Biochemistry

 Volume 42, Number 37 September 23, 2003

Current Topics

Highly Fluctuating Protein Structures Revealed by Variable-Pressure Nuclear Magnetic Resonance

Kazuyuki Akasaka*

Department of Biotechnological Science, School of Biology-Oriented Science and Technology, Kinki University, Wakayama 649-6493, Japan, and Cellular Signaling Laboratory, RIKEN Harima Institute, Hyogo 679-5148, Japan

Received May 5, 2003; Revised Manuscript Received July 28, 2003

ABSTRACT: Although our knowledge of basic folded structures of proteins has dramatically improved, the extent of our corresponding knowledge of higher-energy conformers remains extremely slim. The latter information is crucial for advancing our understanding of mechanisms of protein function, folding, and conformational diseases. Direct spectroscopic detection and analysis of structures of higher-energy conformers are limited, particularly under physiological conditions, either because their equilibrium populations are small or because they exist only transiently in the folding process. A new experimental strategy using pressure perturbation in conjunction with multidimensional NMR spectroscopy is being used to overcome this difficulty. A number of rare conformers are detected under pressure for a variety of proteins such as the Ras-binding domain of RalGDS, β-lactoglobulin, dihydrofolate reductase, ubiquitin, apomyoglobin, $p13^{MTCP1}$, and prion, which disclose a rich world of protein structure between basically folded and globally unfolded states. Specific structures suggest that these conformers are designed for function and are closely identical to kinetic intermediates. Detailed structural determination of higher-energy conformers with variable-pressure NMR will extend our knowledge of protein structure and conformational fluctuation over most of the biologically relevant conformational space.

A protein in solution is a thermodynamic entity existing, in principle, as a dynamic equilibrium of conformers (*1*). Generally, function is considered to be coupled to structural fluctuations or multiple conformational changes (*2*). Various fluctuating conformations are involved in folding (*3*) and in misfolding events leading to prion and amyloid diseases (*4*). Historically, the multiple-conformation view of proteins started with induced fit (*5*) and allosteric concepts (*6*). In accordance with this, crystal structural variations of a protein in different environments have been shown (*7*). Experimental evidence for conformational fluctuations involving multiple conformations or higher-energy conformers of proteins in solution is rapidly improving from a number of sources, including enzyme reaction kinetics (*2*), hydrogen exchange studies (*8*−*11*), kinetic studies of folding (*12*, *13*), NMR[1] analyses of spin relaxation (*14*) and residual dipolar couplings (*15*), and single-molecule spectroscopy (for example, see ref *16*). Multiple conformations are universal rather than ex-

* To whom correspondence should be addressed. E-mail: akasaka8@spring8.or.jp. Fax: +81-736-77-4754.

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; HSQC, heteronuclear single-quantum correlation; δ, chemical shift; $\Delta\delta$, pressure-induced chemical shift; DHFR, dihydrofolate reductase; BPTI, basic pancreatic trypsin inhibitor; protein G, immunoglobulin-binding domain of protein G; HPr, histidine-containing protein; RalGDS-RDB, Ras-binding domain of the Ral guanine nucleotide dissociation stimulator; $P13^{MTCP1}$, oncogenic protein involved in T-cell leukemia.

10.1021/bi034722p CCC: $25.00
Published on Web 08/29/2003

ceptional, and there is a need for a general method for detecting higher-energy structures at a level of detail comparable to the average folded conformation.

Although NMR may be considered in detecting signals from all fluctuating conformers in solution, in reality it reports a single structure, close to the one in the crystal (*17*), neglecting coexisting minor conformers. Moreover, even within the subensemble of the major conformer, any fast fluctuation with a frequency of $\gg 10^3$ s^{-1} is averaged out, leaving almost no trace in the spectrum. Direct structural characterization of higher-energy conformers by NMR is only possible by sufficiently increasing their equilibrium populations. This is attained, in some cases, by varying the pH or concentration of a denaturant (*18)*. However, it is not generally assured that the conformers under extreme pH or denaturant represent intrinsic conformers in the absence of chemical perturbants.

Pressure shifts the population distribution on the simple ground that it favors a smaller volume for the system. Because the partial volume of a protein in solution fluctuates amply in accordance with the conformational fluctuation, pressure provides a simple, efficient means of shifting the population distribution among fluctuating conformers. The idea of using pressure perturbation to study conformational fluctuations in proteins is not new (*19*−*21*), and its combination with NMR has gained much attention in recent years (*22*−*26*). A technical breakthrough is attained by the introduction of the on-line cell variable-pressure NMR method using a pressure-resistant cell on a commercial high-frequency NMR spectrometer (*27*−*29*). In particular, the method using the quartz cell (*28*) has proven to be suitable for monitoring structural changes at essentially all individual amino acid residues on ^{15}N- and/or ^{13}C-labeled protein samples (MW < 30 000) under variable pressure from 1 bar to ~4 kbar (*30*). In this article, I emphasize the conceptual aspect of this new method as applied to proteins, and review its application to the study of a wide range of conformational fluctuations in proteins, leaving purely technical aspects for literature (*27*−*29*). For earlier pioneering works using high-pressure probe methods, see refs *24* and *31*−*33*.

The basic principle of the method is rather straightforward. A protein in solution consists of an ensemble of fluctuating conformers associated with a range of fluctuating partial molar volumes. However, the fluctuation is hardly visible in the NMR spectrum, either because the fluctuation is too rapid on the NMR time scale ($\gg 10^3$ s^{-1}) or because the fluctuation is slow but rare. Information about the fluctuation is only partly recovered by spin relaxation measurements. To revive the conformational fluctuation so that it can be explicitly observed in the NMR spectrum, we apply pressure. Pressure causes a shift of population in the ensemble of fluctuating conformations so that the system assumes a smaller partial molar volume. The resultant spectral difference between 1 bar and the pressure p in the NMR spectrum represents the fluctuating part of the structure. If the pressure is varied sufficiently (up to ~4 kbar) (1 bar = 10^5 Pa = 0.9869 atm), a considerable range of conformational fluctuation may be explored, the range of which depends critically on the protein itself (stability and volume) and the experimental condition (temperature, pH, etc.). For some proteins, only the fluctuation within the folded manifold may be monitored, but for many other proteins, one can explore much of their allowed conformational space close to full unfolding.

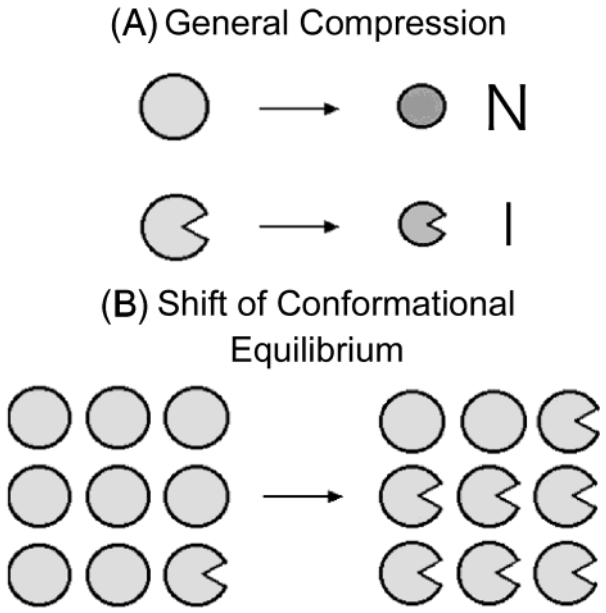

FIGURE 1: Schematic representation showing how a protein in solution can attain a lower effective volume (A) by a general compression within the same subensemble of conformer N or I and (B) by a shift of the I equilibrium (N to I) in favor of a lower-volume subensemble I. The volume here refers to an effective volume, including the contribution from hydration (i.e., partial molar volume) (*34*).

General Response of Protein Volume to Pressure

The effective volume of a protein molecule in an aqueous environment is represented by the partial specific or partial molar volume, because the volume of the protein and that of water are inseparable. We start with a simplified view of the fluctuation of a partial molar volume of a protein. Namely, the partial molar volume of a protein fluctuates in two typical ways (*34*), one within the same subensemble of conformers and the other among different subensembles of conformers having different average energies. Under pressure, a protein may shift to a smaller partial molar volume through these two kinds of fluctuations.

General Compression within the Same Subensemble of Conformers. In a simple case where fluctuation is within a single subensemble of conformers (the case in which higher-energy conformers are far distant), e.g., a native subensemble [as in the compression of ice, approximately elastic response (*20*), Figure 1A], the mean-square fluctuation of the volume [$\langle(\delta V)^2\rangle$] of a protein is intimately related to the compression of the volume (βV) under pressure through the relation

$$\langle(\delta V)^2\rangle = \beta V k T \qquad (1)$$

where V is the volume of a system, β is the isothermal compressibility coefficient, k is Boltzmann's constant and T is the absolute temperature (*35*). Equation 1 states that we acquire information about the volume fluctuation by measuring compressibility, i.e., compression per unit pressure. Microscopically, the volume compression is attained by a shift of the population among microscopic states within the subensemble in favor of lower-volume microscopic states.

The shift will be manifested as NMR spectral changes under applied pressure.

For many globular proteins, rms fluctuations of the volume are estimated to be on the order of ~0.3% of the total volume (*36*).

Compression Accompanied by a Shift of the Equilibrium between Different Subensembles. In general, a protein molecule in solution may exist as an equilibrium mixture of subensembles of conformers differing, in general, in their topology of folding (e.g., between the native subensemble N and an intermediately folded subensemble I, cf. Figure 1), in partial molar volume ($\Delta V_0 = \Delta V_I - \Delta V_N$), and in thermodynamic stability ($\Delta G_0 = \Delta G_I - \Delta G_N$). In this situation, the equilibrium constant K between N and I may change with pressure according to the relation

$$K = [\mathrm{I}]/[\mathrm{N}] = \exp(-\Delta G/RT) \quad (2)$$

where

$$\Delta G = G(\mathrm{I}) - G(\mathrm{N}) = \Delta G_0 + \Delta V_0(p - p_0) - {}^1/_2\Delta\beta V_0(p - p_0)^2 \quad (3)$$

where ΔG and ΔG_0 are the Gibbs energy changes from N to I at pressure p and p_0 (1 bar), respectively, ΔV_0 is the partial molar volume change, $\Delta\beta$ is the change in compressibility coefficient, R is the gas constant, and T is the absolute temperature (*30*, *37*). At or near physiological conditions, ΔG_0 is normally positive (i.e., N is more stable than I), and the population of I is too low to be detected. If pressure is applied, the protein may assume a lower partial molar volume by a shift of equilibrium between subensembles in favor of a lower-volume subensemble [like melting of ice, approximately inelastic response (*20*), Figure 1B]. For many proteins, the $\Delta V_0(p - p_0)$ value is on the order of −2 to −10 kJ/mol at 1 kbar and −4 to −20 kJ/mol at 2 kbar for a typical ΔV_0 of −20 to −100 mL/mol (*38*), which may be sufficient to compensate for a marginally positive ΔG_0. This enables NMR assessment of subensemble I stably trapped under pressure. When a protein consists of a series of subensembles differing in volume, higher and higher pressure would populate subensembles with smaller and smaller volumes, each of which could be a target of NMR analysis.

Conformational Fluctuations Revealed by Variable-Pressure NMR

Given the general agreement that protein function involves fluctuations of the protein structure, variable-pressure NMR provides a means of investigating the structural fluctuations that are necessary for function by determining structural changes under pressure in microscopic detail. In NMR, structural changes are manifested as changes in chemical shift, J coupling, nuclear Overhauser effect (NOE), or spin relaxation.

Conformational Fluctuations within the Folded Subensemble. Chemical shifts are extremely sensitive to subtle conformational differences, and are handy reporters of conformational changes under pressure. In all the proteins that have been investigated, melittin α-helix (*39*), gurmarin (*40*), BPTI (*41*−*44*), the immunoglobulin-binding domain of protein G (*45*), lysozyme (*46*, *47*), HPr (*48*), DHFR (*49*), RalGDS-RBD (*50*), the RalGDS-RBD−Rap1A complex (*51*), β-lactoglobulin (*52*), apomyoglobin (*53*), $P13^{MTCP1}$ (*54*), α-lactalbumin (*55*), prion (*56*), and ubiquitin (*57*, *58*), chemical shifts were found to change reversibly with pressure within the folded manifold (general compression) before a discrete conformational transition such as local or global unfolding takes place. In a typical two-dimensional NMR spectrum, e.g., $^1H-^{15}N$ HSQC, each amino acid residue of a folded protein gives a spot (cross-peak) at a specific position (chemical shift) (Figure 2). A single and sharp cross-peak for each residue does not mean a "single structure", but rather, it means that any conformational heterogeneity is averaged out rapidly ($\gg 10^3$ s^{-1}) over the ensemble of conformers (microstates) within the folded subensemble (macrostate). General compression of the protein by pressure is detected, however, as continuous shifts of individual cross-peaks (or ^1H, ^{15}N, or ^{13}C signals) (see Figure 2), meaning that the *averaged* conformation changes continuously within the macrostate with pressure. This means that the shift arises from a change in the population distribution among the microstates comprising the folded macrostate with increasing pressure. It gives a direct experimental demonstration that the folded macrostate consists of an ensemble of conformers (microstates) differing in volume.

The amide ^1H shift is mainly determined by the magnetic anisotropy from neighboring peptide groups and is closely correlated with the hydrogen bonding state of the NH group, in particular, the H···O hydrogen bond distance, thereby giving a measure of the fluctuation of the hydrogen bonding state (*41*−*44*). The ^{15}N pressure shift critically depends on changes in the local electronic environment, and gives a qualitative measure of fluctuation in torsion angles ϕ, ψ, and χ in addition to the fluctuation in the hydrogen bonding state (*43*). Here the magnitude of the shift reflects, among other things, the magnitude of the change in the average conformation at each site. Equation 1 implies that the conformational change is strongly correlated to the amplitude or extent of conformational fluctuation within the folded subensemble. Therefore, the magnitude of the pressure-induced chemical shift in Figure 2 may be used as a qualitative measure of the conformational fluctuation at that site. The continuity of the shift requires that the frequency range of the fluctuation be roughly $\gg 10^3$ s^{-1}.

In Figure 3, panels A and B depict magnitudes of pressure-induced chemical shifts at individual residues of intact hen lysozyme. Both the magnitude and the sign vary significantly for different residues, qualitatively demonstrating heterogeneous conformational fluctuations centered on the loop region. In contrast, the variation is much more uniform over residues in denatured lysozyme (Figure 3, panels C and D).

In BPTI (*41*−*44*), gurmarin (*40*), and the immunoglobulin-binding domain of protein G (*45*) as well as in simple helical peptides (*39*, *59*), the ^1H and ^{15}N chemical shifts of most amide groups are surprisingly linear with pressure at least up to 2 kbar. From the empirical correlation of the amide ^1H shift to hydrogen bond distance and the observed pressure-induced ^1H shifts, an average H···O distance shrinkage of ~1% is estimated for these proteins at 2 kbar (*41*). The linearity of the amide ^1H and ^{15}N shifts versus pressure, meaning a constant compressibility coefficient β in eq 1, must indicate that in the pressure range of 1−2000 bar, these

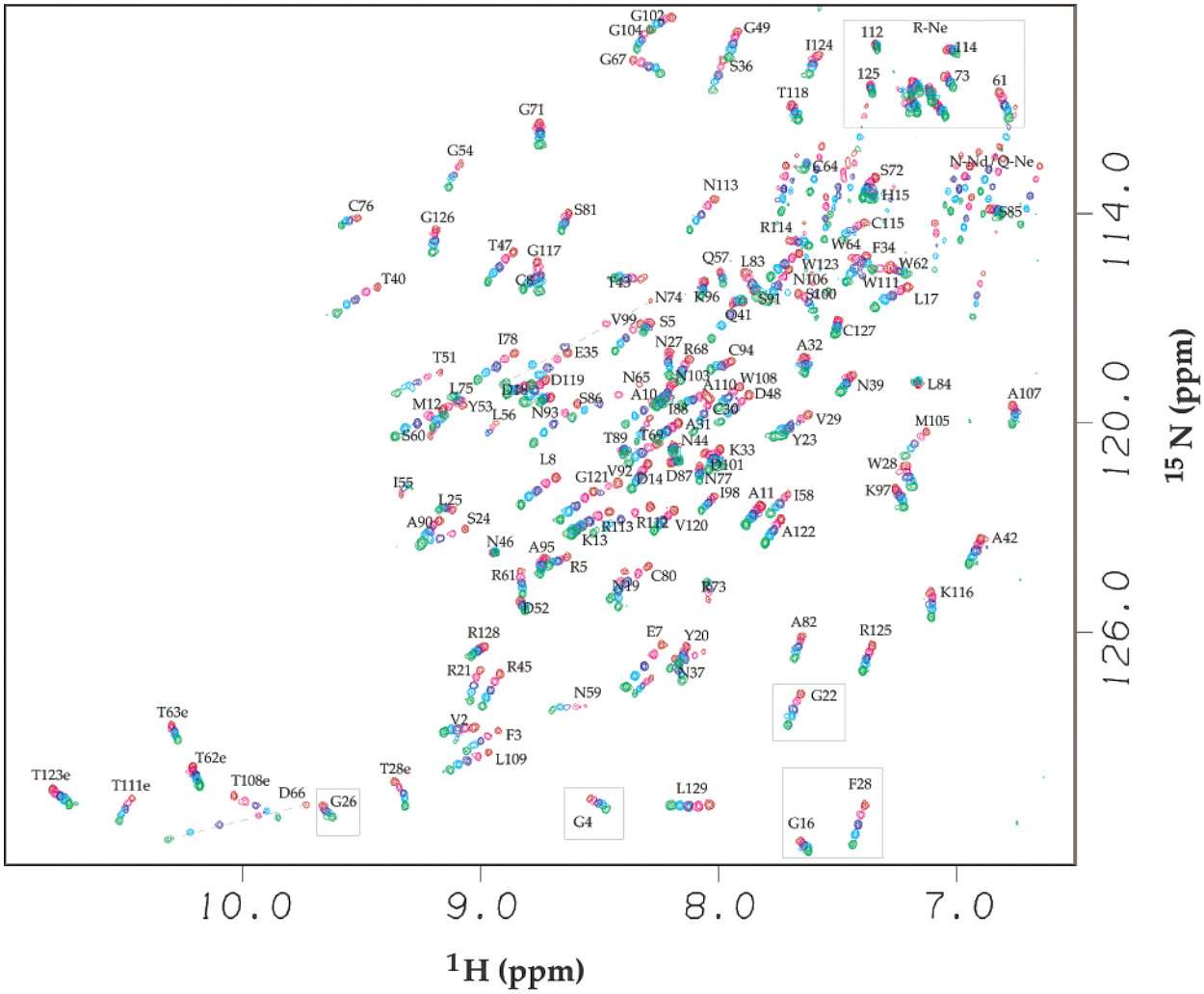

FIGURE 2: Overlay of $^{15}N-^{1}H$ HSQC spectra of native hen lysozyme at pH 2 recorded at variable pressure. Cross-peaks from spectra recorded at 30, 500, 1000, 1500, and 2000 bar are shown in red, magenta, purple, cyan, and green, respectively. Enclosed in boxes are "folded-over" cross-peaks from outside the spectral range shown. (from ref *47*, reprinted with permission).

proteins are largely confined to the basic folded state (Figure 1A).

Fluctuation in the Three-Dimensional Structure

Much about structural fluctuations of proteins has been studied and discussed, yet we know little about actual changes in three-dimensional structure or "shape changes" of the protein molecule associated with the putative motions. We may access such structural or shape changes by performing a quantitative analysis of the pressure-dependent NMR spectral changes and expressing the result in atomic coordinates under appropriate structure constraints. In fact, apart from changes in amide ^{15}N and ^{1}H chemical shifts, significant changes in chemical shift and NOE intensities were observed in most side chain protons of hen lysozyme (*46*) and BPTI (*41*−*44*) at 2 kbar, indicating rearrangements in side chain packing or changes in tertiary structure under pressure.

A novel strategy for elucidating structural changes of a protein using the highly sensitive experimental chemical shifts as structure constraints has been developed recently by Williamson et al. (*39*). In the bottom panel of Figure 4 (*60*), the average conformational changes in hen lysozyme induced by pressure (2 kbar) are depicted as difference distance plots for Cα atoms. The result indicates that the α-helical domain is compressed by approximately 1% at 2 kbar, as a result of tighter packing of helices. In contrast, the *β*-domain undergoes both expansion and compression, resulting in almost no overall compression. These findings agree, in essence, with those reported earlier from the X-ray crystallography of hen lysozyme at 1 kbar (*61*). One of the most intriguing findings in the solution work is that the largest volume changes occur preferentially around the hydrated cavities, which are found in the *β*-domain and in the interface of the two domains (Figure 4, top, colored regions). The same prediction was made previously by Kamatari et al. (*47*) on the basis of unusually large pressure-induced ^{15}N chemical shifts of residues surrounding water molecules in the cavities (Figure 3A).

The finding that fluctuations are larger around the water-containing cavities suggests that these fluctuations are coupled with water molecules penetrating into and out of cavities, consistent with the "mobile defects" hypothesis, proposed many years ago by Lumry (*62*). A large contribution to the fluctuation of a protein molecule from atom packing defects or cavities was initially suggested by Gekko from compressibility measurements (*36*, *63*) but has not been proven directly for a long time. It now appears to be certain that water-containing cavities are sources of local confor-

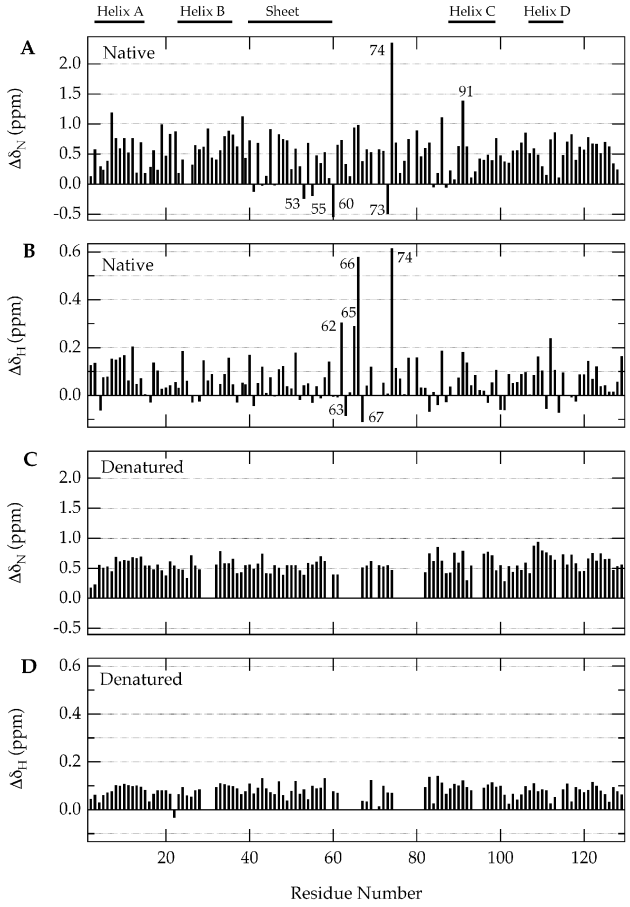

FIGURE 3: Pressure-induced ^{15}N and ^{1}H chemical shifts ($\Delta\delta_N$ and $\Delta\delta_H$, respectively) for the main chain amide groups of native hen lysozyme (A and B) and hen lysozyme denatured in 8 M urea (C and D) [$\Delta\delta = \delta$(2000 bar) − δ(30 bar)]. The numbers indicate unusually shifted residues (from ref *47*, reprinted with permission).

mational fluctuations in lysozyme. It is conceivable that these local fluctuations are evolutionarily designed to facilitate the substrate binding and/or product dissociation.

Nonlinear Pressure Shifts Suggest Cavity-Based Fluctuations

In contrast to BPTI and protein G, etc., the amide ^{1}H and ^{15}N chemical shifts of many other proteins, including HPr (*48*), DHFR (*49*), RalGDS-RBD (*50*), the RalGDS-RBD−Rap1A complex (*51*), β-lactoglobulin (*52*), apomyoglobin (*53*), P13^{MTCP1} (*54*), α-lactalbumin (*55*), prion (*56*), and ubiquitin (*57*, *58*), including a part of lysozyme (*46*, *47*), show distinctly nonlinear pressure shifts. The distinct nonlinearity appears to be explained only by assuming a rapid ($\gg 10^3$ s^{-1}) mixing of higher-energy conformers or low-lying excited states with smaller partial molar volumes into the basic folded conformer with increasing pressure (*64*) (the case of Figure 1B). Interestingly, the extent of nonlinearity, summed up over all the amide residues of a protein, is strongly correlated with the *density* of the cavities with a volume exceeding 20 Å^3 (a size likely to hold a water molecule) within the interior of the protein; the larger the density of such cavities in a protein, the larger the nonlinearity of chemical shifts for the protein (*64*). This finding has led to a notion that the low-lying excited states are associated with hydration of water-accessible cavities. It is

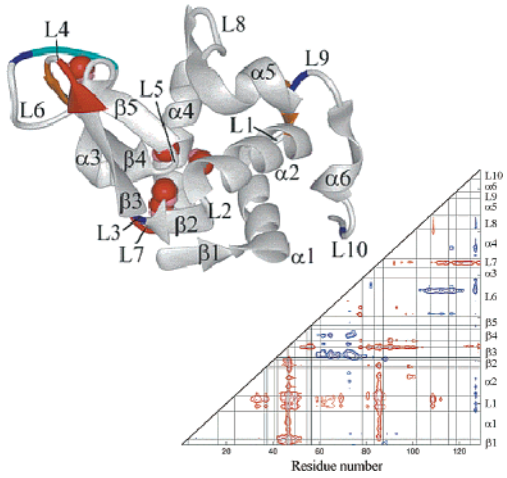

FIGURE 4: Pressure-induced changes in the structure of hen lysozyme at 2 kbar. At the bottom is a difference distance plot for Cα atoms. Orange and red contours denote parts of the structure that have moved closer together, while cyan and blue contours denote parts that have moved apart. Regions of secondary structure are indicated as follows: α, α-helix; β, β-sheet; and L, loop. At the top is a structure of lysozyme, color coded as in the difference distance plot to show regions of the structure that have moved most relative to the rest of the structure. Data are averaged over all distances between Cα atoms for each residue. Regions are coded as follows: $d < -0.12$ (blue), $-0.12 < d < -0.06$ (cyan), $0.16 < d < 0.22$ (orange), and $d > 0.22$ (red). The α-domain is at the right of the figure and the β-domain at the left. The five buried water molecules are shown in red or pink space-filling representation (from ref *60*, reprinted with permission).

conceivable that a protein may have a number of different hydration stages of the cavities, giving a thermally accessible excited state with higher local conformational flexibility. This notion is consistent with the observation that many of these proteins undergo locally unfolding at higher pressures in regions close to the cavities. Although no direct proof has been obtained, it is highly probable that these low-lying excited states and the associated conformational dynamics are closely correlated with the functions of these proteins.

Conformational Fluctuations beyond the Folded Subensemble: Alternately Folded, Partially Folded, and Globally Unfolded Subensembles. In many proteins such as DHFR (*49*), RalGDS-RDB (*50*), β-lactoglobulin (*52*), apomyoglobin (*53*), P13^{MTCP1} (*54*), α-lactalbumin (*55*), prion (*56*), and ubiquitin (*57*, *58*), increasing the pressure to 1−4 kbar range can shift their conformational equilibrium far beyond the basic folded ensemble, in many cases nearly to global unfolding. As pressure is varied upward from 1 bar, a fluctuation involving a conformational subensemble beyond the folded one is normally detected by the appearance of two separate groups of cross-peaks [meaning slow ($\ll 10^3$ s^{-1}) fluctuation] in two-dimensional NMR spectra. (In some cases, some cross-peaks belonging to the higher-energy subensemble may become undetectably broad because of insufficient averaging of signals among heterogeneous conformers.) If this occurs for the entire cross-peaks of the native subensemble (N) simultaneously, it means cooperative unfolding. If loss of intensities occurs only for selected cross-peaks of N, it means a local conformational change. Whether the conformational change is within the folded manifold, or involves unfolding, is judged from the chemical shift position of the cross-peaks after the conformational change. From the relative intensities of each pair of the cross-peaks, we

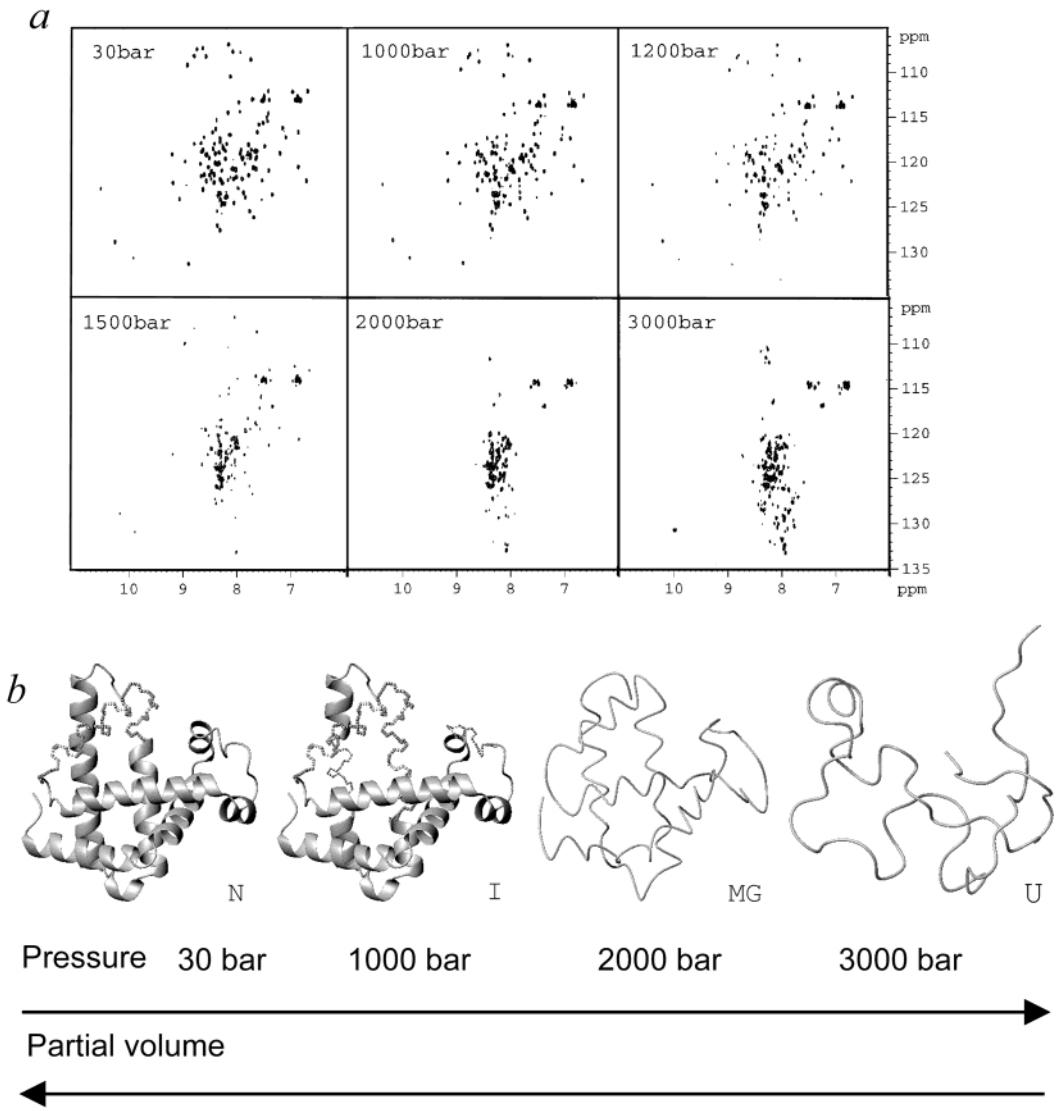

FIGURE 5: Apomyoglobin under variable pressure. (a) $^{15}N-^{1}H$ HSQC spectra of uniformly ^{15}N-labeled sperm whale apomyoglobin in 20 mM MES buffer (pH 6.0) at 35 °C in the pressure range of 30−3000 bar. Only the spectrum at 3000 bar contains 10% ethanol to avoid aggregation. Each cross-peak represents a contribution from each amide group of apomyoglobin. A dramatic change in the spectra indicates a reversible conformational change of apomyoglobin with pressure from the folded to the totally unfolded. At 2000 bar, cross-peak intensities decrease dramatically (to ~20% of that of the native state), showing that MG becomes dominant. At 3000 bar, full intensities are regained, showing that the fully hydrated and extended conformer U becomes dominant (from ref *53*, reprinted with permission). (b) Major conformational subensembles found in apomyoglobin. N is the native state, I the locally unfolded, MG the molten globule, and U the fully unfolded subensemble. Qualitative structures of N and I are based on the structure of holomyoglobin with iron removed. Thin lines represent loop and disordered segments. Note that the conformational order and the order of volume are parallel to each other.

obtain an equilibrium constant (and ΔG) at individual amino acid sites as a function of pressure, and through extrapolation to 1 bar (eq 3), the stability difference (ΔG_0) and the volume difference (ΔV_0) between the two conformers are obtained at 1 bar.

Volume Theorem: Volume Parallels Conformational Order

Figure 5 shows an example of such events in apomyoglobin in 20 mM MES buffer (pH 6.0) at 35 °C in the pressure range from 30 to 3000 bar (*53*). A dramatic spectral change in two-dimensional $^{15}N-^{1}H$ HSQC spectra (essentially reversible, Figure 5a) indicates that a large-scale conformational change takes place from the native to the globally unfolded. The analysis of individual cross-peak intensities as a function of pressure indicated the preferential loss of some cross-peaks, showing that local conformational transitions are involved before a global unfolding. Namely, the native conformer (N) at 1 bar changed into a locally disordered intermediate conformer (I) at 500−1200 bar. At 2000 bar, all the cross-peak intensities decrease dramatically (to less than ~20% of that of N) due to line broadening, indicating heterogeneous disorder of the entire polypeptide chain conformation that undergoes intermediate exchange. The spectral characteristics share a common feature with the classical acid-induced denatured state at high salt, termed "molten globule" (MG) (*18*), and therefore, we retain the word MG for this state. At 3000 bar, full intensities are regained in a well-hydrated, mobile unfolded conformation (U). Namely, the equilibrium is shifted from N to I, I to MG, and MG to U in sequence, as the pressure is increased, as shown schematically in Figure 5b.

From eq 3, this means that the partial molar volume V of apomyoglobin decreases in the following order: N > I >

MG > U. This order coincides with the decreasing order of the tertiary structure ("conformational order") of apomyoglobin. In various other globular proteins examined at or below physiological temperatures, there is a parallel between the partial molar volume V of a protein and its conformational order, suggesting that this correlation is common to globular proteins. We have put this into a theorem. The partial molar volume of a protein decreases in parallel with the decrease in its conformational order (in short, volume parallels conformational order) (the volume theorem of protein) (*53*). This theorem can be used as a general guide in the application of variable-pressure NMR to proteins. The partial molar volume V refers here to a volume under pressure. However, we expect that the theorem similarly applies to the partial molar volume at 1 bar (V_0) assuming $\Delta\beta$ is small (*38*). The negative volume change with unfolding arises from two sources: the increase in the level of hydration of the chain and the loss of cavities from the folded protein. The hydration effect contains both positive and negative contributions, and its absolute contribution increases with temperature (*65*); the loss of a cavity always contributes negatively to the volume (*19*, *38*, *66*). Therefore, if a local conformational change involves a partial loss of a cavity, then the partial volume may decrease considerably. This has been effectively confirmed in a number of proteins for which a pressure-induced alternate state was observed before global unfolding is reached.

Figure 5 shows that, as we decrease the pressure from 3000 bar, the protein folds into its beginning apo conformational ensemble. This intuitively suggests that the conformers between N (apo) and U are likely to be folding intermediates that also appear in the kinetic folding process. The relationship between the kinetic intermediates and the equilibrium intermediates stabilized by pressure will be discussed more generally later.

Higher-Energy Conformers Are Designed for Function

Besides apomyoglobin, in various other proteins having important cellular functions, such as DHFR (*49*), RalGDS-RBD (*50*), β-lactoglobulin (*52*), apomyoglobin (*53*), $P13^{MTCP1}$ (*54*), α-lactalbumin (*55*), prion (*56*), and ubiquitin (*57*, *58*), peculiar intermediate conformers, often with distinct local conformational changes, are found by variable-pressure NMR. Their structures may be depicted qualitatively with regions of local unfolding marked on their known folded structures. Here local unfolding occurs normally as local disorder or local melting of part of the three-dimensional structure rather than as melting of a secondary structure unit. These intermediate conformers are apparently designed by nature for functional purposes. For apomyoglobin, the equilibrium intermediates N′ and I are considered to be functionally relevant conformers, because their open structures would be needed for binding heme to make the holoprotein, myoglobin. A highly fluctuating nature of protein conformation will be shown below in several other proteins based on the results of the variable-pressure NMR experiments, and their relevance to function will be discussed.

Active Site Hinge Motion in an Enzyme, Dihydrofolate Reductase (49). Enzyme catalysis normally involves multiple conformational changes, the structural elucidation of which is seldom carried out. Catalysis normally involves a process of binding of a substrate or a cofactor in the active site pocket, and therefore, the protein may undergo some "open−close motion" in the reaction.

Dihydrofolate reductase (DHFR) catalyzes a reduction of dihydrofolate to tetrahydrofolate with the aid of a cofactor NADPH. DHFR has two active site pockets, one for substrate (folate) and the other for cofactor (NADPH). In the folate-bound form of DHFR from *Escherichia coli*, only one cross-peak is found for each amino acid in the $^{15}N-^{1}H$ two-dimensional NMR spectrum at 1 bar; however, but with increasing pressure, some of them split into two cross-peaks, and the new cross-peaks increase in intensity at the expense of the old ones with increasing pressure. The analysis according to eq 3 indicates that two conformers coexist at all pressures and that the population of the rare conformer increases to ∼50% at 2 kbar, because it has a smaller partial molar volume than the first ($\Delta V_0 = -25$ mL/mol at 15 °C). Linear extrapolation of ΔG to 1 bar (eq 3) gives a ΔG_0 of 5.2 kJ/mol, predicting the population of the rare conformer at 1 bar to be ∼11%. The two conformers differ largely in the hinge parts of the M20 loop (making a flap to the cofactor binding pocket), the C-helix, and the F-helix, surrounding the cofactor-binding pocket. Line width analysis indicates that the folate-bound DHFR undergoes a hinge motion between the "closed" and "open" forms of the NADPH binding pocket at a rate of $<20\ s^{-1}$. It appears certain that the fluctuation of the M20 loop to the open conformer is necessary for NADPH to bind.

Does the open conformation found at high pressure really exist at 1 bar at the predicted population of ∼11%? A separate NMR measurement was carried out on the DHFR solution at 1 bar in a normal sample tube containing a sufficient quantity of the protein solution to gain sufficient signal intensities. The result showed the presence of the signals of the open conformer nearly at the expected population of (∼13%) at positions of predicted chemical shifts. The experiment verifies that the open conformation is actually sampled by the proteins in the "native" condition.

An open conformer would be generally required for an enzyme to bind a substrate, a cofactor, or an allosteric effector. However, the open conformers must be designed to be minor, as they are more "reactive" than the closed conformers. Variable-pressure NMR is expected to be widely applicable for studying structures of these open conformers in various enzymes by stably trapping them under pressure as they generally have smaller partial molar volumes.

Barrel Opening Fluctuations in a Lipocalin Family Protein, β-Lactoglobulin (50). In other proteins, more extensively open conformers are often needed for function. Bovine β-lactoglobulin, a major component of cow's milk, is an 18 kDa protein belonging to the lipocalin superfamily with a variety of biological functions related to the binding and transport of metabolites. Lipocalin family proteins commonly have a β-barrel topology. Uniquely, a fatty acid is known to bind within the central cavity of β-lactoglobulin (*67*). Pressure dependencies of ^{1}H and ^{15}N chemical shifts and cross-peak intensities were analyzed at more than 80 independent atom sites of β-lactoglobulin between 30 and 2000 bar. Besides large and nonlinear pressure shifts, suggesting the existence of low-lying excited states within the folded manifold (N′), some $^{1}H-^{15}N$ cross-peaks are preferentially lost with increasing pressure, showing local conformational disorder. Analysis of the cross-peak intensi-

ties as a function of pressure indicates that the protein has large-amplitude conformational fluctuations, represented by disorder in either side of the β-barrel, i.e., the hydrophobic core side (βF−βH) and the noncore side (βB−βE), producing intermediates I_1 and I_2. They are higher-energy conformers with ΔG_0 values of 6.5 ± 2.0 and 4.6 ± 1.3 kcal/mol and ΔV_0 values of −90.0 ± 35.2 and −57.4 ± 14.4 mL/mol, respectively. Altogether, the variable-pressure NMR experiment revealed that β-lactoglobulin has large-amplitude fluctuations to N′, I_1, I_2, and the totally unfolded conformer U. Fluctuations to I_1 and I_2 with partially disordered barrel structures are considered necessary for small hydrophobic ligands such as palmitic acid to enter into the central cavity of the β-barrel to be transported.

Locally Disordered Conformer of Prion Protein. A Crucial Intermediate to PrP^{Sc} (56)? Conformational pathologies such as prion disease and various amyloid diseases occur as a result of accumulation of respective protein molecules in peculiar non-native structures. The reaction should inevitably involve a fluctuation of the protein into some non-native form, which is reactive enough to form final aggregates. The infectious form of prion protein, PrP^{Sc}, is formed, through a post-translational process, from the normal, cellular isoform PrP^{C} in some form of aggregates rich in β-structure. The formation of PrP^{Sc} requires the substrate PrP^{C} to bind to the product PrP^{Sc} at an intermediate stage in the conversion process. Therefore, knowledge about the structure of the metastable intermediate of PrP^{C} and the conformational equilibrium that is involved are critical for understanding the mechanism of PrP^{C}−PrP^{Sc} transformation.

By carrying out ^{15}N−^{1}H two-dimensional NMR measurements under variable pressure and temperature on Syrian hamster prion protein rPrP(90−231), Kuwata et al. found a metastable intermediate conformer of PrP^{C} (PrP^{I}) in which helices B and C are preferentially disordered (*56*). The intermediate PrP^{I} coexists with normal PrP^{C} at a population of ∼1% under a closely physiological condition (pH 5.2 and 30 °C). The structural characteristics of this metastable intermediate PrP^{I} having disorder in helices B and C and being more reactive than PrP^{C} are consistent with available immunological and pathological information about the prion protein, and it is likely to be a crucial intermediate to PrP^{Sc}. Elucidation of the more detailed structure of PrP^{I} and how it leads to PrP^{Sc} is an interesting question to be followed.

Preferential Disorder in the Interactive Domain of Ubiquitin (57, 58). Ubiquitin is a small (8565 Da) protein present in all eukaryotes and has crucial roles in many important cell functions, its primary role being to control ATP-dependent proteolysis of damaged proteins. Despite a general understanding that ubiquitin is a uniquely folded stable protein, variable-pressure ^{15}N−^{1}H two-dimensional NMR from 1 to 3700 bar has revealed that it fluctuates rather extensively involving four major conformers (N_1, N_2, I, and U) at pH 4.5 at 0 °C (*58*). The rate of fluctuation between N_1 and N_2 is rapid ($\gg 10^3$ s^{-1}) on the NMR time scale, and the rates of fluctuation involving the rest of the species are slow ($\ll 10^3$ s^{-1}). From the analysis of chemical shifts and cross-peak intensities as a function of pressure, the average structures of the N_1, N_2, I, and U conformers are schematically depicted on a hypothetical energy landscape of ubiquitin in Figure 6 with red depicting disordered segments and pink depicting distortion; the distinction of proline isomers into

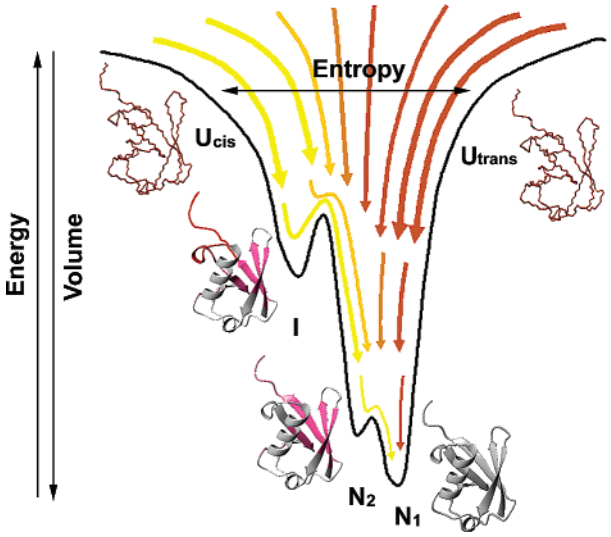

FIGURE 6: Schematic representation of the energy landscape of folding for ubiquitin, based on the results of variable-pressure NMR (*58*) and a kinetic folding experiment (*68*). Here, the vertical axis of the landscape (representing the conformational order) is determined by the internal energy of the protein plus the hydration free energy, whereas the horizontal axis represents the conformational entropy of the polypeptide chain. According to the volume theorem, the vertical axis is represented effectively by the partial molar volume. The conformers detected by variable-pressure NMR are arranged in the following order of decreasing conformational order as well as in partial molar volume: $N_1 > N_2 > I > U$. Ribbon model (gray) for the folded part, ribbon model (purple) for the distorted part, and wire model (red) for the unfolded part. Arrows indicate folding pathways: one directly from U_{trans} to N_1 and the other from U_{cis} to N_1 via a *cis*-proline-trapped transiently populated intermediate (*68*), closely identical to I.

U_{cis} and U_{trans} was made by referring to an earlier kinetic study by Briggs and Roder (*68*). The partial molar volume decreases in the following order: $N_1 > N_2 > I > U$ (ΔV of −24, −58, and −27 mL/mol, respectively, for each step). The parallelism between the conformational order ($N_1 > N_2 > I > U$) and the partial molar volume ($N_1 > N_2 > I > U$) is again clear, supporting the volume theorem.

To provide a signal for the ATP-dependent proteolysis of a damaged protein, the C-terminal carboxyl group of ubiquitin must react with the activating enzymes, with the damaged protein, and with another ubiquitin molecule. It is intriguing to find that the C-terminal domain close to the C-terminal reactive site is partially and fully disordered in conformers N_2 and I, respectively. The disorder in the C-terminal domain surrounding the reactive site would be highly advantageous if ubiquitin were to bind and react with various target proteins. In support of this view, the disordered domain coincides with the enzyme-binding region of ubiquitin, identified from NMR shift perturbation (*69*, *70*).

Variable-Pressure NMR and Energy Landscape for Folding

The above examples of apomyoglobin, β-lactoglobulin, prion, and ubiquitin show that by scanning pressure, one can detect a series of higher-energy conformers of a globular protein from the basic folded conformer even to the fully unfolded within a relatively mild pressure range of a few kilobars. This suggests that the experiment may cover the entire conformational space allowed for a protein. To understand the nature of the variable-pressure NMR experi-

ment in a wider perspective, we consider a generalized conformational space of a protein as expressed by the energy landscape for folding (Figure 6). The vertical axis of the funnel is determined by the solvent-averaged internal (bonding) energy such as that of hydrogen bonding and torsion potentials and the horizontal axis by conformational entropy (*71*). First, the effect of pressure on the energy landscape itself is considered. This is equivalent to considering the effect of general compression (Figure 1A). In general compression, relatively small changes in hydrogen bonding and torsion angles (~1% shortening of O···H distances and ~4° changes in ϕ and ψ angles on average) are found at 2 kbar (*60*), which fall within equilibrium fluctuations at 1 bar [ca. ±20° in ϕ and ψ at ~300 K (*72*)]. It may be assumed, therefore, that the energy landscape is relatively unaffected by pressure.

Then, the major effect of pressure would be the rearrangement of the population by a shift of conformational equilibria (Figure 1B); namely, pressure increases the population of higher-energy conformers according to eq 2 with the efficiency depending on $p\Delta V_0$. According to the volume theorem, the vertical axis of the funnel representing the conformational order is parallel to the partial molar volume of a protein. Therefore, the vertical axis may also be expressed effectively with a partial molar volume, as shown, though not to scale. As the pressure is increased, the major population moves upward on the funnel driven by the term $p\Delta V_0$ in eq 3 until the population reaches the top of the funnel. By choosing an appropriate pressure, we may trap an intermediate conformer stably in a local free energy minimum and examine its structure in detail using multidimensional NMR techniques. The NOE-based structure determination protocol, well established (*73*) for proteins at 1 bar, can be employed for the structure determination at high pressure, along with the protocol using the chemical shift constraints (*60*). The application of the former method is only limited by low signal-to-noise ratios, which are anticipated to be improved in the near future.

How Similar Is the Structure Determined at High Pressure to the Structure at 1 bar?

Higher-energy conformers are rare at 1 bar, but their populations can be increased by applying pressure. In DHFR, the experiment verified that the open conformation is also present at 1 bar. However, are the structures the "same"? How similar is the structure determined at high pressure to the structure at 1 bar?

To answer this question, one must first redefine what is meant by the word "structure". The conventional NMR representation of a structure of a protein gives an average over an ensemble or a subensemble of structures such as the basic folded subensemble. In general compression (Figure 1A), a small shift of population would take place toward a smaller averaged volume among microscopic conformers within the same subensemble, giving chemical shift changes in the NMR spectrum. In some proteins such as BPTI and protein G, these chemical shift changes are surprisingly linear over the range of pressure employed (1−2000 bar). The linearity means that the compressibility is constant over the pressure range, which would be expected only when the microscopic conformers belong to the same subensemble at both pressures, in this case, the basic folded subensemble. In such a case, the two structures are "the same" in the macroscopic sense, any difference in the average conformation at two pressures representing fluctuation within the subensemble. However, the two structures are "different" in the microscopic sense because they are sampling different sets of microscopic conformations. Figure 4 visualizes the overall structural differences of hen lysozyme between 1 bar and 2 kbar, representing fluctuations within the folded subensemble, normally identified as the "native state".

The same argument is expected to apply to any subensemble energetically (as well as in terms of signal) well separated from others, such as conformer I of ubiquitin, for which mixing between subensembles is unlikely with increasing pressure. In case two or more subensembles are energetically close and may mix with increasing pressure, the situation is more complex, as the structure at high pressure may belong to a different subensemble than that at 1 bar. In such a case, individual assessment is required. Whatever the case, pressure would not create a new state, but simply create a new population distribution among the existing conformational microstates.

The Pressure-Stabilized Intermediate Mimics a Kinetic Intermediate

Given that the energy landscape is not significantly altered under mild pressure, the relation of the equilibrium intermediates stabilized by pressure to the kinetic intermediates in the folding reaction at 1 bar also becomes clear on the basis of the common energy landscape for the two experiments (*58*). A kinetic folding process usually starts with the major populations placed at the top of the funnel. Then the folding reaction is simply a process of redistributing the population over the entire funnel until a thermal equilibrium, determined by a factor $\exp(-\Delta G^\circ/RT)$, is reached (Figure 6). During this process, a significant population may be trapped transiently in a local free energy minimum, producing a subensemble of conformers in a local equilibrium within the trap, which may be detected by a method such as pulse-labeling hydrogen−deuterium exchange. The effect of pressure is also to redistribute the population over the entire funnel, but the redistribution is made by ensuring the equilibrium condition given by eq 2. In this equilibrium, a significant population may be trapped stably in the same local free energy minimum described above, producing the same kind of equilibrium subensemble of conformers as that in the kinetic process. If the two experiments are performed on the same energy landscape (i.e., under the same experimental condition), essentially the identical subensemble of conformers should be trapped in the same local free energy minima in the two experiments. Thus, the averaged structure of the conformers in the subensemble determined by the variable-pressure NMR technique will represent closely the transiently trapped subensemble in the kinetic experiment.

In the case of ubiquitin, a *cis*-proline-trapped intermediate is transiently populated (*68*) before thermal equilibration, which is nearly identical in structure with the pressure-stabilized intermediate I. The near identity of pressure-stabilized intermediates and kinetic intermediates has been recognized also in β-lactoglobulin and $p13^{MTCP1}$. The near identity of the kinetic folding core (kinetic intermediates)

and the stable core (stable intermediates) formed at equilibrium of both native and partially folded proteins has been pointed out for a number of proteins by Woodward and her associates using hydrogen−deuterium exchange monitored by NMR (*74*).

Concluding Remarks

One reason variable-pressure NMR spectroscopy is powerful in studying a wide range of conformations of a protein is that it utilizes the volume property of a protein, which is directly coupled to conformation. Specifically, the order of conformation and the partial molar volume of a protein are usually parallel (the volume theorem) and decrease as pressure is increased. For many proteins, it offers a means of searching and detecting higher-energy conformers of a protein from the bottom nearly to the top of the folding funnel, disclosing the highly fluctuating nature of a protein conformation in solution. The biological importance of these higher-energy conformers is that they are more reactive than the basic folded conformer and are often directly related to function or in certain cases to aggregation. The NOE and chemical shift constraints are being used successfully to determine structures of proteins under pressure, disclosing "shape changes" of the protein molecule associated with the fluctuation. In the near future, it may be possible to express the conformational fluctuation of a protein in a series of three-dimensional structures with average coordinates determined by NMR at different pressures, each representing a "snapshot of fluctuation".

ACKNOWLEDGMENT

I would like to acknowledge the collaboration of many international colleagues who contributed to the cited works. In particular, I am grateful to Kunihiko Gekko, Clare Woodward, Fumio Hirata and Hans-Robert Kalbitzer for variable discussions and suggestions and Shigeyuki Yokoyama for his interest and support, and Ryo Kitahara for his help with the figures.

REFERENCES

1. Anfinsen, C. B. (1973) *Science 181*, 223−230.
2. Hammes, G. G. (2002) *Biochemistry 41*, 8221−8228.
3. Pain, R. H. (2000) *Mechanisms of Protein Folding*, Oxford University Press, New York.
4. Dobson, C. M. (1999) *Trends Biochem. Sci. 24*, 329−332.
5. Koshland, D. E., Jr. (1960) *Adv. Enzymol. 22*, 45−97.
6. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88−118.
7. Sawaya, M. R., and Kraut, J. (1997) *Biochemistry 36*, 586−603.
8. Woodward, C. K., Simon, I., and Tuechsen, E. (1982) *Mol. Cell. Biochem. 48*, 135−160.
9. Englander, S. W., and Mayo, L. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 243−265.
10. Kim, K.-S., Fuchs, J., and Woodward, C. (1993) *Biochemistry 32*, 9600−9608.
11. Bai, Y., Sosnick, T. R., Mayne, L., and Englander, S. W. (1995) *Science 269*, 192−197.
12. Roder, H., and Colon, W. (1997) *Curr. Opin. Struct. Biol. 7*, 15−28.
13. Dobson, C. M., Sali, A., and Karplus, M. (1998) *Angew. Chem., Int. Ed. 37*, 868−893.
14. Mulder, F. A. A., Mittermaier, A., Hon, B., Dahlquist, F. W., and Kay, L. E. (2001) *Nat. Struct. Biol. 8*, 932−935.
15. Meiler, J., Peti, W., and Griesinger, C. (2003) *J. Am. Chem. Soc. 125*, 8072−8073.
16. Wazawa, T., Ishii, Y., Funatsu, T., and Yanagida, T. (2000) *Biophys. J. 78*, 1561−1569.
17. Berndt, K. D., Guntert, P., Orbons, L. P. M., and Wuethrich, K. (1992) *J. Mol. Biol. 227*, 757−775.
18. Eliezer, D., Yao, J., Dyson, J., and Wright, P. E. (1998) *Nat. Struct. Biol. 5*, 148−155.
19. Mozhaev, V. V., Heremans, K., Frank, J., Masson, P., and Balny, C. (1996) *Proteins: Struct., Funct., Genet. 24*, 81−91.
20. Frauenfelder, H., Alberding, N. A., Ansari, A., Braunstein, D., Cowen, B. R., Hong, M. K., Iben, I. E. T., Johnson, J. B., Luck, S., Marden, M. C., et al. (1990) *J. Phys. Chem. 94*, 1024−1037.
21. Robinson, C. R., and Sligar, S. G. (1995) *Methods Enzymol. 259*, 395−427.
22. Wagner, G. (1980) *FEBS Lett. 25*, 446−445.
23. Morishima, I. (1987) *Current Perspectives of High Pressure Biology*, pp 325−333, Academic Press, New York.
24. Jonas, J., and Jonas, A. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 287−318.
25. Fuentes, E. J., and Wand, A. J. (1998) *Biochemistry 37*, 9877−9883.
26. Silva, J. L., Foguel, D., and Royer, C. A. (2001) *Trends Biochem. Sci. 26*, 612−618.
27. Urbauer, J. L., Ehnhardt, M. R., Bieber, R. J., Flynn, P. F., and Wand, J. A. (1996) *J. Am. Chem. Soc. 118*, 11329−11330.
28. Yamada, H., Nishikawa, K., Honda, M., Shimura, T., Akasaka, K., and Tabayashi, K. (2001) *Rev. Sci. Instrum. 72*, 1463−1471.
29. Arnold, M. R., Kremer, W., Luedemann, H.-D., and Kalbitzer, H. R. (2002) *Biophys. Chem. 96*, 129−140.
30. Akasaka, K., and Yamada, H. (2001) *Methods Enzymol. 338*, 134−158.
31. Jonas, J., and Jonas, A. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 287−318.
32. Jonas, J., Ballard, L., and Nash, D. (1998) *Biophys. J. 75*, 445−452.
33. Jonas, J. (2002) *Biochim. Biophys. Acta 1595*, 145−159.
34. Akasaka, K. (2003) *Pure Appl. Chem. 75*, 927−936.
35. Cooper, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2740−2741.
36. Gekko, K., and Hasegawa, Y. (1986) *Biochemistry 25*, 6563−6571.
37. Weber, G., and Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89−112.
38. Royer, C. (2002) *Bioochim. Biophys. Acta 1595*, 201−209.
39. Iwadate, M., Asakura, T., Dubovskii, P. V., Yamada, H., Akasaka, K., and Williamson, M. P. (2001) *J. Biomol. NMR 19*, 115−124.
40. Inoue, K., Yamada, H., Imoto, T., and Akasaka, K. (1998) *J. Biomol. NMR 12*, 535−541.
41. Li, H., Yamada, H., and Akasaka, K. (1998) *Biochemistry 37*, 1167−1173.
42. Akasaka, K., Li, H., Yamada, H., Li, R., Thoresen, T., and Woodward, C. K. (1999) *Protein Sci. 8*, 1946−1953.
43. Li, H., Yamada, H., and Akasaka, K. (1999) *Biophys. J. 77*, 2801−2812.
44. Marchi, M., and Akasaka, K. (2001) *J. Phys. Chem. B 105*, 711−714.
45. Li, H., Yamada, H., Akasaka, K., and Gronenborn, A. M. (2000) *J. Biomol. NMR 18*, 207−216.
46. Akasaka, K., Tezuka, T., and Yamada, H. (1997) *J. Mol. Biol. 271*, 671−678.
47. Kamatari, Y. O., Yamada, H., Akasaka, K., Jones, J. A., Dobson, C. M., and Smith, L. J. (2001) *Eur. J. Biochem. 268*, 1782−1793.
48. Kalbitzer, H. R., Goerler, A., Dubowskii, P., Li, H., Hengstenberg, W., Kowolik, C., Yamada, H., and Akasaka, K. (2000) *Protein Sci. 9*, 693−703.
49. Kitahara, R., Sareth, S., Yamada, H., Ohmae, F., Gekko, K., and Akasaka, K. (2000) *Biochemistry 39*, 12789−12795.
50. Inoue, K., Yamada, H., Akasaka, K., Herrmann, C., Kremer, W., Maurer, T., Doeker, R., and Kalbitzer, H. R. (2000) *Nat. Struct. Biol. 7*, 547−550.
51. Inoue, K., Maurer, T., Yamada, H., Herrmann, C., Horn, G., Kalbitzer, H. R., and Akasaka, K. (2001) *FEBS Lett. 506*, 180−184.
52. Kuwata, K., Li, H., Yamada, H., Batt, C. A., Goto, Y., and Akasaka, K. (2001) *J. Mol. Biol. 305*, 1073−1083.
53. Kitahara, R., Yamada, H., Akasaka, K., and Wright, P. E. (2002) *J. Mol. Biol. 320*, 311−319.
54. Kitahara, R., Royer, C., Yamada, H., Boyer, M., Saldana, J. L., Akasaka, K., and Roumestand, C. (2002) *J. Mol. Biol. 329*, 609−628.

55. Lassalle, M. W., Li, H., Yamada, H., Akasaka, K., and Redfield, C. (2003) *Protein Sci. 12*, 66−72.
56. Kuwata, K., Li, H., Yamada, H., Legname, G., Prusiner, S. B., Akasaka, K., and James, T. L. (2002) *Biochemistry 41*, 12277−12283.
57. Kitahara, R., Yamada, H., and Akasaka, K. (2001) *Biochemistry 40*, 13556−13563.
58. Kitahara, R., and Akasaka, K. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 3167−3172.
59. Orekhov, V. Y., Dubovskii, P. V., Arseniev, A. S., Yamada, H., and Akasaka, K. (2000) *J. Biol. NMR 17*, 257−263.
60. Refaee, M., Tezuka, T., Akasaka, K., and Williamson, M. P. (2003) *J. Mol. Biol. 327*, 857−865.
61. Kundrot, C. E., and Richards, F. M. (1987) *J. Mol. Biol. 193*, 157−170.
62. Lumry, R., and Rosenberg, A. (1975) *Col. Int. CNRS L'Eau Syst. Biol. 246*, 55−63.
63. Pain, R. H. (1987) *Nature 326*, 247.
64. Akasaka, K., and Li, H. (2001) *Biochemistry 40*, 8665−8671.
65. Chalikian, T. V. and Breslauer, K. J. (1996) *Biopolymers 39*, 619−626.
66. Lassalle, M. W., Yamada, H., Morii, H., Ogata, K., Sarai, A., and Akasaka, K. (2001) *Proteins: Struct., Funct., Genet. 45*, 96−101.
67. Ragona, L., Fogolari, F., Zetta, L., Perez, D. M., Puyol, P., Kruif, K. E., Loehr, F., Rueterjans, H., and Molinari, H. (2000) *Protein Sci. 9*, 1347−1356.
68. Briggs, M. S., and Roder, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2017−2021.
69. Miura, T., Klaus, W., Gsell, B., Miyamoto, C., and Senn, H. (1999) *J. Mol. Biol. 290*, 213−228.
70. VanDemark, A. P., Hofmann, R. M., Tsui, C., Pickart, C. M., and Wolberger, C. (2001) *Cell 105*, 711−720.
71. Dill, K. A. (1999) *Protein Sci. 8*, 1166−1180.
72. Sareth, S., Li, H., Yamada, H., Woodward, C. K., and Akasaka, K. (2000) *FEBS Lett. 470*, 11−14.
73. Wuethrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley and Sons, New York.
74. Li, R., and Woodward, C. (1999) *Protein Sci. 8*, 1571−1590.

BI034722P