would suppress or expand such fluctuations. Alternatively, there could be a small number of alternative conformers, one of which might correspond to the conformation favored by phosphorylation at Ser46. In the energy landscape picture, these two hypotheses are only slightly different, the distinction being whether or not the alternative conformers are true local free energy minima. In either case, one expects associated higher and lower volume conformations, so that the active site should have increased compressibility.

Indeed, pressure-induced changes of the ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) NMR spectra were largest near Ser46 and His15. Unexpectedly, the pressure-induced backbone amide ^{1}H–^{15}N peak shifts of Ser46 and Ile14 were distinctly different from those of their structural neighbors. Ile14 has one of the lowest shifts in the entire molecule, which the authors speculated may indicate it forms a conformational pivot or nucleation point. They did not specifically consider Ser46, but based on the tabulated data, its amide peak changes are smaller than typical for surrounding residues, and the ^{1}H shift is opposite in sign from its neighbors. The rigidity of Ser46 and Ile14 may help transmit phosphorylation-induced displacements to the active site His15.

Dihydrofolate reductase. Perhaps the best characterization of how high-pressure NMR

could be used to characterize the energy landscape and important dynamic modes of a protein was the study by Kitahara et al. (34) on dihydrofolate reductase (DHFR). From ambient pressure X-ray crystallography, DHFR was known to adopt different conformations when binding cofactors and substrate. A model had been developed using those X-ray structures to explain the conformational changes associated with enzymatic action, but solution NMR work had revealed only one conformation in the folate-bound state, whereas crystallography had indicated two.

Prior ambient pressure NMR indicated substantial fluctuations. Thus, high-pressure NMR was an excellent tool to isolate CS involved in the fluctuations and to tie those substates to enzymatic action based on the crystal structures. Those structures revealed a potential motion of the M20 loop that could occlude or open the active site. Kitahara et al. examined both the pressure and temperature dependency of ^{1}H–^{15}N HSQC spectra, showing that on either end of this loop the cross-peaks shifted and split into doublets, indicating two conformational states. They measured the free energy, enthalpy, entropy, and volume differences between the two states. In particular they showed that at ambient pressure, with a folate substrate, and without the NADH cofactor, only 10% of the population would be in the open state, functionally significant, but largely undetectable by ambient pressure NMR. High pressure was necessary to identify this state in solution.

Compared with earlier work on BPTI (56), the authors used the sensitivity of the ^{1}H–^{15}N HSQC spectra to amide NH group orientations to estimate changes in backbone torsion angles. Roughly 4° changes at the ends of the M20 loop contrasted sharply with no observable torsion angle changes in the rest of the loop. The high-pressure NMR work conclusively demonstrated a continuous hinge-like motion, confirming the hypothesis generated from X-ray structures. Clearly, the challenge was to generate high-pressure structures and directly observe such changes with the complete structural information available from X-ray crystallography.

DHFR: dihydrofolate reductase

Diamond anvil cell (DAC): a kind of high-pressure cell for X-ray and optical studies

Methods of Performing High-Pressure Protein Crystallography

It is useful at this point to digress from the historical flow to summarize the major methods that have been developed to perform high-pressure protein crystallography. These include ambient temperature beryllium and diamond anvil cell (DAC) methods and cryogenic liquid and gas charged methods.

Ambient temperature methods. Urayama et al. (53) modified Kundrot & Richards' (37) beryllium cell to reach 200 MPa by repositioning an O-ring between the threaded beryllium rod and a steel seat to act as a Bridgman seal. Beryllium cells have limitations. The required high-strength beryllium material is difficult to obtain and machine. The typical one-quarter-inch rod thickness limits pressures to about 200 MPa; in principle a thicker rod and higher energy X-rays can be used to widen the pressure range while mitigating increased X-ray absorption. It is difficult to align the crystal in the X-ray beam because the rod is opaque. Most seriously, intense beryllium powder diffraction rings are superposed on the protein diffraction spots. The lowest order rings begin at an atomic spacing of ∼2.3 Å, limiting the collection of higher resolution protein diffraction data.

Diamond anvil cells are alternative X-ray pressure cells capable of reaching higher pressures and resolutions. Early experiments (29) have evolved considerably, as reviewed recently (16). The diamond optical transparency facilitates aligning the protein crystal in the X-ray beam, but X-ray absorption and limited sample size require a high-energy beam line delivering an intense, small-area beam. The diamond cell mount limits the rotation angles of the crystal; this is partly compensated for by short X-ray wavelength reduction of scattering angles into the forward direction. Wider angle DAC

designs exist but have not yet been applied to protein crystallography.

Cryogenic methods. The method pioneered by Thomanek et al. (51) uses liquid isopentane as a pressure medium. The tedium of removing the pressure cryocooled crystal from isopentane at liquid nitrogen temperature led us to develop a convenient method using helium gas (30). The crystal is pressurized in a column of helium gas and then dropped, still at high pressure, into a zone at liquid nitrogen temperature. Once the crystal is frozen, gas pressure is released and the crystal is removed from the apparatus under liquid nitrogen and then handled as any normal cryocooled crystals. Normally, no penetrating cryoprotectants are required; the only fluid present is mother liquor. The method requires special equipment, but once in place it is relatively easy to use. At Cornell, several crystals per hour may be processed, and dozens of different types of protein crystals have been successfully pressure cryocooled (30).

Crystals cryocooled in this manner tend to produce superior Bragg diffraction. It is also possible to produce bulk vitreous cryocooled mother liquors: The method has been used to cryoprotect crystals and crystallization solution in thick-walled polycarbonate capillaries, which is not possible at ambient pressure without adding high concentrations of chemical cryoprotectants (31). Not only do cryoprotectants add complexity, they frequently compete with ligands for binding sites, compromising structural experiments. A good example of these advantages is a recent study of ligand bound to the RCK domain of the KtrAB K^+ transporter (1), which proved difficult in the presence of cryoprotectants and standard ambient pressure methods.

Kim et al. proposed (30), and subsequently confirmed (32), that the method mitigates cooling damage by inducing formation of high-density amorphous ice in the crystal. The glassy water exhibits unusual liquid-like behavior when warmed from 80 K to 160 K (see figure 4 of Reference 33). Although it is beyond the scope of this review, this behavior may be advantageous in studying protein fluctuations or using temperature dependency to separate entropic contributions to conformational free energies.

Conformational Substates of Myglobin

In 2002, Urayama et al. (53) solved multiple structures of sperm whale myoglobin with two goals in mind. The first goal was technological: At the time, high-pressure crystallography on proteins required beryllium cells that limited both the diffraction resolution and the pressure range available. Urayama et al. quantitatively demonstrated that the structural effects of high pressure at room temperature could be "frozen in" if the crystal was cooled to 77 K while under pressure in isopentane. The observation that the diffraction quality was excellent, even if the crystals were slowly cooled without added cryoprotectants, motivated the development of the high-pressure cryocooling technique (30).

The second goal was to map out conformational substates of myoglobin. In a landscape picture, effects that are trapped upon cooling under high pressure represent free energy minima that are linked to each other in a continuous free energy landscape. The low-energy substates will be populated some definite fraction of the time and can be functionally relevant. To show that the frozen-in effects of pressure had in fact shifted the equilibrium population of two or more CS, Urayama et al. began from the observation that increasingly acidic pH and increasing high pressure had similar effects on the relative populations of two spectroscopically defined CS. The structures of sperm whale myoglobin at representative pH values were available, and the object was to compare structures solved at high pressure against those solved at acidic pH to determine whether the conformational changes were similar, as measured by the displacements of individual peptide backbone atoms. Urayama et al. showed that the changes due to pH and pressure are highly correlated. This was the first clear demonstration that pressure could be used to identify and characterize detailed shifts

in CS populations that would be functionally relevant to the study of a given protein.

The RMS deviations of the α-carbon backbone positions upon pressurization of myoglobin were typically sub-angstrom in magnitude. Although crystallographic resolutions are usually poorer than an angstrom, deviations of groups of atoms can be determined to higher accuracy. Urayama et al. (53) determined that the experimental uncertainty in the myoglobin experiments was on the order of one-tenth of an angstrom. A related issue is how best to superimpose two distorted structures so that the RMS deviations may be measured (for more detail see References 3 and 10).

Energy Landscapes by Crystallography

An important caveat is that a crystallographic experiment yields a structure representing the ensemble of states. Any side chain or structural subunit of the protein that normally fluctuates between two CS will likely appear as a region of increased thermal *B*-factors. *B*-factors are difficult to interpret quantitatively because structural contributions are convolved with crystal mosaicity and other imperfections.

Furthermore, the implied large conformational and volume fluctuations can also be explained by a relatively flat free energy landscape. To demonstrate conclusively the existence of true CS requires careful observation of the complete transition and, ideally, a model of the CS that describes changes in volume, free energy, and so on, as was done in the pioneering NMR examples described above. The remaining examples below demonstrate how various kinds of CS can be mapped out in detail using X-ray crystallography, either directly under high pressure or in parallel with other high-pressure experiments.

T4 lysozyme, the hydrophobic core, and buried water in proteins. Collins et al. (9, 10) used a beryllium cell to solve the room-temperature, high-pressure structure of the T4 lysozyme (T4L) mutant L99A. Brian Matthews' laboratory (59) had shown that this mutant contained a buried and empty (i.e., vacuum!) cavity of almost 150 $Å^3$. The cavity is hydrophobic, lined by aliphatic and aromatic residues, buried in the C-terminal helical bundle of the protein, and is formed by a leucine-to-alanine substitution. Defying expectations, the cavity failed to collapse under pressures as high as 200 MPa and instead steadily filled with water, whereas the structure of the protein remained mostly unchanged. This presented an unusual opportunity to closely follow a clear multistate system as pressure altered CS populations, i.e., the cavity water occupancies.

T4L: the lysozyme from bacteriophage T4

The issue of water buried in proteins is fascinating and sometimes contentious. Many proteins (including T4L) contain buried pockets of water inaccessible to the bulk solvent; this buried water is frequently coordinated with buried polar or charged amino acid side chains (47, 55). The more interesting cases include interleukin-1β, certain cytochromes, and even membrane ion channels, where water is proposed to occupy more hydrophobic pockets. What effect do such waters have on protein function and stability?

Collins et al. (9) examined T4L and determined the water occupancy by integrating the excess electron density inside the L99A cavity. Increasing electron density with pressure indicated that the cavity water content increased slowly up to pressures of about 100 MPa, and then rose steeply above that, suggesting the midpoint of a sigmoidal transition (**Figure 2**).

Molecular dynamics (MD) modeling yielded additional insight and suggested a transition between two cavity states—one empty and one filled with up to four hydrogen-bonded water molecules (**Figure 2**). The protein itself was extraordinarily rigid with a cavity volume that decreased by less than 3%, suggesting that pressure did not alter the energetics of water interaction with the cavity walls. Assuming this, it was estimated that the free energy difference between the cavity's empty and flooded states was only ~1.4 kT/water molecule (Boltzmann's constant, k, times the absolute temperature, T, equal to ~2.5 kJ mol^{-1} at room temperature). This difference is sufficiently small that even

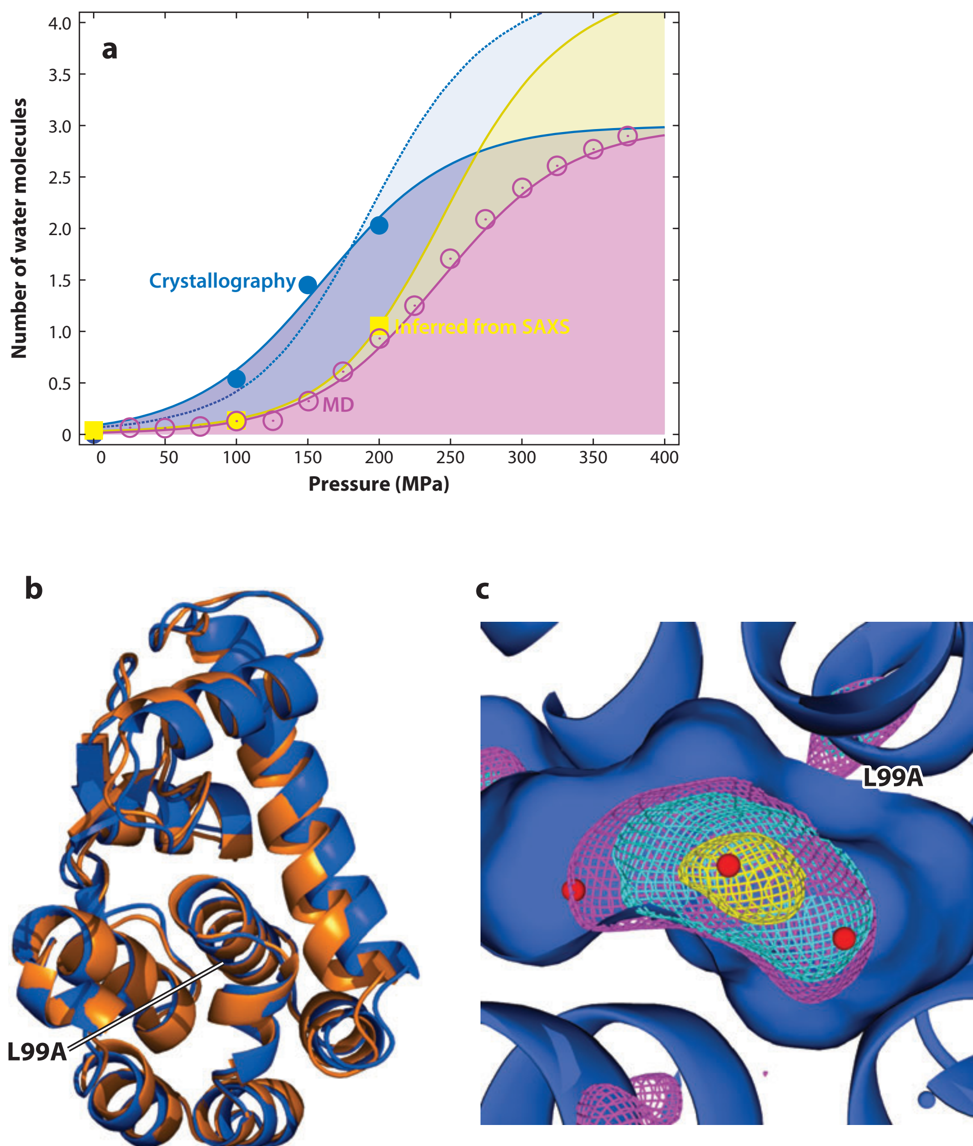


Figure 2

T4 lysozyme (T4L) mutant L99A at high pressure. (*a*) Number of water molecules observed crystallographically (*solid blue circles*), in molecular dynamics simulations (*yellow squares*), and inferred from small-angle X-ray scattering (SAXS) measurements on pressure unfolding (*open purple circles*). For comparison, the SAXS data are shifted horizontally (*blue dotted line*) to have the same midpoint pressure. Adapted from Reference 2 and 9. (*b*) Structural changes in T4L from the ambient (*blue*) to the 200 MPa (*orange*) structure. Differences have been magnified 5X for clarity, and the structures are aligned using the helical bundle that contains the L99A cavity mutation. (*c*) Crystallographic electron density maps show water filling the L99A cavity at 100 (*yellow*), 150 (*blue*), and 200 (*magenta*) MPa. Adapted from Reference 9.

Erratum

under ambient conditions one would expect this state to be populated around 2% of the time, assuming the cavity had access to the bulk water.

Water diffuses even through very hydrophobic materials such as Teflon™ or lipid bilayers at measurable rates. A protein's hydrophobic core fluctuates constantly and is only angstroms removed from bulk water, so one must expect a slow diffusion of water through the volume of all proteins. In the case of T4L, the effect of pressure is to increase the water occupancy in the cavity by making the free energy sufficiently favorable that the waters remain long enough to be detected crystallographically.

The T4L studies provided crystallographic evidence connecting detailed structural changes in the protein and transitions involved in unfolding at high pressure. It has been long hypothesized that pressure-induced unfolding involves water penetration into the protein core, a subtly different concept than the protein core becoming exposed to bulk water (24). The T4L measurements showed that such penetration by water molecules into the hydrophobic protein core was not only possible, but probably quite common.

Studies of pressure unfolding of staphylococcal nuclease (SNase) had shown that the radius of gyration of pressure-unfolded SNase was smaller than that of thermally or chemically denatured SNase (45). This finding suggested that pressure only partially unfolded SNase. Paliwal et al. (44) used small-angle neutron scattering and MD simulations to infer that pressure unfolding of SNase was a progressive process involving loosening of tertiary contacts, leaving much secondary structure relatively intact. This suggested that water was involved in protein unfolding at high pressures, but direct evidence of water entering at specific sites of the protein was lacking. An important challenge in the T4 lysozyme experiments was to connect the crystallographic result that water penetrated into the protein's hydrophobic core under pressure with partial unfolding.

Ando et al. (2) followed with a careful study of L99A T4L unfolding by high pressure. Using a newly designed small-angle X-ray scattering (SAXS) chamber, Ando et al. determined the volume change in a pressure-induced, unfolding-like transition. This volume change could be substantially accounted for by the empty cavity volume of the ambient pressure structure, supporting an unfolding scenario in which these cavities become hydrated and thereby loosen tertiary contacts. Furthermore, the SAXS data demonstrated the relatively compact structures of the progressively unfolded states with pressure.

SAXS: small-angle X-ray scattering

Pressure-induced unfolding highlights that there is neither one unfolded state nor one folded state of a protein. High-pressure structural studies, building on mutagenic studies of proteins, help to show this more clearly by allowing the experimenter to slowly tune a perturbation of the structure. There is increasing evidence that the complicated free energy landscapes of proteins, and rarely populated CS, may be involved in diseases such as Alzheimer's and type II diabetes (22). The studies on T4L suggest that high-pressure crystallography may allow such states to be studied in detail.

Ubiquitin and T4L: How rigid is an α helix?

In 2005, Kitahara et al. (36) published the structure of the important small protein ubiquitin, one of the first complete high-pressure structures solved by NMR. Using nuclear Overhauser effect (NOE) spectra, backbone dihedral angle constraints from chemical shifts, and simultaneous fitting of the two structures to the data, the authors generated averaged structures at 3.0 and 300 MPa and independently determined their relative populations. The largest difference between the two structures was a 9° rotation of the main helix (residues 23–40). This helix's internal structure was unaltered by pressure, but it rotated and displaced relative to the rest of the protein. This contrasts with T4L, in which a helix in a similarly exposed position bent by several degrees roughly about its midsection, suggesting that helices might require some stabilization from their surroundings to be truly rigid (10). In ubiquitin, a smaller protein with presumably fewer side chain contacts to stabilize this helix, it appears that 300 MPa

was not enough to deform the helix. On the other hand, in T4L, the midpoint of the deformed helix rests against a dense network of contacts that has also been implicated as a nucleation site in folding. It would be especially interesting to examine the issue of side chain contacts in ubiquitin.

Ubiquitin and T4L: Water penetrates the core in high-pressure unfolding. Similar to Collins et al. (9, 10), Kitahara et al. (36) found that the protein core opened up at high pressure, visible both by its three-dimensional structure and by an increasing solvent-accessible surface area even as the molecular volume decreased. While this may lead to unfolding, this core opening is directly related to the shift between the two observed conformational states.

It would be especially interesting to better constrain side chain conformations and any changes in crystallographically observable water in this pressure range and to compare the results with the T4L study (9, 10). Not long after the study by Kitahara et al. on ubiquitin structure was published, MD simulations (15) confirmed an increase of water hydrating small, hydrophobic cavities that opened up in ubiquitin, largely validating hypotheses of pressure-induced denaturation (2, 9, 24). It remains unclear whether these cavities become hydrated before unfolding or if they become completely open to the surrounding water as a result of pressure (57).

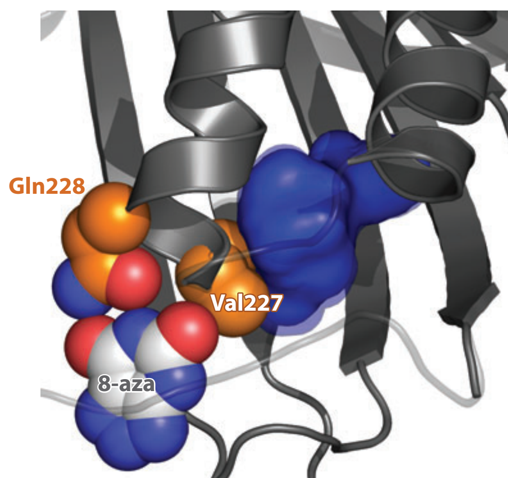


Figure 3

The urate oxidase active site and accessory cavity. Nearby residues Val227 and Gln228 are shown with their carbon atoms in orange. The active site blocker 8-azaxanthine (8-aza) is shown with its carbons in white. The accessory cavity of the 150 MPa structure (PDB code 3F2M; Reference 19) is shown in blue, with the ambient pressure cavity (PDB code 2IBA; Reference 12) shown in transparent blue around it.

A VIEW INTO THE FUTURE OF PROTEIN ENGINEERING?

Thus far, we have discussed how NMR and crystallography at high pressure have been used to map out regions of the protein free energy landscape to understand protein action. Now we turn to examples more directly related to practical protein engineering.

Urate Oxidase

The enzyme urate oxidase (Uox) is unusual in that it requires no cofactors or metal ions to break down its target substrate, uric acid's purine ring. It can be used medically to manage hyperuricemia, for instance, due to renal failure or chemotherapy, but is avoided because it requires intravenous administration and can be anaphylactic. Reengineering such enzymes has obvious merit.

Colloc'h and colleagues (11) reported the Uox structure at 140 MPa using DAC X-ray crystallography. Consistent with other work, the Uox β-sheet structure is remarkably rigid, with the all-atom RMS deviation only 0.14 Å, which is barely resolvable. The ambient pressure structure contains a 136 Å^3 hydrophobic cavity devoid of water (**Figure 3**). Unlike the T4L cavity, its volume decreases by 24% and it remains empty under pressure. By titrating the pressure of oxygen from 1 to 4 MPa (13), the authors found that this accessory cavity did not fill with oxygen, ruling out its proposed role as an oxygen reservoir for the enzyme. Does this cavity have a purpose?

A recent paper combines SAXS, functional assays, and crystallography at high pressure

(19) to show that Uox undergoes two pressure-induced transitions leading to reduced activity, one of which is most likely dissociation of the functional tetramer into monomers. The nature of the other transition is less clear. The kinetics of the enzyme-catalyzed reaction itself changes little with pressure. However, the substrate affinity for the binding pocket decreases with pressure, reducing overall activity.

The authors (19) made two important observations based on the high-pressure structure. First, thermal *B*-factors among amino acids lining the active site entrance actually increase, consistent with residues gating other cavities (9, 10). Although these side chains become more flexible, the kinetic data suggest that access to the active site is more restricted at high pressure. The authors speculate pressure-induced flexibility might allow a normally open side chain gate to close. Such a gate could be a novel drug target.

Second, the accessory cavity collapses with pressure (**Figure 3**), whereas the active site cavity expands—the accessory cavity acts as ballast for the reaction products, which require a different binding pocket geometry. Pressure disfavors the cavity geometry that binds substrate.

High pressure stabilized a state that likely represents the conformation of the active site after the oxidation reaction has occurred. No complicated chemistry or kinetic trapping was needed to observe this conformation. Moreover, the allosteric interaction between these cavities suggests a general approach to identifying and exploiting allosteric interactions in proteins.

Morphinone Reductase: Multiple Conformational Pathways of an Enzyme

Although pressure was not used directly in morphinone reductase crystallography, we include this example because it suggests how other high-pressure techniques could be used together with crystallography to identify and map out multiple reaction pathways in an enzyme. Capping a remarkable set of papers, Scrutton and colleagues (21, 46) identified three kinetically distinct pathways in morphinone reductase, a hydride transfer enzyme. An aspartic acid–to-alanine substitution (N189A) increased disorder in the active site and increased usage of the three pathways, whose rates spanned three orders of magnitude.

This mutation demonstrates that enzymes can and do have multiple kinetically distinct reaction pathways. To characterize these pathways, Pudney et al. (46) used pressure to alter the populations of the three different NADH-enzyme conformations.

Crystallographic structures of the wild-type and N189A enzymes identified multiple active site substrate conformations. MD simulations of wild-type enzyme indicated high-pressure-induced changes in active site geometry corresponding to CS of the N189A active site. Further simulations of N189A showed that pressure shifted the equilibrium between these CS, decreasing the hydride donor-acceptor distances in the same way as that observed in simulations of the wild type.

The high-pressure kinetic results are equally intriguing. Pressure essentially has no effect, or speeds up the reaction, depending on the pathway. On the other hand, pressure significantly alters each pathway's utilization. The occupancy of the initial CS changed owing to their different volumes. The overall enzyme activity slows because of the underlying conformational flexibility of the protein, and not because pressure alters the pathways' underlying kinetics.

High-Pressure Crystallography as a Predictive Tool: The Case of Citrine

The most direct suggestion of how to use the ideas developed above comes from Barstow et al. (3, 4), who analyzed high-pressure-induced changes in the pressure-sensitive yellow fluorescent protein, citrine, using the high-pressure cryocooling method (30). Citrine differs from the green fluorescent proteins (GFPs) by a threonine-to-tyrosine substitution at residue 203; the Y203 phenol ring interacts with the main chromophore common to this

superfamily of fluorescent proteins. Y203 is anchored to a structurally distinct part of the protein, on the β barrel rather than on the central 3_{10} helix containing the main chromophore, the autocatalytically fused residues called Cro66.

In their first paper, Barstow et al. (3) detailed pressure-induced changes near the active site, where Cro66 and Y203 overlap. The overlap of the two ring structures is thought to result in the distinct yellow color of citrine's fluorescence. If so, then fluorescence should be exquisitely sensitive to small, pressure-induced changes in separation and orientation of the two rings. Indeed, as pressure is increased from 50 to 300 MPa, the fluorescence peak shifts from ~530 nm to 510 nm, the GFP peak, and the fluorescent yield drops by roughly a factor of two. The effect appears to be reversible, and no such shifts occur in engineered GFP, where the perturbing Y203 is absent.

In experiments on crystals, Barstow et al. unambiguously assigned fluorescence spectral changes to high-pressure protein structures derived from X-ray crystallography. Above 50 MPa, the main chromophore motion is essentially confined to a plane parallel to the Y203 phenol ring. Over ~300 MPa, it moves in a direction about 45° to the axis formed by the phenolic oxygen and the fourth carbon of the phenyl ring, by almost 1 angstrom. With this small shift, the influence of the Y203 ring is eliminated.

From atmospheric pressure to 50 MPa, the results are in some ways much more interesting. Here, increasing pressure red-shifts the fluorescence peak. This suggests that perturbation by Y203 could be red-shifted, and potentially more efficient, by stabilizing the pressure-induced conformation at ambient pressure. One wonders whether the same could be said for other enzymes such as those discussed above. But how would one take advantage of this knowledge?

Barstow et al. (4) showed a way forward by discussing the global citrine structure. The key finding is that the changes observed in the active site are really due to subtle changes in large, essentially rigid structural domains linked to the active site.

A cluster analysis was used to identify groups of atoms that move together with increasing pressure. The analysis identified two distinct regions, the borders of which are not obvious when looking at the citrine structure. One region contains Y203, with Cro66 and most of the 3_{10} helix in the other region (**Figure 1**).

The two parts of the active site sit in different structural regions, which explains why they move relative to each other. But their relative motion is not what one might first suppose. The centers of mass move at most a couple tenths of an angstrom relative to each other, at the noise limit of the measurement. Instead, small relative rotations of the two structural elements cause Cro66 and Y203 to move relative to each other. The two elements of the active site sit at the ends of long lever arms, an observation beautifully demonstrated by high-pressure crystallography.

This example demonstrates the globally connected nature of proteins. In citrine, pressure resulted in relative shifts of two segments of the protein. These, in turn, pulled on the residues responsible for fluorescent activity, which act as an active site in the molecule. This concrete example gives a plausible explanation for why many enzymes are very pressure sensitive: Similar global distortions of enzymes result in small deformations of active sites. As was the case with citrine, most enzymes involving electrostatic or quantum mechanical interactions will be sensitive even to sub-angstrom deformations of its active site.

SUMMARY: THE FREE ENERGY LANDSCAPE REVISITED

The last few examples in particular illustrate the goal of this review: to map out the free energy landscape of proteins in a way that connects their thermodynamics to their structure. The example of citrine is particularly relevant because it has a conformational substate about which we can ask questions.

What are the conformational substates—those configurations readily grouped together to simplify our representation? For citrine,

it is the folded state. The pressure-induced deformations appear smoothly and without jumps, indicating that pressure is simply perturbing this conformational substate from its ambient pressure conformation. What are the coordinates? The analysis by Barstow and coworkers showed that the description of this system can be reduced to two quasi-rigid bodies, with a relative center of mass and a relative orientation. Because this is a pressure experiment, we can determine how much work is done—how much energy is added to the system—from the overall compressibility of the system, determined independently or from the crystallographic experiment. In simplified form, **Figure 1** depicts an illustrative cross section through the free energy landscape, with one position coordinate and one orientation coordinate.

Morphinone reductase is more complicated. MD simulation, X-ray crystallography of the N189A mutant, and high-pressure kinetics experiments indicated the existence of three distinct conformational substates, each having its own distinct kinetics in the enzymatic reaction. These, along with kinetic intermediates, and any final states constitute the set of conformational substates.

One can next attempt to identify a set of coordinates that adequately describes the atomic displacements associated with switching between these conformational substates. With improved structural information, from crystallography, NMR, or the available MD data, one may be able to reduce the number of necessary coordinates using rigid body analysis, as Barstow et al. (3, 4) did with citrine.

The pressure-dependent kinetic data can then be utilized to determine the energy landscape as a function of those coordinates. This case is somewhat unusual in that the branching ratios are here giving information about the relative population of conformational substates. Nonetheless, with that population information in hand, we can determine the relative free energies of those CS. The structural information here allows us to put those into context on their underlying free energy landscape.

In summary, high-pressure structural experiments now allow detailed questions about protein function to be posited and answered. Pressure provides an experimentally accessible variable as potent as, e.g., temperature or pH, but more readily related to measurable atomic positions. Pressure experiments can be used to guide mutation studies and to help engineer proteins. Pressure experiments have the drawback of requiring equipment that, at the moment, is specialized and home constructed. This is likely to change over time.

SUMMARY POINTS

1. High-pressure methods, especially crystallography, are now routine and mature. Any protein that can be crystallized can be subjected to high pressure.
2. High pressure gives access to protein conformations that may not be visible in other ways, providing new insight into the conformational substates and dynamics.
3. High-pressure methods allow us to perturb the structure in a smooth, continuous fashion, making it easier to connect the dots when correlating structural changes to functional changes.
4. A detailed understanding of how protein deforms under high pressure may provide additional insight for advanced structure-based protein engineering.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Erratum

Figure 2 was mislabeled with corresponding errors in the caption. In Figure 2*a* the yellow squares should be labeled "MD," and the open purple circles should be labeled "Fluorescence." The correct caption should read:

T4 lysozyme (T4L) mutant L99A at high pressure. (*a*) Number of water molecules observed crystallographically (*solid blue circles*), in molecular dynamics simulations (*yellow squares*), and inferred from fluorescence measurements on pressure unfolding (*open purple circles*). For comparison, the molecular dynamics predictions are shifted horizontally (*blue dotted line*) to have the same midpoint pressure as the crystallographic data. Adapted from References 2 and 9. (*b*) Structural changes in T4L from the ambient (*blue*) to the 200 MPa (*orange*) structure. Differences have been magnified 5X for clarity, and the structures are aligned using the helical bundle that contains the L99A cavity mutation. (*c*) Crystallographic electron density maps show water filling the L99A cavity at 100 (*yellow*), 150 (*blue*), and 200 (*magenta*) MPa. Adapted from Reference 9.

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