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Review

Exploring the folding energy landscape with pressure

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ABSTRACT

The unique role of pressure in protein folding studies is emphasized. Variable-pressure NMR experiments carried out under equilibrium conditions give unique opportunities to explore the energy landscape for protein folding. Intermediate conformers that may appear transiently in the kinetic folding experiments may be stably trapped under pressure, allowing examination of their conformations in site-specific detail with modern NMR spectroscopy. The intimate relationship between the kinetic folding experiment and the equilibrium pressure experiment is described with examples from ubiquitin and hen lysozyme.

Pressure "reverses" protein folding

To detect transiently produced intermediates upon T-jump or concentration jump (stopped-flow), a synchronized spectroscopic detection such as absorption, fluorescence and X-ray scattering is usually required. On the other hand, to examine structures of transient intermediates in the folding process in details with spectroscopy, we must trap the intermediates stably under equilibrium conditions with a suitable means. To mimic transient intermediates, a common technique has been to destabilize the protein structure with artificial additives such as urea or by changing the hydrogen ion concentration of the solution. There would be no assurance, however, that the intermediates stabilized in the presence of such "chemical agents" are the same kind as those found in the process of folding. Then, is there any method that can trap kinetic intermediates stably under equilibrium conditions and perform direct spectroscopic analysis of their structures?

Here pressure gives the answer. The utility of pressure in protein folding studies is simply that it "reverses" the folding reaction with least chemical perturbation to the system. To understand this statement, we may go back to the energy landscape for protein folding [1]. As we go back to the classics of protein folding, the Anfinsen's dogma infers that folding is nothing but a thermodynamic equilibration process of the protein system under physiological conditions, where the folded state is thermodynamically most favored (lowest Gibbs energy state) [2]. This means, in essence, that, when the folding reaction starts, the system is far from equilibrium, but when folding is complete, equilibrium is reached between the folded species N and the unfolded species U (including any intermediates in between) in such a way that the former is much more populated than the latter.

The conformational equilibrium of a protein in solution N ⇄ U between the unfolded species U and the basic folded conformer N is given by Eq. (1) [3], *both before and after the folding reaction.*

$$K = [\mathrm{U}]/[\mathrm{N}] = \exp(-\Delta G/RT), \tag{1}$$

where ΔG is the relative thermodynamic stability of U and N, which is given in the simple case by

$$\Delta G = G_{\mathrm{U}} - G_{\mathrm{N}} = \Delta G_0 + p\Delta V, \tag{2}$$

where ΔG_0 is the Gibbs energy difference between U and N under standard conditions, p is the pressure, and $\Delta V = V_{\mathrm{U}} - V_{\mathrm{N}}$, the difference in partial molar volume (PMV)[1] between U and N. ΔV itself may vary with pressure, but for a small range of pressure within a few kbar, the variation is considered small and, for simplicity, is neglected in the discussion below.

Under most physiological conditions on earth, ΔG_0 is positive, assuring Anfinsen's dogma for protein folding. Importantly and interestingly, the term $p\Delta V$ is negative in most situations, because ΔV is negative for most proteins under physiological conditions, meaning that the PMV of the unfolded species U is smaller than that of the fully folded conformer N [4]. This means that, if pressure is applied, the term $p\Delta V$ counteracts the term ΔG_0, the essential term for Anfinsen's dogma, and may even overcome the positive surplus from ΔG_0 in Eq. (2). As a result, the most striking effect of pressure is a reversal of the folding reaction by making

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[1] *Abbreviation used:* PMV, partial molar volume.

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conformer U more stable than N, a complete opposite of what the Anfinsen's dogma predicts.

On the other hand, the rate constant for a conformational transition depends also on pressure in the following manner.

$$k(p) = k(0)\exp(-p\Delta V^{\neq}/\mathrm{RT}) \quad (3)$$

Here $\Delta V^{\neq}$ is the activation volume of the reaction and is assumed to be independent of pressure in Eq. (3), which is considered a reasonable approximation within a few kbar range.

Exploring the energy landscape with variable-pressure NMR spectroscopy

Pressure, when combined with the site-specific spectroscopy such as NMR, namely high pressure (variable-pressure) NMR spectroscopy, does much more than this. Despite the prevailing concept that protein folds into a single unique structure called "the native structure", we often find semi-stable intermediates between the fully folded and the fully unfolded in many proteins under various perturbations including temperature, pH and denaturant. There may be a case that the intermediate is on the pathway of folding or a case that the intermediate is off the pathway. In relatively large proteins, which may consist of several domains, formation of intermediate conformers on the pathway would be a necessity to attain correct folding rapidly to avoid any misfolding events. The energy landscape, defined with the vertical axis representing the solvent-averaged energy and the horizontal axis representing the conformational entropy of the polypeptide chain, should remain the same before and after the folding event. And thus any semi-stable intermediate kinetically trapped in the folding process should also be present as minor population after the equilibrium is reached. However, the equilibrium populations of the intermediates are usually so low under physiological conditions that their spectroscopic detection is not normally possible. Although relaxation dispersion NMR Spectroscopy has been introduced to circumvent the situation [5], the method is limited in the time range of fluctuations involved.

To enable direct spectroscopic analysis of intermediates, it is usually required that their equilibrium populations be increased actually to a level of direct spectroscopic detection. Temperature, pH and denaturants are the perturbations that may cause significant changes in the "energetic" and "chemical" environment of the protein and therefore the energy landscape. In contrast, pressure, as applied under isothermal conditions, gives little changes in internal energy and in chemical bonding of *pre-existing* intermediates besides N and U, thus altering least the structures of the intermediates. On the other hand, it can increase populations of *pre-existing* intermediates exponentially with increasing pressure according to Eqs. (1) and (2).

In most actual proteins having various alternate substates, we have an empirical rule (the "volume rule") that the PMV decreases ($\Delta V < 0$) in parallel with the loss of conformational order, namely as we go up the folding funnel (Fig. 1) [6,7]. The volume rule (previously termed the volume theorem [6–11]) describes an essential character of the volume behavior of proteins, which predicts the unidirectional conformational variation of a protein with pressure [12], which has been experimentally confirmed for a number of proteins so far studied [6–11].

The actual values of ΔV between U and N may vary from a few tens of ml/mol to a few hundreds of ml/mol in negative values [4]. By applying higher and higher pressure, in general, one can increase more and more the populations of conformers with higher and higher up the funnel, but with smaller and smaller volume (Fig. 1). The major underlying mechanism for the volume rule is considered the hydration of cavities (or atom defects) in the interior of protein architecture [12], along with other factors.

The effect of pressure on the energy landscape itself is considered small. Apart from major transitions between substates, pressure affects weak chemical bonding, including torsional potential, hydrogen bonding potential, van der Waals and ionic interactions within the protein molecule itself and between the protein atoms and water (hydration space). Average changes in these interatomic distances are rather small. For the NH–OC hydrogen bonds, an average of -0.029 ± 0.12 Å is found for BPTI at 2 kbar [13], which generally lie within the range of their thermal fluctuation under physiological conditions. Thus the effect of pressure on the solvent-averaged internal energy constituting the vertical axis of the energy landscape is likely to be small, lying within the range of its thermal fluctuation. Thus we consider that pressure is one of the most neutral perturbations for changing equilibrium populations among sub-states of a protein, giving basis for detailed structural studies of folding intermediates stably trapped under pressure.

Although the situation depends on the depth of the funnel and the roughness of the energy landscape, one may be able to increase sufficiently the populations of intermediates (or more generally "high-energy" conformers) existing over the entire energy landscape from the bottom to the top, by varying pressure, within a few kbar in favorable cases (Fig. 1). A number of proteins have been investigated in this way for possible intermediate states by

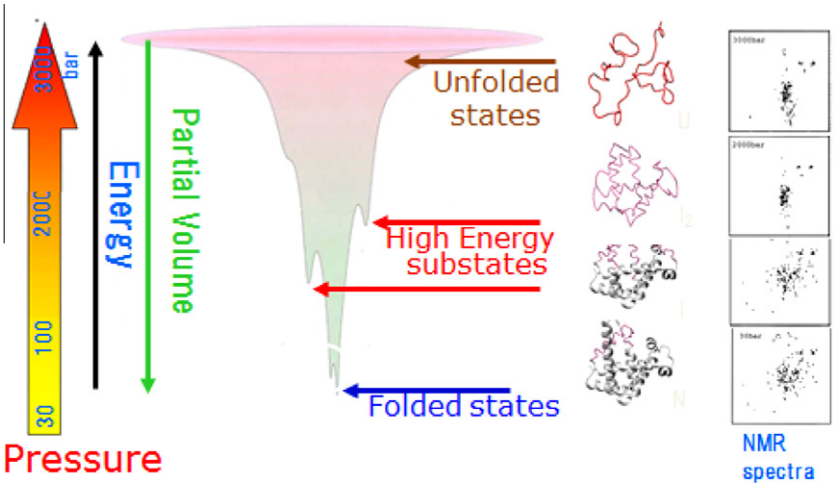

Fig. 1. Exploring the protein energy landscape with high pressure NMR. The horizontal axis represents the conformational entropy of the polypeptide chain. The vertical axis represents the conformational order and is determined by the solvent-averaged conformational energy [1].