

Errata:

page 16670, left-hand column, fourth line from the bottom, Ser-116 should Ser-117.

Figure 2, on the horizontal axis the pressure ranges from 0 to 500 MPa.

# Cooperative water filling of a nonpolar protein cavity observed by high-pressure crystallography and simulation

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Contributed by Brian W. Matthews, September 20, 2005

**Formation of a water-expelling nonpolar core is the paradigm of protein folding and stability. Although experiment largely confirms this picture, water buried in “hydrophobic” cavities is required for the function of some proteins. Hydration of the protein core has also been suggested as the mechanism of pressure-induced unfolding. We therefore are led to ask whether even the most nonpolar protein core is truly hydrophobic (i.e., water-repelling). To answer this question we probed the hydration of an  $\approx 160\text{-}\text{\AA}^3$ , highly hydrophobic cavity created by mutation in T4 lysozyme by using high-pressure crystallography and molecular dynamics simulation. We show that application of modest pressure causes approximately four water molecules to enter the cavity while the protein itself remains essentially unchanged. The highly cooperative filling is primarily due to a small change in bulk water activity, which implies that changing solvent conditions or, equivalently, cavity polarity can dramatically affect interior hydration of proteins and thereby influence both protein activity and folding.**

hydrophobic effect | T4 lysozyme

Internal protein hydration, its contribution to folding mechanisms, and the polarity of the protein interior in general are fundamental to protein structure and function. Despite the general success of hydrophobic models (1–3), attempts to determine the interior “hydrophobicity” of proteins have often given conflicting results (2, 4–10). Some proteins seem to require water at least transiently in nonpolar cavities for their function (11, 12). Moreover, the responses of proteins to pressure are not in accord with simple hydrophobic models (13); as a result, hydration of the protein core has been suggested as the mechanism of pressure-induced unfolding (14).

Additional progress will require direct measurement of the free energy needed to insert water molecules into a nonpolar protein cavity. Such an experiment requires sufficient structural resolution to unambiguously locate water molecules, the ability to resolve small changes in structure and occupancy, and the means to bias the occupancy while minimally perturbing the protein. Protein crystallography can detect changes in cavity hydration directly, but the temperature or chemical perturbations useful in solution thermodynamic measurements frequently damage crystals. We have found that high pressures of up to several hundred megapascals generally do not damage crystals (15, 16). High-pressure crystallography makes it possible to shift the equilibrium to interesting, unexplored protein states.

The  $\approx 160\text{-}\text{\AA}^3$  cavity containing mutant L99A of T4 lysozyme studied here was originally produced to probe the stabilizing interactions between buried nonpolar residues (2, 17). The Leu-99  $\rightarrow$  Ala mutation creates a large cavity that destabilizes the folded protein because of lost interactions between side chains and the reduced free-energy cost of exposing alanine (instead of leucine) to water (17). L99A T4 lysozyme has also been the subject of ligand- and noble-gas-binding studies (18–20) and is believed to be entirely empty under ambient condi-

tions. Under pressure, one may expect a large cavity, similar to that produced by the L99A mutation, to collapse. Indeed, this was the original impetus to study this mutant under pressure. Instead, we find this cavity to be remarkably rigid and to fill with water at  $\approx 150$  MPa. It provides an excellent system in which to examine the interactions of water with surrounding protein.

## Methods

**High-Pressure Crystallography.** Crystals were grown in space group  $P3_221$  as described (2). Crystallographic experiments were performed by using a beryllium cell described by Urayama *et al.* (15). The cell is a rod that is 6.35 mm in diameter and 25 mm long with a  $1 \times 19\text{-mm}$  bore on axis. The bore is sized to hold a glass capillary containing 0.7- to 1-mm crystals surrounded by a mixture of Sephadex and mother liquor, which supports and transmits pressure to the crystals while keeping them hydrated. Pieces of copper wire separate the crystals; x-ray shadows of the wires are useful for locating crystals once in the optically opaque cell. The cell is connected with a homemade adapter and standard high-pressure fittings to a hand-cranked liquid high-pressure press (High Pressure Equipment Company, Erie, PA) that is capable of reaching 200 MPa. The system is sealed and pressurized at least 30 min before data collection. Reversibility was ensured by examining the data collected on crystals at atmospheric pressure and on pressurized crystals after pressure release. In all cases, data at a given pressure refine to models more similar to each other than refined models from other pressures. Data were otherwise collected by using standard protocols at the F1 station of the Cornell High Energy Synchrotron Source and refined with the CCP4 suite (21) from Protein Data Bank structure 1L90. Resolution was limited to  $\approx 2.1$  Å by beryllium powder diffraction. Table 1 lists refinement statistics and unit-cell parameters at each pressure.

## Cavity Measurement and Construction of Difference-Density Maps.

Observed difference electron-density maps are constructed as  $(F_{o, \text{hp}} - F_{o, 0.1 \text{ MPa}})\alpha_{\text{calc}}$  for each pair of data sets.  $F_{o, \text{hp}}$  and  $F_{o, 0.1 \text{ MPa}}$  are the experimentally measured structure factors at high pressure and 0.1 MPa.  $\alpha_{\text{calc}}$  are phases calculated from the ambient pressure structure. Observed structure factors are scaled to calculated structure factors to put them on an absolute scale. Because the unit cell decreases in size by as much as 1 Å as pressure increases, the highest-resolution data yield only noise

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Abbreviation: MD, molecular dynamics.

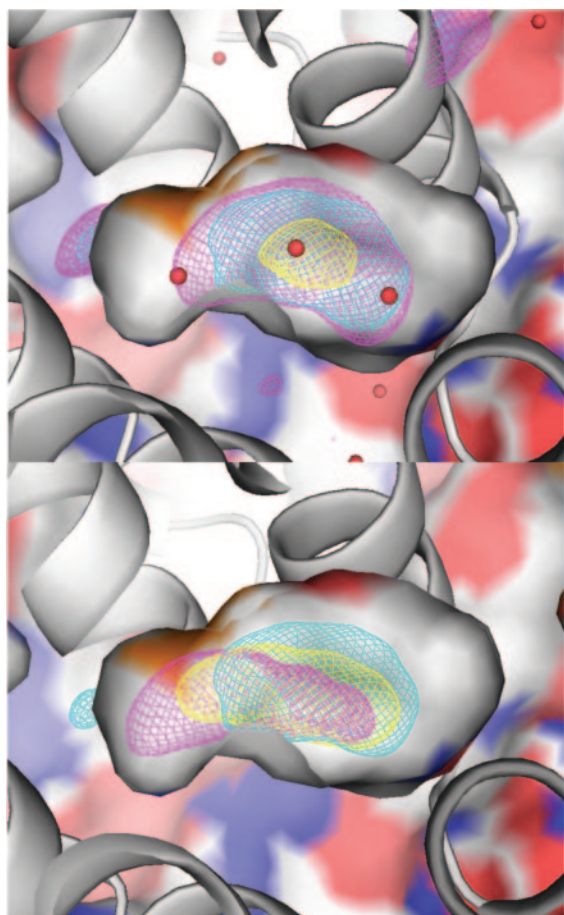
Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID codes 2B6T, 2B6W, 2B6X, 2B6Y, 2B6Z, 2B70, 2B72, 2B73, 2B74, and 2B75).

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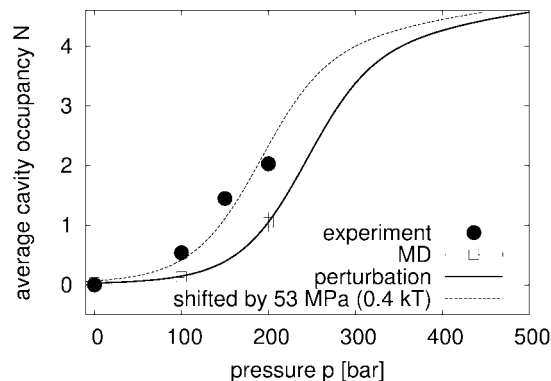


**Fig. 1.** Electron density in the main cavity of T4 lysozyme mutant L99A at high pressure. Helix E is shown behind a cut-away view of the  $\approx 160\text{-}\text{\AA}^3$  cavity. (A) Experimental density at 100 MPa (yellow), 150 MPa (cyan), and 200 MPa (magenta) is contoured at 0.1 electrons per  $\text{\AA}^3$ . (B) Experimental electron density at 150 MPa (cyan) compared with simulation density at 200 MPa (magenta), contoured at 0.1 electrons per  $\text{\AA}^3$ , viewed as described above. The distribution of atoms at 100 MPa (using the occupancies of  $N = 1, 2, 3, 4, 5$  at 200 MPa) is shown in yellow for comparison. [The figures were made with Pymol (<http://pymol.sourceforge.net>).]

shown in Figs. 2 and 3, the calculations indicate a sharp transition from a predominantly empty cavity at pressures below  $\approx 100$  MPa to a cavity cooperatively filled by approximately four water molecules at pressures above  $\approx 200$  MPa. The calculated electron densities (Fig. 1) and average occupancies (Fig. 2) are in good agreement with the experiments after shifting the chemical potential of the bulk water phase (or, equivalently, the average interaction energy of water with the protein cavity) by  $\approx 0.4 k_B T$  (1 kJ/mol).

The MD simulations indicate that water molecules confined to the cavity fluctuate between one-dimensional wires and closed hydrogen-bonded rings of four and five water molecules, as seen in experiments on gas-phase water clusters and simulations of confined water (26). Additional hydrogen bonds are formed with the backbone carbonyl oxygen of Ala-99.

During two simulations a water molecule escaped the cavity, one after  $\approx 0.5$  ns in the occupancy  $N = 1$  simulation and one in the  $N = 5$  simulation after 0.9 ps, both at atmospheric pressure (105 Pa). In each case, water escaped through a transient opening between the side chains of Phe-114, Ser-116, Asn-132, and Leu-133. The escaping water molecule transiently occupied the crystallographic water site Wat-196. Water escape is coupled to protein dynamics, in particular, motions of Phe-114. Indeed,

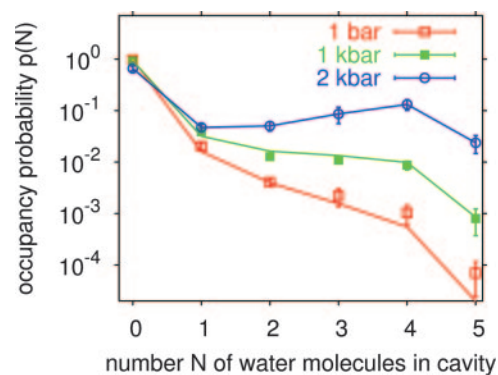


**Fig. 2.** Average number of water molecules inside the cavity as a function of pressure. Solid circles show the results from x-ray crystallography, dividing the integrated electron density by 10 electrons per water molecule. The open squares show the average occupancies calculated from the MD simulations. The solid line is the result of perturbation theory about the  $\approx 200$ -MPa simulation reference state. Shifting the pressure by 53 MPa (or  $\approx 0.4 k_B T$ ) produces the dashed line.

the Phe-114 side-chain crystallographic B factors, a measure of atomic fluctuations, are almost twice as large as those of the four other phenylalanines in the L99A crystal structure. The water-escape route from our simulations corroborates earlier NMR data that suggested a pathway for binding of nonpolar molecules near the F and G helices (19).

We find that a small change of the chemical potential of water in the bulk phase ( $\Delta\mu_{\text{wat}} \approx 1.4 k_B T$  at 293 K between 0.1 and 200 MPa) is sufficient to induce  $\approx 50\%$  filling of the cavity, on average. Such biphasic, environment-dependent behavior finds support in the coexistence of liquid and vapor water under ambient conditions (27). Consequently, filling under near ambient conditions could also be induced by lowering the average interaction energy of water with the surrounding protein through changes in the electrostatic environment of the cavity. The free energy of dissolving water into oil is remarkably high and largely an entropic penalty because of the formation of a water-sized cavity in oil. What contributes to the much more favorable free energy of transferring a water molecule into the protein?

Our MD simulations suggest that a significant part of the free energy comes from hydrogen bonds between multiple water molecules in the cavity, explaining the cooperativity of the transition. This cooperativity is reflected in the average potential energy of water in the cavity, which decreases from  $-23$  kJ/mol



**Fig. 3.** Probability distribution (logarithmic scale) of the number  $N$  of water molecules in the cavity from computer simulations. Symbols show results from MD simulations at 0.1, 100, and 200 MPa. Lines are the results of perturbation theory using the 200-MPa simulations as a reference point. Error bars indicate statistical uncertainties corresponding to one estimated standard deviation.

( $N = 1$ ) to  $-60$  kJ/mol ( $N = 4$ ) per molecule, or approximately  $-10$  kJ/mol per water–water hydrogen bond. Van der Waals interactions with the surrounding medium account for an additional approximately  $-10$  kJ/mol per water molecule. Other electrostatic interactions with the protein should also contribute, as will entropy from the static-free volume of the preexisting cavity. The subtle thermodynamics of filling show that a simple description of the interior as hydrophobic fails to capture all of its relevant features.

Our results have direct implications for pressure unfolding of proteins. The observation that pressure unfolds most proteins is seemingly at odds with predictions from the hydrophobic effect (13), which suggest that the transfer of hydrocarbons into water should be unfavorable at typical unfolding pressures. The paradox can be resolved by noting that the pressure-unfolded state is relatively compact compared to its thermally denatured counterpart (14). It was predicted that the pressure-unfolded state corresponded to extensive hydration of the protein interior rather than transfer of buried hydrophobic residues into bulk water. Our experimental evidence of pressure-induced filling of an existing nonpolar cavity supports this hypothesis by demonstrating that the free-energy penalty of filling a cavity is small or even zero at low pressures. At higher pressures, cavity filling should become so favorable that new cavities will grow to accommodate water and the protein will partially unfold. Even at lower pressures, our work supports the idea that collapsed intermediate states in protein folding may be hydrated internally (4, 28, 29) and that dehydration may be a rate-limiting step in folding (4, 29–31).

Pressure-induced hydration may help explain why many proteins undergo large changes in activity under pressure (32). Interactions between water and protein hydrophobic surfaces is particularly important for molecular interfaces in ligand or substrate binding and complex formation (33) and in multidomain protein folding (30). Changes in the interior hydration also affect enzymatic function. Weakly screened electrostatic interactions in the protein interior provide a strong coupling between charge sites and internal water. Consequently, changes in protein conformation and charge states during enzyme turnover directly influence the free energy of water in the low-polarizability protein interior, with implications for protein function. Cytochrome P450 (11) and bacteriorhodopsin (12) are examples in which changes in redox states and amino acid protonation determine the presence or absence of functional water.

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