Novel Aspects of Protein Conformational Fluctuations Revealed by Variable Pressure NMR

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Received 1 January 2007/Accepted 8 January 2007

Abstract

Variable-pressure NMR spectroscopy is capable of probing conformational fluctuations of proteins from within the folded structure to nearly the total unfolding with pressure as major variable. It allows one to examine directly the structures of high-energy conformers resulting from thermal fluctuations in atomic detail or at least in residue-specific detail. Some of these conformers are found widely deviated from folded structures in crystals. Importantly, their structures are unique to individual proteins, many of which are apparently designed for function. Furthermore, the self-association reaction of a hen lysozyme mutant forming amyloid protofibrils is shown to be reversible with pressure, suggesting that amyloidosis is also correlated with an intrinsic fluctuation of the protein.

Keywords: pressure, NMR, conformational fluctuation, energy landscape, amyloidosis

1. Introduction

The three-dimensional structure of a protein is considered to provide a basis for understanding its function. While in a crystalline environment the protein conformation is “immobilized”, in a cell environment it is more dynamic-namely, folding, unfolding and interaction with other molecules or with themselves. In general, protein structure is fluctuating over a wide range of conformational space between folded and unfolded even under physiological conditions (Fig. 1), but the slow fluctuations in a wider range of conformational space (Fig. 1, \( \tau > \mu s \)) has not been explored by any means in detail, because of the lack of availability of suitable experimental techniques. It is apparent that we need further information beyond a simple “static” structure of a protein given from X-ray analysis in crystal.

NMR spectroscopy, in principle, can provide such information. However, the target of current NMR spectroscopy is highly biased toward the basic folded states and rapid and small-amplitude fluctuations within the folded states (Fig. 1, \( \tau < \mu s \)). In fact, we know very little about the reality of conformational fluctuations of proteins outside the folded conformation, which may be crucially important not only in understanding folding, and misfolding, but also in understanding physiology and macro molecular and/or drug interactions.
In fact, to reliably detect the fluctuation and to know the *structure* that has deviated from the original by fluctuation, we need to amplify the population of higher energy conformers without changing their original structures. Perturbation by temperature, pH and denaturant is usually too strong for detecting subtle differences in protein conformation, as they involve relatively large change in interaction energy of the target protein, which would smear out any subtle conformational differences. We found that the most suitable perturbation in this regard is *pressure*.

![Fig. 1](image)

**Fig. 1** A schematic illustration showing conformational fluctuations in proteins that span a wide time-range. There are those occurring mostly within the folded conformer ($\tau > \mu$s), while there are those occurring outside the range of the folded conformer ($\tau < \mu$s), spanning the wide conformational space.

2. Methods

Pressure within a few kbar causes little change in weak chemical bonding, e.g., hydrogen bond [1], while it can dramatically change the equilibrium populations of existing conformations 1 and 2 through the direct thermodynamic perturbation given by $p\Delta V$ in the following equation [2],

$$\Delta G = \Delta G^0 + p\Delta V \quad (1)$$

where $p$ is pressure and $\Delta V$ is the difference in partial molar volume between conformers 1 and 2 in equilibrium

$$\Delta V = V_2 - V_1 \quad (2)$$

The value of $\Delta V$ is always decreasing as the structure of the protein is less and less folded, giving the basic folded state the largest volume. This rule, called the *Volume Theorem* [3], is
generally observed for most globular proteins at physiological temperatures or lower [4]. Thus increasing the pressure of the protein solution, the population of the less-folded conformer 2 increases at the expense of the population of the more-folded conformer 1.

The conformational difference between the two conformers could be very subtle, and we may need an atomic level resolution to differentiate them. Nuclear magnetic resonance (NMR) spectroscopy, especially with multi-dimensional capability, is the best and probably the only spectroscopy to perform this aim. Thus the combination of pressure perturbation with NMR is inevitable. We have realized this combination through the introduction of the on-line cell variable-pressure NMR spectroscopy by employing hand-made pressure-resisting quartz-cells in the range of 1~3000 bar or higher [2, 5]. This method turned out to be the method of choice for the purpose mentioned above.

The pressure effect on protein structure is unique in that it works as “mechanical” as well as “thermodynamic” perturbation. This enables detection of conformers in a wide range of conformational space from the bottom to practically the top of the folding funnel [6, 7] (Fig. 2). Furthermore, pressure within a range of a few kbar affects little inter atomic potentials, thereby leaving the internal energy or the entropy of the system practically unchanged. In other words, the method enables one to explore the energy landscape of a protein from the bottom nearly to the top. This in turn will assure the close identify of the structure of a semi-stable intermediate determined at elevated pressure to the intermediate which would be

![Diagram](image_url)

**Fig. 2** Two parts of a figure side-by-side. They should be labelled A and B either in the figure or adjacent to it. Such figures are not left indented. Two figures of similar size with consecutive numbers may be arranged in the same way, with separate captions underneath each figure.
present in a small population in equilibrium or as a transient species in folding [8] at ambient pressure.

3. Applications

3.1 Conformational fluctuations leading to high-energy conformers

A number of proteins studied by the variable-pressure NMR spectroscopy showed the existence of high energy conformers considerably deviated in structure from those found in crystals, often with disorder or unfolding in selected segment of the molecule (See Fig. 2 in ref. 7). They exist as “rare” conformers in equilibrium with the major conformer in solution, the “native” conformer, which is usually close to the conformer found in crystal environment. The results indicate that proteins are fluctuating in solution far more than anticipated from the crystal structure alone. They are difficult to be detected under normal pressure, as their populations are slim (usually a few percent or less). However, the Gibbs energy difference between these high energy conformers and the “native” conformer is very marginal indeed, on the order of a few kcal/mol. The marginal balance is one of the intrinsic nature of naturally selected proteins.

The unique property of pressure perturbation opens up the possibility of performing the NOESY-based structure determination of higher energy conformers of proteins by trapping them stably under pressure. Indeed, this approach has gained success in giving the first “NMR snapshots” of a fluctuating protein structure in atomic level detail [9].

3.2 Conformational fluctuations leading to amyloidosis

Another novel aspect of pressure application to conformational fluctuations in proteins is in the field of amyloid fibrillation. We found that pressure can reversibly dissociate and reassemble the intrinsically denatured variant of hen lysozyme (the disulfide-deficient mutant, OSS) into protofibrils as monitored by $^{15}$N/$^1$H two-dimensional NMR spectra [10]. As the process is relatively slow, one can study even the time-dependent dissociation and association processes with pressure-jump NMR [12] (Fig. 3). With pressure-jump fluorescence spectroscopy, one can study the pressure dependence of the rate of dissociation in detail. Together with the result of atomic force microscopy, we could not only determine that the fibril grows by linear polymerization mechanism, but also estimate the intrinsic rate of monomer dissociation from the protofibril [12].
Fig. 3 Pressure-jump $^1$H NMR spectra (left) and the time-dependent signal intensity change (of the peak at 0.9 ppm) upon pressure jump (right). In both figures, the increase in signal intensity is considered to arise from dissociation of amyloid protofibrils (inset AFM figure) into monomeric species in the disulfide-deficient hen lysozyme mutant [11].

References: