Proteins under pressure

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Today, 3D models of proteins are often constructed entirely by a computer. Building old-fashioned wire models may have been time-consuming, but it was not drudgery. To the contrary, it was a labor of love. One could not fail to marvel at the way in which the successive amino acids fitted together, with all the interactions making physical sense. However, the packing was not perfect. Here and there, small unfilled spaces remained, and as shown by Roche et al. (1) in PNAS, these cavities come at a price, particularly with regard to pressure denaturation.

Given the current interest in exploring the deepest regions of the ocean, it might be noted that the pressure at the bottom of the Mariana Trench is about 1,100 bar (~1,100 atm). Early studies showed that a number of proteins remain folded at substantially higher pressures. Therefore, hydrostatic pressure, of itself, is not expected to preclude life in the deep. The present experiments (1) use mutants of the small monomeric protein staphylococcal nuclease (SNase). These proteins do not unfold within the working range of the instrument (up to 3,000 bar), but unfolding transitions can be monitored by adding supplementary denaturant (0.8–1.5 M GuHCl). Clearly, SNase would be perfectly stable under ambient conditions in the Mariana Trench.

It is well known that the creation of an artificial cavity in the core of a folded protein (e.g., by replacing a large nonpolar residue with a small one) is destabilizing. The larger the volume of the created cavity, the greater is the loss in protein stability (2). Although such cavities destabilize proteins against heat and denaturants, the mechanism by which pressure unfolds proteins has been somewhat contentious. An especially informative experiment was carried out by Akasaka and coworkers (3) using a subdomain of the *c*-Myb transcription factor. This folded 52-residue domain has a naturally occurring cavity with a volume of 33.1 $Å^3$. Using pressure alone, the domain becomes almost entirely unfolded at 3,700 bar. There is a mutation, valine 103 to leucine (V103L), which fills the naturally occurring cavity and also greatly increases the resistance of the protein to pressure denaturation. At 3,700 bar, the mutant has only begun to unfold and appears to require pressure in excess of 8,000 bar to denature completely. The apparent reduction in volume associated with unfolding of the native (cavity-containing) domain ($\Delta\Delta V_u$ = 35.3 $Å^3$) agrees well with the volume of the cavity, suggesting that it is the collapse of the cavity that is responsible for protein unfolding.

Are the Cavities Empty?

A key question in the putative importance of cavities is whether they are empty. If a cavity is filled or partly filled with water or some other ligand, its destabilizing effect will be reduced. In the case of SNase, the evidence for the lack of water molecules in the engineered cavities comes from crystal structure analyses. As stated by Roche et al. (1), "not even a trace of electron density was found." This agrees with protein crystal structures in general, which rarely, if ever, show evidence for solvent in nonpolar cavities, either in native proteins or in cavity-containing mutants (2, 4, 5). Nevertheless, there has been a school of thought that solvent molecules are present in nonpolar cavities but are sufficiently disordered or diffuse that they cannot be seen in X-ray crystallographic electron density maps (6). Key evidence for this idea came from an NMR study of interleukin-1β, a small globular protein with a central nonpolar cavity with a volume around 80 $Å^3$. In the experiment (6), NMR nuclear Overhauser effect (NOE) signals showed that there were water molecules close to the methyl groups of the side chains lining the walls of the cavity. These water molecules were assumed to be in the cavity; however, this interpretation has been revised recently (7). It is now thought that the NOEs seen in the NMR experiment come from water molecules elsewhere in the protein (8) and that the occupancy of water in the cavity is low.

The use of NMR makes it possible to monitor the pressure-dependent unfolding at multiple sites throughout the protein.

Volume Change on Unfolding

Roche et al. (1) use a variety of experimental and theoretical approaches to follow the pressure-dependent unfolding of each of the cavity-containing SNase mutants. The apparent change in volume of the protein on unfolding, ΔV_u, was obtained in three different ways: (*i*) by using pressure perturbation calorimetry, (*ii*) by monitoring the fluorescence of the tryptophan residues, and (*iii*) by following the change in intensity of the NMR HSQC peaks. As explained by Roche et al. (1), the ΔV_u values obtained calorimetrically cannot readily be compared either among themselves or with the other measurements. The ΔV_u values obtained by the other two techniques are summarized in table 1 and figure 2*B* in ref. 1. In every case, the apparent reduction in volume on unfolding the cavity-containing mutant protein is greater than that for the reference protein. This strongly suggests that the introduction of cavities plays a dominant role in the unfolding process.

At the same time, the ΔV_u values obtained using NMR are numerically about 40% larger than those obtained from tryptophan fluorescence. It is not altogether clear why this should be the case. Also, it is not shown whether the ΔV_u values from pressure denaturation correspond to the volumes of the cavities that are observed in the individual proteins, as was the case with the *c*-Myb domain mentioned above (3). An isoleucine-to-alanine variant, for example, is expected to create or expand an existing cavity by about 57 $Å^3$, assuming that the protein structure remains otherwise unchanged (cf. 2). For the I92A variant of SNase relative to the reference protein, the apparent change in volume on unfolding ($\Delta\Delta V_u$) is 49 mL/mol or 82 $Å^3$ per molecule, based on fluorescence. This is reasonably close to what one might expect. The $\Delta\Delta V_u$ based on NMR, however, is considerably higher (127 $Å^3$). A detailed accounting of the $\Delta\Delta V_u$ values relative to the cavity volumes present in each of the mutants would have been instructive. For example, the authors hypothesize that cavities introduced closer to the surface of the protein are likely to contribute less to ΔV_u because solvent is less likely to be excluded from these regions owing to their close proximity to bulk water. An alternative explanation might be that putative cavity-creating mutations made close to

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the surface are prone to collapse when the bulky residue is replaced with the smaller one, thus reducing the volume of the incipient cavity (9). In this scenario, the ΔV_u values would be smaller because the cavity volumes are smaller.

One of the exciting aspects of the analysis of Roche et al. (1) is that the use of NMR makes it possible to monitor the pressure-dependent unfolding at multiple sites throughout the protein. For SNase, the ΔV_u values could be determined for 101 of the 143 residues in the protein and suggested that there are deviations from simple two-state unfolding that are unique for each variant. Further exploitation of this approach is awaited with interest.

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