The Nuclear Magnetic Resonance Society of Japan *Editor*

Experimental Approaches of NMR Spectroscopy

Methodology and Application to Life Science and Materials Science

Springer

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Preface

This book describes the advanced developments in methodology and applications of NMR spectroscopy to life science and materials science. Experts who are leaders in the development of new methods and applications of life science and materials science have contributed an exciting range of topics that cover recent advances in structural determination and dynamic characterization of biological and material molecules, and development of novel NMR techniques, including resolution and sensitivity enhancement. First, this book particularly emphasizes the experimental details for new researchers to use NMR spectroscopy and also to pick up the potentials of NMR spectroscopy. Second, the book is designed for those who are involved in either developing the techniques or expanding the NMR application fields by applying them to specific samples. Third, the Nuclear Magnetic Resonance (NMR) Society of Japan has organized this book not only for NMR members of Japan but also for worldwide readers who are interested in using NMR spectroscopy extensively.

This book consists of two parts: methodology and application to life science and materials science. In Part I (Chaps. 1–9: Methodology), first, new concept of NMR experiment such as high pressure NMR and isotope-aided NMR methods are described. Second, advances in NMR data acquisition and processing methods are introduced. Third, advances in NMR hardware such as dynamic nuclear polarization (DNP), photoirradiation and microwave irradiation NMR spectroscopies are emphasized. Fourth, recently developed solid-state NMR spectroscopy under ultrafast magic angle spinning (MAS) is focused in this book. Fifth, dynamics of biological molecules using relaxation dispersion NMR spectroscopy is particularly emphasized. Sixth, structure-determination experiments of biological molecules utilizing paramagnetic lanthanide probe methods and solid-state NMR spectroscopy are particularly highlighted.

In Part II (Chaps. 10–22: Application to Life Science and Materials Science), advanced application to life science and materials science is described. First, as applications of solid-state NMR spectroscopy to materials science, studies on silk materials, polymer materials, functional materials and gaseous molecules in polymer materials are extensively described. Second, as applications of advanced NMR

techniques to life science, studies of natural products, glycoproteins and ribonucleic acid (RNA) are widely accounted. Third, metabolic profiling for small molecular complexity is introduced. Fourth, this book focuses on NMR studies of paramagnetic compounds. Fifth, NMR spectroscopy of quadrupole nuclei in organic compounds and inorganic materials is described. Sixth, advanced NMR spectroscopies are focused on characterization of protein–ligand interaction and determination of protein structure and dynamics.

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We would also like to acknowledge the council member of the NMR Society of Japan for their devoted help in the process of editing this book.

Finally, we would like to thank Ms. Risa Takizawa and Mr. Shinichi Koizumi of the Springer Japan for their encouragement and assistance in editing the book.

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Chapter 1
Protein Studies by High-Pressure NMR

Kazuyuki Akasaka

Abstract High-pressure NMR spectroscopy is a method that allows NMR measurements of any target samples under variable pressure. When applied to proteins, the method extends the conformational space that NMR spectroscopy can handle, from the space limited to the basic folded paradigm into the extensive conformational space that spans the entire high-energy paradigm of protein structure from the folded to the unfolded. The success of the experiment is ensured by the strong coupling between the volume and the conformation of a protein such that *the partial molar volume of a protein decreases in parallel with the loss of its conformational order* (the "volume theorem of protein") as advocated by Akasaka). The validity of this principle arises from the fact that a globular protein generally has a significant vacant space (sometimes called "void" or "cavities") inside its folded architecture into which water molecules may penetrate to reduce its partial molar volume and its conformational order simultaneously. Thanks to this principle, pressure provides a simple, clean, systematic and often efficient means of investigating the high-energy conformers of a protein, which is essential for understanding its folding, function, interaction, fibrillation, adaptation and evolution.

Keywords Partial molar volume · Conformational order · Cavity hydration
High-energy paradigm · Protein function and evolution

1.1 Introduction

This chapter draws attention to the new paradigm of protein structure that has not hitherto been the direct target of NMR spectroscopic observation, but could be crucially important for function, adaptation and evolution of proteins, namely the "high-energy paradigm" of proteins. To explore this paradigm with NMR, the author found, a couple of decades ago, that the best means to advance our

K. Akasaka (✉)
Kyoto Prefectural University of Medicine, Kyoto, Japan
e-mail: akasakak@koto.kpu-m.ac.jp

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knowledge in this respect is the combination of NMR spectroscopy with pressure perturbation. In this chapter, I begin with reviewing the relevant thermodynamics that proteins obey, emphasizing their unique volumetric properties that are decisive for the successful high-pressure NMR experiments on proteins. Techniques and applications will follow.

As unit of pressure, both "bar" and "MPa" will be used in this text, which are related by

1 bar = 0.1 MPa, 1 kbar = 100 MPa, or 1 MPa = 10 bar, 100 MPa = 1 kbar, 1 GPa = 10 kbar.

The pressure in our natural environment is at most $\sim$1 kbar or 0.1GPa at the deepest part of the ocean, while our high-pressure NMR experiments are performed in the pressure range within several kbars.

1.1.1 The NMR Spectroscopy and Its Limitation

Since its discovery in 1946 [1, 2], the NMR spectroscopy has made several dramatic transformations into high-resolution spectroscopy of all kinds of molecules in solid and in solution. In particular, thanks to the slow spin relaxation of spin 1/2 nuclei, the time-domain spin technology [3] has revolutionized NMR spectroscopy into unprecedented versatility and utility where the information is selected or enhanced to meet the variety of needs and choice of the experimentalist [4]. A variety of combination with external perturbations such as rapid sample rotation (see Chap. 6 by Nishiyama), photo-illumination (see Chap. 5 by Naito), concentration-jump and temperature-jump have also been employed along with the spin technology and the isotope-labeling techniques (see Chap. 2 by Kainosho) to enhance or select the information to meet the individual needs.

With regard to the NMR application to protein studies, following the discovery of "Anfinsen's dogma" [5] such that "the functional structure of a protein is thermodynamically determined in solution to be in the Gibbs energy minimum state," the determination of protein structures in solution by expressing them in average atomic coordinates has gained the major attention and become the major accomplishment in NMR spectroscopy of proteins [6]. The consequence gives an overwhelmingly "static" image of protein structure such that "the polypeptide chain is folded to give a tightly interlocking, rigid molecule" [7], the small fluctuations of which give rise to function.

On the other hand, the life as we see daily in nature is distinctly dynamic and this dynamism depends largely on the dynamism of protein molecules (see Fig. 1.1), namely the "kicking and screaming stochastic molecules in which fluctuations are frequent and dramatic" [7], which, in turn, is closely connected to the dynamism of water molecules filling the earthy environment, even a tiny space within a protein molecule [8]. The necessity for overcoming the limitation of the "single conformer" concept and "static" picture of protein structure, as unduly enhanced by the astonishing elegance of X-ray crystallography and modern NMR spectroscopy,

must be replaced with a new dynamic concept of protein structure to face *directly* with the dynamic reality of life.

In an effort to gain information on the dynamics of a protein structure, a number of important contributions have been made from different experimental approaches, including those from compressibility [9], enzyme kinetics [10] and hydrogen exchange, to the recent relaxation dispersion method (see Chap. 7 by Sugase), a major conflict still remains between the two concepts of proteins; the one confined largely to a "single" major conformer and the other basically allowing "multiple" conformational transitions over the entire allowed conformational space. Now we wish to overcome the conflict with a new experimental approach based possibly on a simple general principle that governs the protein conformation and its fluctuation in the entire allowed conformational space from the folded to the unfolded.

Before going into our strategies in solving the conflict, one should first realize the basic property of NMR spectroscopy per se, which places strong limitation on our visibility of dynamic events in proteins, in two ways both originating from the small magnetism of nuclear spins:

(1) Limitation due to the *long* spin relaxation times:
NMR is intrinsically a "slow" spectroscopy with long spin relaxation times, so that the effects of any "*rapid fluctuations*" with $\tau \ll$ ms disappear during acquisition of free induction decay signals, leaving practically "no trace" in the resultant spectrum.

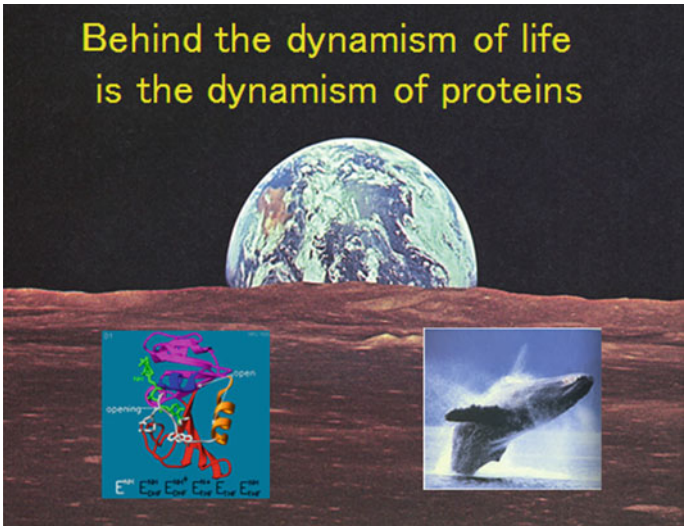

Fig. 1.1 Astonishing "dynamism of life" on earth has its origin in the "dynamism of protein molecules," which is only realized in collaboration with water molecules

(2) Limitation due to the *low* sensitivity of detection:
The current NMR spectroscopy per se would fail to detect or identify signals from "rare" or "minor" conformers that might exist in equilibrium with the major conformer and the "transient" species that might appear transiently in protein folding and/or unfolding.

1.1.2 Overcoming the Limitation with Pressure

Over the past decades, the new inventions in NMR spectroscopy have been made primarily in spin technologies producing advanced spectroscopy, such as multi-dimensional NMR spectroscopy and high-resolution solid-state NMR. This all done, but apparently has not been sufficient to reach the dynamic entirety of proteins controlling the dynamism of life. Proteins are the molecules not just with chemical bonding, but are *the molecules of life* that have adapted themselves to the natural environment on earth for billions of years, long before the human evolution. Namely they are the molecules that have survived by fitting to the natural environment on earth, obeying the laws of thermodynamics and statistics, so that they function under the control of these laws, namely under the choice of *temperature* and *pressure*, in addition to the choice of other conditions.

Of the two, temperature is a "reality," to which proteins have delicately adjusted themselves to function, from below the freezing point in anti-arctic to the boiling bath of a hot spring. What about pressure? It varies from 1 bar to 1.1 kbar in the deepest ocean, but most proteins on earth have adapted themselves to the low-pressure environment close to 1 bar. Thus, pressure over 1 kbar is a "non-reality" for most proteins, as they would have seldom experienced it in their long history of evolution, but the general belief that *pressure at kbars is energetically a much more strong perturbation to proteins than temperature, is wrong*. In most pressure experiments where the experiments are performed after the temperature rise due to adiabatic compression is removed, the energetic contribution is very subtle compared to that by temperature, so that the structures in high-energy sub-states remain practically unchanged from those at 1 bar. Furthermore, in contrast to temperature, the rewarding properties of pressure perturbation on protein solution are that (1) pressure is transferred uniformly over the entire sample solution upon applying pressure (Pascal's rule) and (2) pressure causes practically no aggregation of proteins under applied pressure or even upon removal of pressure if experiments are carefully done.

In 1972 and 1974, Yamada proposed high-pressure NMR up to $\sim$200 MPa on solution samples by using a "pressure-resisting cell" made of glass capillaries [11, 12]. By utilizing techniques, Morishima [13] and Wagner [14] performed some pioneering and stimulating works on selected topics of protein dynamics in early 1980's before the two-dimensional NMR techniques came into use.

In 1990s, Jonas pioneered the field of high-pressure NMR studies of proteins with his "autoclave type" high-pressure NMR apparatus operable up to 850 MPa and started protein structure and unfolding studies under pressure with 1D ^{1}H NMR spectroscopy successfully [15, 16]. However, because of the lack in sufficient field homogeneity and versatility in pulse sequence, the extension of his "autoclave" technique into two-dimensional high-pressure NMR spectroscopy of proteins turned out to be unsuccessful.

A few years later in 1990s, Akasaka and Yamada came up with hand-made pressure-resistive synthetic quartz cells, in conjunction with a high-field NMR spectrometer (750 MHz for ^{1}H) [17], started high-pressure NMR spectroscopy of proteins with excellent high resolution in the pressure range up to 200–400 MPa. Since then, Akasaka and co-workers and later independently Kalbitzer and coworkers [18] have opened a "new paradigm" of NMR spectroscopy of proteins with pressure as variable, as summarized in a number of review articles hitherto published [19–34] that describe high-pressure NMR techniques for the study of protein dynamics, folding and aggregation. In recent years, easy-handling ceramic cells made of zirconia became commercially available, which has changed the world landscape of high-pressure NMR studies of proteins for its expansion to the next decades [34].

1.1.3 Turning NMR "Invisible" Conformers into NMR "Visible" with Pressure

To simply illustrate what the high-pressure NMR spectroscopy can do in contrast to the conventional NMR spectroscopy limited to 1 bar, we show below two typical cases of *turning the NMR* "*invisible*" *conformers to NMR* "*visible*" by pressure perturbation.

Case 1. ^{1}H NMR spectra: hen lysozyme between 3 and 400 MPa [35].
Take a look at Fig. 1.2 for the case of 1D ^{1}H NMR spectrum of hen lysozyme (pH 2.0) at −5 °C using a quartz cell (low temperature is chosen to enhance the pressure effect). The bottom spectrum is the only spectrum that we can observe at 0.1 MPa (1 bar), assignable to the basic folded structure of the protein. When we apply pressure at 0.1–300 MPa, the spectrum starts to undergo dramatic changes (*chemical shift changes with some broadening*) until finally at 400 MPa (the highest pressure available in this experiment) the high-field shifted methyl proton signals (0 to −1 ppm) coming from the folded protein core disappear and are replaced by a new combined methyl signals at around 0.9 ppm, characteristic of unfolding. Chemical shift positions of most other signals are also closer to those of fully unfolded conformer (U) in 8 M urea, but the broadness of their signals at 400 MPa suggests that their side-chain motions are restricted, characteristic of a molten globule state (*MG*).

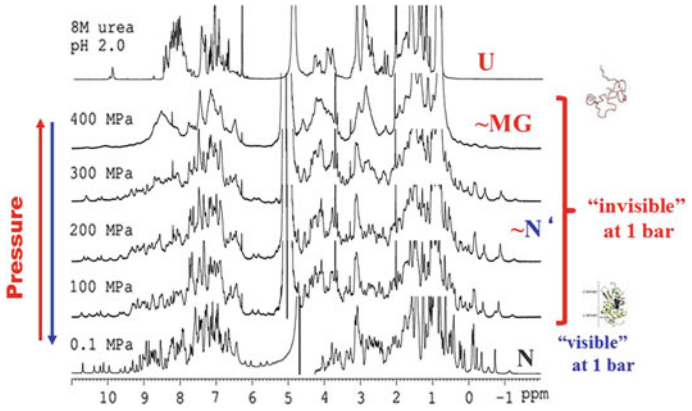

Fig. 1.2 Pressure turns the "invisible" conformer to "visible" in hen lysozyme [35]

Qualitatively, the spectral changes with continuous chemical shift changes occurring in the pressure range of 0.1–300 MPa indicate a rapid ($\tau \ll$ ms) conformational fluctuations (compaction of the core) within the folded manifold (*N–N′*), whereas the jumping of signal positions represented by intensity changes at 300–400 MPa indicates the slow ($\tau \gg$ ms) transition of the folded conformation (*N–N′*) into the unfolded, molten globule (*MG*) state at 400 MPa.

The important point here is that *the entire spectral changes at 0.1–400 MPa are fully reversible with pressure*: It is not likely that pressure has produced newly conformers like *N′* and *MG* from *N*; rather, the conformers *N′* and *MG* preexist before applying pressure. Pressure merely increases their populations to such a level that they can be detected by NMR. This supports the notion that all the conformational species from *N* to *MG* (at least) exist in equilibrium in the entire pressure range studied (0.1–400 MPa), despite the fact that at 0.1 MPa, only the signals from the dominant conformer *N* are observed. Here we see *"the magical power of pressure" that turns the "invisible" to "visible" in NMR spectroscopy*. In other words, it "dramatically increases the NMR sensitivity" of "rare conformers" in proteins.

A series of 1D ^{1}H NMR spectra of hen lysozyme (1.7 mM protein, pH 2.0) observed under applied hydrostatic pressures between 0.1 and 400 MPa [35]. The spectra between 100 and 400 MPa were obtained at −5 °C at 800 MHz using a pressure-resisting quartz capillary tube [17] whereas the reference spectra at the bottom (0.1 MPa) and the top (in 8 M urea) were obtained at 600 MHz at 25 °C. At 0.1 MPa, only the basic folded conformer *N* is "visible" in the NMR spectrum, while the conformers between 100 and 300 MPa are likely the folded conformers with different degrees of compaction of the core, here designated by *N*. The conformer at 400 MPa may be assigned to MG (molten globule) (cf. the fully unfolded conformer U in 8 M urea at 0.1 MPa). All the spectral changes are reversible with pressure, suggesting that the *N′* and *MG* conformers preexist at 0.1 MPa in

equilibrium with *N* at "invisibly small fractions," but becomes "visible" at higher pressures simply because the equilibrium is shifted to *N′* and *MG* at elevated pressures (adapted from Fig. 2 in [35]).

Case 2. ^{1}H/^{15}N HSQC spectra: apomyoglobin between 3 and 300 MPa [36]. A more delicate and dramatic spectral change occurs for the case of two-dimensional NMR spectroscopy (^{15}N/^{1}H HSQC) of apomyoglobin. We observe here an entire process of its transition from the folded *N* at 3 MPa to the fully unfolded conformer *U* at 300 MPa. At the lowest pressure (here 30 bar), only the folded species *N* is observable. By increasing pressure (3–120 MPa), individual signals undergo continuously chemical shift changes e ($\tau \ll$ ms), and, by further increasing the pressure (150–300 MPa), individual cross-peaks jump to new positions ($\tau \gg$ ms). When the pressure is reduced back to 3 MPa, the spectrum returns essentially to that of the original, suggesting again that the *I* and *MG* conformers preexist at 0.1 MPa in equilibrium with *N* at "invisibly small fractions," but becomes "visible" at higher pressures simply because the equilibrium is shifted to I and MG at elevated pressures, as exactly in the case of hen lysozyme.

^{1}H/^{15}N HSQC spectra of apomyoglobin at various pressures from 3 to 300 MP at 35 °C (3 MPa instead of 0.1 MPa to avoid bubbling in the solution). Adapted from Fig. 5 of Ref. [21].

The observation is again consistent with the notion that all different conformers of apomyoglobin, designated tentatively by the symbol *N*, *I*, *MG*, *U*, coexist in equilibrium at any pressure between 3 and 300 MPa. Namely the equilibrium

$$N \rightleftarrows N' \rightleftarrows I \rightleftarrows \mathrm{MG} \rightleftarrows U. \tag{1.1}$$

holds at whatever the pressure chosen. At 3 MPa, only the basic folded conformer *N* is "visible" by NMR spectroscopy, while, at elevated pressures, all conformers *I*, *MG* and *U*, which are "invisible" at 3 MPa, become visible on NMR at high pressures. In this way, we can observe all conformers existing between *N* and *U* directly with NMR under pressure. Here, *pressure does not produce any new conformers, but rather simply shifts the equilibrium* (1.1) to the right by increasing the populations of conformers already existing at 0.1–3 MPa according to the Le Chatelier's principle. Or alternately, *an equilibrium structure stabilized at high pressure should correspond to one of the fluctuated structures at the ambient pressure* [8].

Thus, the essential new feature that these two experiments depict is that, in reality, *a proteins protein exists in solution as a fluctuating equilibrium mixture of "multiple-conformations"* rather than confined to a "single conformation" as hitherto has been believed. This feature, generally observed in globular proteins under variable pressure, should be accepted as a reality of proteins existing in nature today.

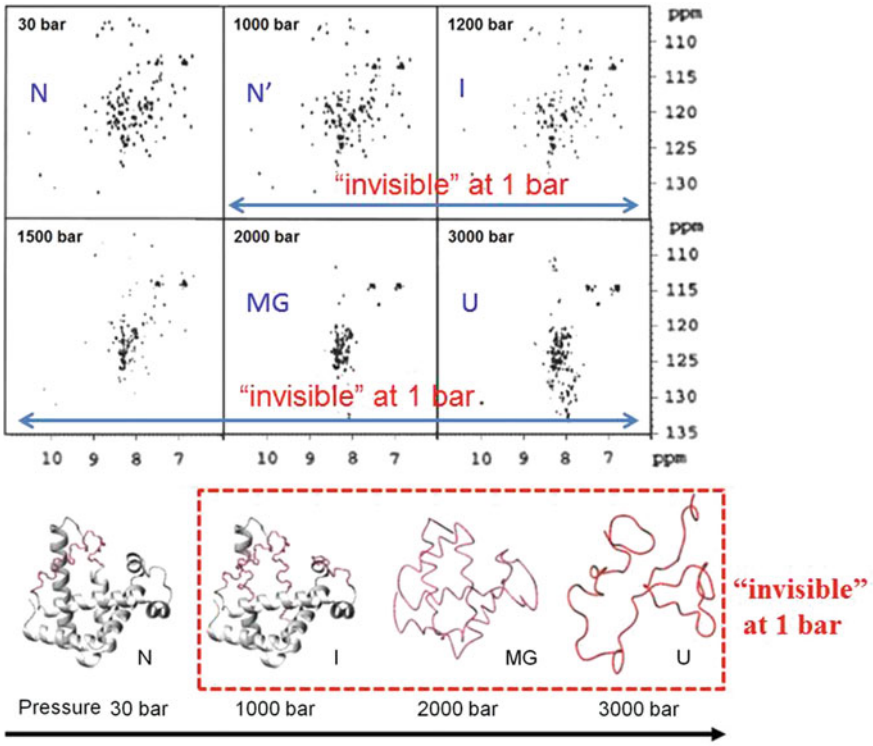

Fig. 1.3 Pressure turns the "invisible" conformer to "visible" in 2D $^{15}N/^{1}HNMR$

In the next section, we will discuss the thermodynamic background on how one can visualize this reality on NMR spectroscopy using pressure perturbation.

1.2 The Thermodynamic Background

1.2.1 Effect of Pressure on the Protein Conformational Equilibrium

Proteins are the molecules for maintaining the dynamism of life on the earth environment at relatively mild temperature and pressure. Thus, their structures must have been made to be sufficiently mobile under the control of thermodynamic laws. Thus, a folded protein molecule under physiological conditions existing mainly in the basic folded state N may actually exist in dynamic equilibrium with other conformational states under physiological conditions, say with the unfolded state U or any conformer other than N. The equilibrium constant $K = [U]/[N]$, *say*

between the two conformers N and U conformational states is governed by the Gibbs energy difference (ΔG) between the two states

$$\Delta G = G_U - G_N = -RT \ln K. \tag{1.2}$$

In most variable–pressure experiments in which we vary pressure while we keep the temperature constant ($T = Tx$), we have the following basic relation to p up to the second order [20, 37, 38]

$$\Delta G = \Delta G_x^0 + \Delta V^0(p - p_0) + \frac{\Delta\kappa}{2}(p - p_0)^2 + \Delta\alpha(p - p_0)(T_x - T_0) \tag{1.3}$$

and

$$\Delta V = V_U - V_N = \Delta V_0 + \Delta\alpha(T - T_0). \tag{1.4}$$

Equation (1.3) depicts that the pressure dependence of the equilibrium between U and N of a protein depends on four parameters ΔG^0, ΔV^0, $\Delta\kappa$ and $\Delta\alpha$, which are

ΔG^0: $\Delta U(T_x) - T_x\ \Delta S(T_x)$; the stability difference at 1 bar at T_x (>0 except at extreme T_x)
ΔV^0: the volume difference under standard conditions (a significantly large negative value <0)
$\Delta\kappa$: the compressibility difference ($\sim$small for low pressures)
$\Delta\alpha$: the expansivity difference (a significantly positive value > 0)

Granted that $\Delta\kappa$ is negligible for $p <$ a few kbar, Eq. (1.4) is simplified to (1.5);

$$\Delta G = \Delta G^o + \Delta V(p - p_0). \tag{1.5}$$

In high-pressure NMR experiments, where pressure is varied at a fixed temperature, the equilibrium constant K between any two conformational sub-states (e.g., N and U) is reduced from the intensities of individual sub-states or from chemical shift changes for each pressure, from which ΔG is calculated from Eq. (1.2). For a series of pressures chosen, we get ΔG values as a function of pressure, which then is fitted to Eq. (1.5) to obtain the best-fit values of ΔG^0 and ΔV. The general procedure is illustrated in Fig. 1.4. Here by fitting Eq. (1.5) to the experimental data [plot of ΔG vs. p], we obtain $\Delta V_{\mathrm{IN}} = -80$ mL/mol from the slope and $\Delta G^0_{\mathrm{IN}} \sim 4$ kcal/mol or $\sim$16 kJ/mol by extrapolating ΔG to $p_0 = 1$ bar. In this case, the excess stability of N over I ($\Delta G^0 > 0$), which turns out to be marginal for most functional proteins, decreases further with increasing pressure, making conformer I to increase by $\sim$1000-fold, making it more dominant than N above $\sim$200 MPa. *The dramatic conversion from N to I occurs as a result of small positive $\Delta G^0 > 0$ and significantly negative $\Delta V_{IN} < 0$.* Thus, ΔV is the decisive factor, along with ΔG^0, in each step of the equilibrium (1.1).

In practice, as long as the experiment is carried out under physiological conditions, ΔV is considerably negative, giving $\Delta V_{\mathrm{UN}} = -30$ to -100 ml/mol for small

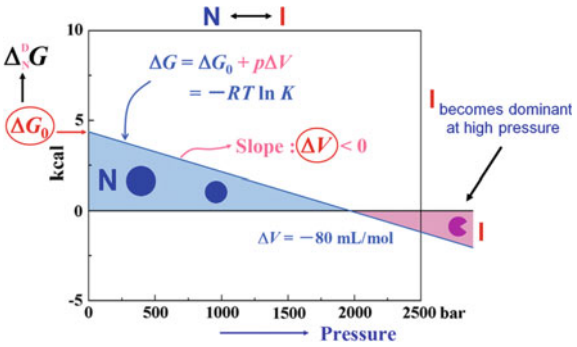

Fig. 1.4 Illustration how the thermodynamic Eq. (1.5) will explain the shift of conformational equilibrium $N \leftrightarrows I$ in equilibrium (1.1.) for a typical case when ΔG^0 *is* $\sim$4 kcal/mol and ΔV is -80 ml/mol

globular proteins and considerably larger for larger proteins. Because in Eq. (1.4) ΔV depends on temperature and $\Delta\alpha$ is known to be positive, the negative ΔV value tends to diminish at higher temperature. Thus, to realize a larger negative ΔV and to explore the high-energy sub-states of proteins with high-pressure NMR effectively, a temperature at physiological or lower is preferred. Furthermore, as the ΔG^0 value in Eq. (1.5) tends to decrease with temperature (toward cold denaturation), the low-temperature effect is further increased.

Our experience tells that, when the temperature is close to 0 °C or even lower, pressure within $\sim$4 kbar is usually sufficient to find the onset of unfolding in the equilibrium (1.1) in a globular protein. Likewise, under pressure of 2 kbar, one can even realize the cold denaturation of ubiquitin, which does not denature within 4 kbar at 25 °C.

1.2.2 "Volume" Decreases as "Cavity" Hydration Increases

We now must understand how the sign of ΔV is determined in each step of the equilibrium (1.1).

The "volume" of the protein we deal with is actually the "partial molar volume" of a protein in solution (water as solvent), which is given approximately by the three major contributions (cf. Fig. 1.5a [39]):

$$V = V_{\text{atom}} + V_{\text{cav}} + \Delta V_{\text{sol}} \tag{1.6}$$

V_{atom} the sum of the *van der Waals volume* of all the constitutive atoms of the protein (Fig. 1.5a)

V_{cav} the solvent-excluded "*void*" *volume* or the space occupied by "*cavities*" (Fig. 1.5a)

ΔV_{sol} the volume change due to the *solvation* of the protein in water; This includes the *volume change* ΔV_{hyd}, *due to "hydration of the protein"* (both the main chain and side chains) as well as *the thermal volume*, V_{T}, which results from

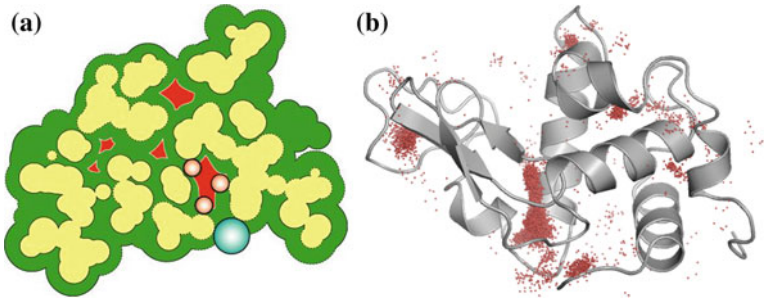

Fig. 1.5 Atoms, cavities and water in proteins. **a** Schematic cross-sectional view of a protein, illustrating the *van der Waals volume* (V_{atom}, green) and *the cavities* (V*cav*, red), estimated by rolling the blue sphere and the pink sphere, respectively, on the van der Waals surface (adapted from Fig. 2 of Ref. [39] by permission of Gekko). **b** Penetration of water molecules into the interior of hen lysozyme at 1 bar as obtained by M.D. simulation (adapted from Fig. 2 in [40] by permission of Elsevier). The figure shows water molecules (*red dots*) getting in and out of the cavities, located mainly in the hinge region between α and β domains and in the loop region of the β domain

thermally induced molecular fluctuation between the solute and the solvent (i.e., the average empty space around the solute due to imperfect packing of the solvent).

Then, the molar volume difference ΔV between any two conformational states of a protein becomes, as V_{atom} largely cancels

$$\Delta V = \Delta V_{cav} + \Delta V_{sol}. \tag{1.7}$$

The term V_{cav} results when a polypeptide chain folds into the N state, the packing of the side chains is not generally perfect and leave some "defect" or extra space unoccupied by atoms within the folded protein architecture. The location and the size of the cavities are found in crystal structures by scrolling a probe of a sphere of $1.0 \sim 1.4$ Å inside the protein molecule and by finding the space left out by the probe. Recent studies indicate that cavities are always found in a variety of globular proteins and further that the size and density of cavities vary greatly from protein to protein apparently related to their function [19]. In the case of lysozyme, cavities are found always in the same strategic positions common to all lysozymes from different biological species [41]. These observations support the notion that cavities, large or small, are the essential elements in globular proteins for creating function.

The cavities are haunted by solvent water even at ambient pressure as the M.D. simulated figure (Fig. 1.5b) shows. As pressure is increased, more water molecules occupy cavities and void inside, eliminating their space further. Namely at each step of the equilibrium (1.1), V_{cav} decreases ($\Delta V_{cav} < 0$).

On the other hand, the term ΔV_{sol} is contributed at least by two factors (ΔV_{hyd} and ΔV_T); the term ΔV_{hyd} is likely to decrease as hydration proceeds, whereas the thermal volume ΔV_T is likely to contribute a considerably larger positive value at higher temperature as $\Delta\alpha > 0$ in Eq. (1.4), and may even cancel the negative

contribution from ΔV_{cav}. Thus, in order to cover a wide range of conformational order of the protein by varying pressure, it would generally be better to perform the experiment at a relatively low temperature. An additional merit of choosing a lower temperature is that we will have decreased ΔG^0 value closer to cold denaturation, so that full unfolding within a few kbar pressure would become easier. For example, one can bring the extremely stable protein ubiquitin to undergo cold denaturation by applying pressure at 2 kbar [42].

As each step of the equilibrium (1.1) is characterized with increased hydration of the polypeptide chain and $\Delta V < 0$. All the globular proteins so far studied with high-pressure NMR have been found to follow this general rule. Values of $\Delta V = -30$ to -100 ml/mol are reported for small globular proteins [43], and significantly larger values ($\Delta V >$ a few hundred ml/mol) for larger proteins and membrane proteins.

1.2.3 *The "Volume Theorem" of Protein and the High-Pressure NMR Experiment*

When a protein in solution exists in a dynamic mixture of various conformeral substates differing in Gibbs energy and in partial molar volume, a conformational transition like that in Fig. 1.4 may occur between any two substates, and, as we increase the pressure, consecutively in the decreasing conformational order $N \rightarrow I \rightarrow \text{MG} \rightarrow U$. Provided that the ΔG^0 value is marginal and the ΔV values

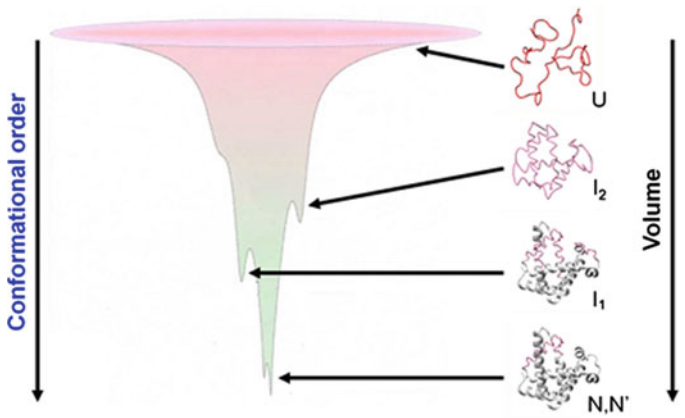

Fig. 1.6 The "volume theorem" of protein-a general rule for globular proteins [20]. The Volume Rule of Protein "the partial molar volume of a protein decreases in parallel with the loss of its conformational order" as advocated by Akasaka [20]. The funnel is drawn schematically for a hypothetical globular protein, with five major conformational sub-ensembles, placed in decreasing conformational order; the native (N), the low-lying excited states (N'), intermediate states (I_1, I_2) and the unfolded state (U), in parallel with decreasing partial molar volume, N > N'>I_1 >I_2 >U

are significantly negative, we may scan the entire conformational space allowed for a protein from the fully ordered native (*N*) to the totally disordered conformer (*U*) *with pressure as sole variable*.

Here the underlying principle that makes this possible is that "*the partial molar volume of a protein decreases in parallel with the loss of its conformational order*" (the "volume theorem" of protein), as advocated by Akasaka globular proteins [20] (Fig. 1.6). The "volume theorem" assures us to observe NMR signals of all relevant high-energy conformers of a protein in decreasing conformational order as we increase the hydrostatic pressure to a sufficiently high level.

In principle, by stably trapping each of these conformers at appropriate pressures, we may determine the average structure of each intervening conformer in detail with NMR, if possible with atom-based resolution, and by extrapolating the signals to 1 bar (as in Fig. 1.4), we may find out its equilibrium population under the physiological condition. This does not necessarily mean that all these intervening species give separately observable spectra as we increase the pressure, but some are separate and others appear mixed, depending on how rapidly they mutually exchange. Only when the exchange rate is much slower than the chemical shift separations of their signals, the two spectra are separately observed. But in general, the exchange rate between conformers becomes slower under pressure, because of the positive activation volume governing the transition. However, when they exchange rapidly, the mixing would be sensed as nonlinear pressure dependence of chemical shift [19].

In this way, *pressure provides a simple, clean and often efficient means of shifting the population distribution among fluctuating conformers of a protein in solution at the will of the experimenter* [24].

1.3 Apparatus for High-Pressure NMR Experiments

Traditionally, there have been two basic methods for performing NMR measurements under variable pressure. One is to use an *autoclave (pressure vessel)* inside of which the NMR detection coil and the sample cell containing the target solution are housed [44]. The other is to use a commercial NMR probe into which a *pressure-resisting sample cell* is inserted, just like the way the conventional NMR measurement at 1 bar is performed, but the pressure within the cell is controlled by a pressure generator (e.g., a pump) placed outside the magnet [18]. The latter method is now almost exclusively used for protein and biopolymer studies, because of the H_0 and H_1 homogeneity that assures high-resolution NMR signals and allows the use of a variety of pulse sequences that standard NMR measurement of protein structure may need, despite the drawback such as the limitation in the applicable pressure to avoid cell burst.

1.3.1 The Autoclave Method

The autoclave (the pressure vessel) method started as early as in 1954 when Benedeck and Purcell [45] measured spin relaxation and self-diffusion of small molecules up to ~1000 MPa (~1 GPa). Jonas extended the technique [16] to study proteins under pressure followed by Markley et al. [46]. The autoclave method utilizes a pressure-resisting vessel (autoclave) made of nonmagnetic materials, inside of which the NMR detection coils and the sample solution are housed. Pressure is applied from the remotely located pump often with, for example, silicone oil as the pressure mediator, and fills the inner space of the autoclave containing the detection coil.

The autoclave method is not extensively used today for studying proteins in solution, because of its low spectral resolution and the inability of carrying out multi-dimensional experiments with sophisticated pulse sequences. However, the utility of the autoclave method continues in studying dynamics of simple liquids and other substances in a wider pressure range (~1 GPa or more).

1.3.2 The Pressure-Resisting Cell Method

The other design is "the pressure-resisting cell method," by which only the sample solution within a specially manufactured cylindrically shaped pressure-resisting cell is under pressure, while the NMR probe itself, usually from a commercial NMR spectrometer, is kept at ambient pressure throughout the measurement [12, 46–49]. The cell is connected with an external pressure line through which the pressure of the sample solution is varied (Fig. 1.7). Pressure is mediated, through a high-pressure line filled with a pressure-mediating liquid, either mineral oil, silicone oil or water, to the protein solution in the sample tube placed in the NMR probe. An example of the block diagram is shown in (Fig. 1.7). To avoid mixing of the sample solution with the pressure-mediating liquid, usually a barrier is provided, either with a synthetic membrane or with a tiny plastic piston [17, 47, 48].

Once the sample solution is set under pressure, measurements can be carried out, just like the measurement at ambient pressure with the versatility of pulse sequences as long as the signal-to-noise permits.

The only concern is how to set up the high-pressure NMR measuring system with a reliable pressure-resisting cell. The crucial factors for the pressure-resisting cells for use in high-pressure NMR is (1) the sufficient pressure-resistance at least 2 kbar or preferably higher (up to 4 kbar or more), besides easy handling and safety and (2) a sufficient magnetic homogeneity to assure high-resolution signals and to allow for multi-dimensional NMR spectroscopy (particularly for protein studies). It is for factor 1 that recently focus is on ceramic materials like zirconia with high shear strength, although factor 2 is not a trivial factor to overcome with ceramic

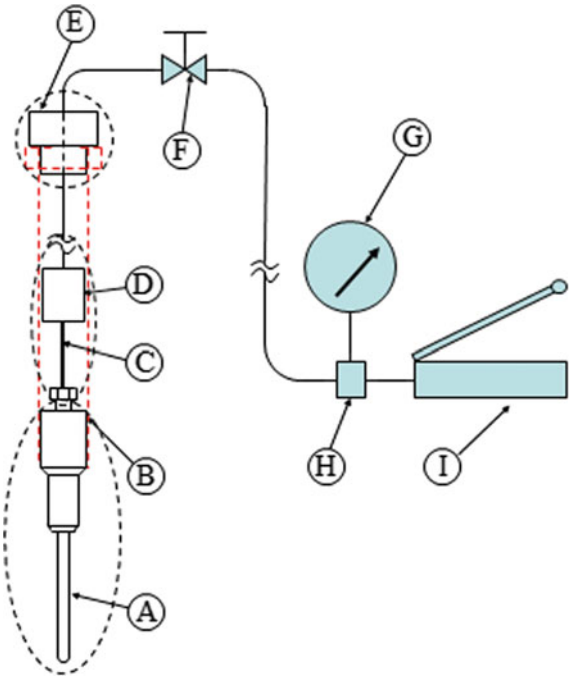

Fig. 1.7 An example of the block diagram of the high-pressure NMR ceramic cell system. *A* ceramic cell; *B* cell holder; *C* connecting tube; *D* tube to tube connector; *E* cell suspender (to be placed on top of the magnet bore); *F* stop valve; *G* pressure gauge; *H* tee; and *I* pressure generator (pump)

cells compared to glass or quartz cells for which high resolution is relatively easily attained.

Cells made of glass

In 1974, Yamada introduced a "hand-made" glass capillary tube that can fit to a commercial NMR probe and endure pressure between 0.1 and 200 MPa for pressure shift measurement of organic compounds [52]. Luedemann employed the method for studying dynamics of simple liquids, and later Wagner applied it for the first time to study protein dynamics (ring-flip motions) in a protein BPTI [14]. Earlier, Yamada had introduced a "one-by-one" type pressure-resisting glass cell (pressure is produced within a sealed glass tube by an expansion of an organic liquid) [11], which Morishima employed for his high-pressure NMR studies of heme proteins [13].

Cells made of synthetic quartz (Fig. 1.8)

In 1996, Akasaka and Yamada, started high-resolution high-pressure NMR studies of proteins at 750 MHz with a hand-made pressure-resisting cell made of synthetic quartz (Fig. 1.8). The synthetic quartz cells extended the resistivity to ~4 kbar and gave extremely high magnetic field homogeneity inside the cell [cf. Fig. 3, 50]. Because of the excellent high resolution along with the high Q value of the detection coil system, the sensitivity was surprisingly high despite of the small quantity (~40 μL) of the sample solution in the NMR active part of the coil.

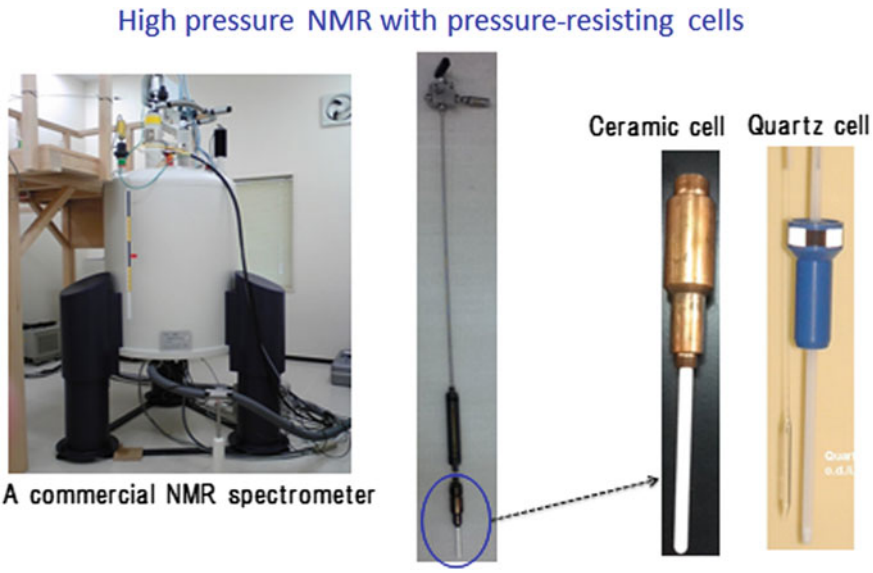

Fig. 1.8 Photograph of a system for the high-pressure NMR measurement, with a ceramic cell or a quartz cell

In 1997, the first paper using the quartz cell was published, showing details of the *diamagnetic* chemical shift changes in the ^{1}H NMR spectrum within the folded manifold of hen lysozyme upon applying pressure [46]. Since then, the technique was applied to a number of proteins, in one-dimensional ^{1}H and heteronuclear two-dimensional NMR spectroscopy under varying pressure up to 200–400 MPa, and produced a number of new findings on protein structure and dynamics extending into the hitherto unexplored *high-energy paradigm of protein structure*, as summarized in a series of review articles by Akasaka and collaborators [18–30, 50] and briefly in [53].

The major drawback of this method is that these cells are not commercially available, but must be hand-made by following the detailed procedure reported by Yamada et al. [17]. Moreover, a special procedure must be followed to introduce the sample solution into the cell also. On the other hand, it has some excellence over other sample cells such that, besides the high resolution of the recorded spectra, an accidental breakage of the cell during measurement causes no damage to the NMR probe a small device, because a special device is designed to protect the sample solution from spilling out of the cell in case of a sudden cell damage.

Cells made of sapphire

For measurements at relatively low pressure range (≤ 2 kbar), cells made of sapphire can also be used. Wand and co-workers report sapphire cell designs for a multi-dimensional NMR study of proteins for a relatively low pressure range ($\sim$up to 1 kbar) [47]. Kalbitzer and co-workers designed sapphire cells to be used for up to ~ 2 kbar [54].

In terms of availability and handiness, sapphire cells may be useful for some applications, where an extreme high pressure is not required.

Cells made of zirconia (Fig. 1.8)
Zirconia cells are currently favored by many researchers because of its sturdiness as well as handiness, and importantly commercial availability, as described below. In general, cells made of zirconia, particularly those of aluminum toughened, are stronger in tensile strength and thus preferred over the other materials for the cell materials for studying proteins. The major question is how to shape them into cylindrical cells with high magnetic homogeneity along with high stability against pressure.

Zirconia cells are now commercially available from Daedalus Innovations, which changed the landscape of high-pressure NMR researches in the world as many new researchers have come into this area by using the commercial cells to study proteins with high-pressure NMR. A few groups have independently developed zirconia cells for their own research [51, 55] in particular. Akasaka et al. [30] have developed zirconia cells based on their own design with a special protection system against an accidental cell burst. One of the merits of the ceramic cell is that, because of its simple cylindrical shape, one can use it for systems other than soluble proteins, for example, the suspension of bacterial spores under pressure [55].

Since the wall of the zirconia cells requires a minimum thickness for keeping the pressure, the inner diameter of the sample cell is limited to 2–3 mm for the 5-mm probe, the filling factor, and therefore the NMR sensitivity is a few to several times lower than that of a glass-made normal sample tube for conventional NMR spectroscopy at 1 bar. Therefore, the use of NMR spectrometer with high sensitivity is preferred, if available, for the high-pressure NMR study of proteins using the ceramic and other pressure-resisting cells. The principles of the design and performance of an aluminum-toughened zirconia cell from Akasaka's group will be reported elsewhere.

1.4 Application to Protein Studies

The NMR spectroscopic study of proteins with pressure as variable over a couple of decades has allowed exploration of protein structures in the conformational space of proteins hitherto unexplored by conventional NMR spectroscopy, namely the paradigm of high-energy conformers having Gibbs energies higher than that of the basic folded conformer N [20, 24, 29, 33]. In contrast to the basic folded paradigm of protein, the high-energy paradigm of protein may consist of a variety of conformers from those close to N to those close to U with varying partial molar

volumes, whose populations may be increased systematically by applying pressure and structures analyzed by employing established NMR techniques.

Application may be divided into three major categories according to the extent of conformational changes involved:

1. Rapid ($\ll \mu s \sim ms$) conformational fluctuations within the folded state: $N \leftrightarrows N'$;
2. Conformational fluctuations (in $\mu s \sim ms$) involving high-energy sub-states: $N1 \leftrightarrows N2 \leftrightarrows I$;
3. Slow conformational fluctuations (in $\gg ms \sim s$) involving the unfolded and fibril or aggregated states: $N \leftrightarrows MG \leftrightarrows U \leftrightarrows$ Fibril.

1.4.1 Fluctuations within the Basic Folded Ensemble

In this section, we discuss the simple case where the fluctuation is restricted largely within a single sub-ensemble of conformers (the case in which higher energy conformers are far distant and the protein remains in the native ensemble "*N*" or "*N* '". On applying pressure on the native ensemble "*N*," its partial molar volume (protein molecule + hydration layer) responds to pressure, but on the NMR spectrum, one can detect signals only from the protein part of the structure as residue-specific chemical shift changes of ^{1}H, ^{15}N or ^{13}C nuclei with pressure.

Macroscopically, the compressibility measurements of folded proteins have long been the primary source of conformational fluctuation of proteins [39]. The compressibility of proteins in their native states is known to be positive [$\kappa > 0$, Eqs. (1.2) and (1.3)] [9], suggesting that the interatomic distances should decrease, on average, within the native fold at high pressure.

Importantly, the *compressibility* (coefficient β) of the volume V of a protein is related to its mean-square *fluctuation* ($\langle\langle(\delta V)^2\rangle\rangle$) through the relation

$$\langle(\delta V)^2\rangle = \beta V k_B T \tag{1.8}$$

where V is the partial molar volume of the protein system, β is the isothermal compressibility coefficient, k_B is the Boltzmann's constant and T is the absolute temperature [7].

This relation indicates that the information on the volume fluctuation of a protein can be estimated from its compressibility measurement, which traditionally has been obtained from ultrasonic sound velocity measurements of proteins, the adiabatic part of which giving RMS fluctuations of volume on the order of $\sim 0.3\%$ for most globular proteins [9]. By using high-pressure NMR, the *compressibility* of the protein part can be measured, in principle, *at individual residue sites*, e.g., at individual hydrogen bonds, through residue-specific chemical shift changes of ^{1}H, ^{15}N or ^{13}C nuclei with pressure, as will be shown later.

Since most covalent bond lengths are practically invariant under pressure at least within kilo-bar ranges, within the protein structure, compression arises from *changes in individual weak chemical bonds* (hydrogen bonds, ionic bonds, van der Waals interactions) accompanied by changes in individual ϕ, Ψ or χ torsional angles [49], which are manifested most sensitively in pressure-induced chemical shifts of individual ^{1}H, ^{15}N and ^{13}C nuclei from the main chain as well as the side chains.

1.4.1.1 Fluctuation in Individual Hydrogen Bonds Detected by ^{1}H Pressure Shifts

Pressure-induced changes in chemical shift of individual amide (^{1}HN) protons can be measured either on ^{1}H/^{1}H two-dimensional spectra (e.g., nuclear Overhauser effect spectroscopy (NOESY) (Fig. 1.9a) and total correlation spectroscopy (TOCSY) or on ^{15}N/^{1}H two-dimensional heteronuclear single-quantum correlation spectra (HSQC) (for the uniformly ^{15}N-labeled samples) in an $^{1}H_20$-rich environment (e.g., 90% $^{1}H_2O$/10% $^{2}H_20$) at neutral to low pH in the pressure range 1–2000 bar [18, 27, 49, 56–57].

The low field shift trend of amide ^{1}H and ^{15}N signals are generally observed (Fig. 1.9b), which may be understood as arising from a decreased shielding of the magnetic field on these nuclei due to an increased polarization of the peptide N–H bond and also from the increased susceptibility effect from the increased proximity of the C=O group at high pressure. Therefore, the pressure-induced shifts of amide ^{1}H and ^{15}N signals give at least a qualitative measure of the pressure-induced changes (compaction in general) of individual N–H···O=C hydrogen bond distances [57].

The pressure-induced shifts of individual amide (^{1}HN) protons are quite linear in BPTI in the pressure range up to 200 MPa, but heterogeneous in magnitude over individual (^{1}HN) protons, indicating that the compressions of the hydrogen bonds occur actually quite heterogeneously over the hydrogen bonds of the protein molecule [57]. The larger the change in distance denotes the larger the fluctuation of the hydrogen bond. The observed low-field shifts of ^{1}HN protons averaged over all of the NH···O hydrogen bonds of BPTI correspond to a shortening of N–H distances on average by $\sim$0.020 Å (or by $\sim$1%) at 2 kbar for those engaged in the internal (NH···O=C) hydrogen bonding, as estimated from the empirical shift–distance relationship (see [23, 27, 57]). The results indicate that the compression is not uniform but rather heterogeneous over the folded molecule.

Despite the heterogeneity in fluctuation among hydrogen bonds within a single protein molecule, the average degree of fluctuations of hydrogen bonds over a protein molecule, as represented by the linear response part of the pressure shift, are similar to each other among different globular proteins. On the other hand, in many