Biochemistry

 Volume 40, Number 30 July 31, 2001

New Concepts

Low-Lying Excited States of Proteins Revealed from Nonlinear Pressure Shifts in ^{1}H and ^{15}N NMR†

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Received February 14, 2001; Revised Manuscript Received May 31, 2001

Proteins are generally thought to fold into a "single" conformer which is thermodynamically most stable under physiological conditions, normally identified as the native structure (*1*). Thanks to the recent advancement in X-ray crystallography and NMR spectroscopy, knowledge of these lowest-energy structures has dramatically improved. Although in solution proteins are known to exist in thermodynamic equilibrium among different conformational states, conformations other than those with the lowest or nearly lowest energy are seldom detected under physiological conditions by spectroscopic techniques. This is largely because they are usually hidden within the overwhelming population of lowest-energy conformers. The only generally useful, though indirect, detection technique of such less-populated conformers has been the hydrogen exchange method (*2*, *3*).

We introduce here a new approach to detection of less-populated conformers of proteins in solution, by combining pressure with multidimensional NMR spectroscopy, namely, the on-line cell, high-pressure NMR technique. This technique combines the site-specific structural information with an external physical parameter, *pressure* (*4*, *5*). Besides compression of the structure of each conformer, the major thermodynamic effect of pressure on proteins in solution is to increase the population of rare conformers by virtue of their smaller partial volumes compared to those of native conformers.

Linear and Nonlinear Pressure Shifts of ^{1}H and ^{15}N Signals

The pressure dependence of ^{1}H chemical shifts in proteins was reported first for heme proteins (*6*). Recently, by utilizing the on-line cell, high-pressure NMR technique at a very high magnetic field (17.6 T), combined with the two-dimensional and multinuclear capability, chemical shifts have become the most sensitive parameter for monitoring subtle structural changes in proteins caused by pressure. The side chain protons of hen lysozyme (*4*) and the amide protons of BPTI[1] (*7*) and gurmarin (*8*) exhibit remarkably linear pressure dependence of chemical shifts between 1 and 2000 bar. Also, ^{15}N and ^{1}H chemical shifts for ^{15}N-labeled BPTI (*9*) and ^{15}N, ^{1}H, and ^{13}C chemical shifts for ^{15}N- and ^{13}C-labeled (doubly labeled) immunoglobulin binding domain of protein G (hereafter called protein G) (*10*) were measured, and essentially all resonances exhibit a linear dependence on pressure. Figure 1A shows an example from BPTI (*9*). Since a change in chemical shift generally corresponds to a change in molecular structure, the linear pressure dependence of the chemical shift means a linear change in protein structure as a function of pressure. This would also mean that compression of the protein volume should be linear with pressure over the studied range (usually 1−2000 bar), and compress-

† This work is supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12480201).

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[1] Abbreviations: BPTI, basic pancreatic trypsin inhibitor; DHFR, dihydrofolate reductase; RalGDS-RBD (or RalFree), ras-binding domain of the guanine-dissociation stimulator of Ral; blgb, β-lactoglobulin; RalCom, RalGDS-RBD−Rap1A complex; HPr, histidine-containing protein.

10.1021/bi010312u CCC: $20.00
Published on Web 07/03/2001

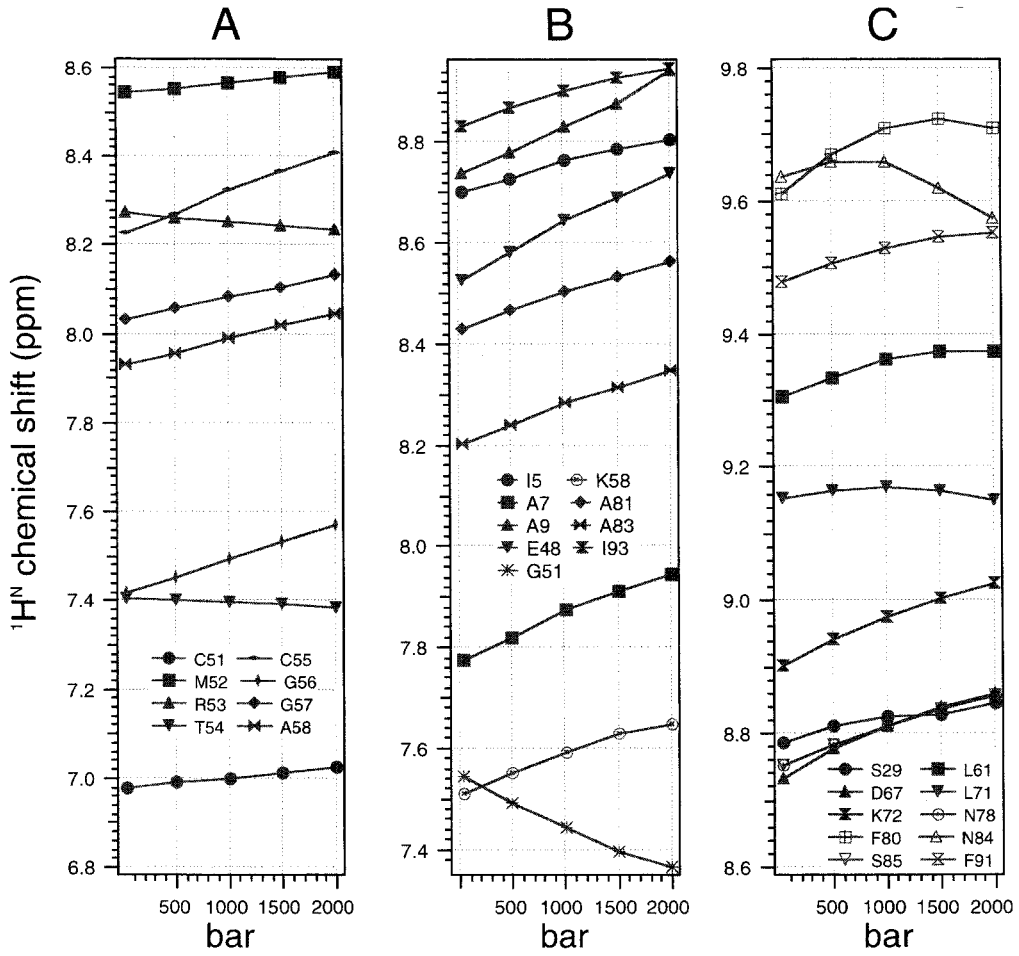

FIGURE 1: Plot of chemical shifts of selected amide protons of BPTI (A) (*9*), DHFR (B) (*16*), and RalGDS-RBD (C) (*15*) as a function of pressure. The experimental conditions were as follows: 2 mM ^{15}N-labeled BPTI, 100 mM acetic acid buffer, pH 4.6, and 36 °C; 1.2 mM ^{15}N-labeled DHFR, 20 mM Tris-HCl buffer, pH 7.0, and 15 °C; and 1.2 mM ^{15}N-labeled RalGDS-RBD, 15 mM Tris-HCl buffer, 150 mM NaCl, 10 mM DTE, pH 7.3, and 25 °C.

ibility should be invariant with pressure for proteins such as BPTI (*7*, *9*).

The origin of amide ^{1}H and ^{15}N pressure shifts has been discussed (*7*, *9*). Both shifts represent site-specific structural changes in the polypeptide backbone. The ^{1}H pressure shift is correlated particularly well with the H···O hydrogen bond distance in NH···O=C groups (*7*, *11*), whereas the ^{15}N pressure shift is more sensitive to variations in main chain torsion angles ψ, ϕ, and probably χ_1 (*9*). Therefore, the dependence of the ^{15}N and ^{1}H chemical shifts on pressure would indicate the linear response of hydrogen bond distance and these torsion angles to pressure. Previous estimates for the average change in the hydrogen bond distance and in ψ for BPTI in an aqueous environment (*9*) and for α-helical melittin in methanol (*12*) are in agreement (on the order of 0.02 Å per 2 kbar for BPTI and a few degrees per 2 kbar for melittin). Comparable degrees of change in ψ, ϕ, and χ_1 angles are reported for crystalline lysozyme (*13*).

More recently, however, we have encountered cases showing distinct nonlinear pressure shifts of amide ^{1}H and ^{15}N signals in proteins such as HPr (*14*), the ras-binding domain of the guanine-dissociation stimulator of Ral (RalGDS-RBD, or RalFree) (*15*), dihydrofolate reductase (DHFR) (*16*), β-lactoglobulin (blgb) (*17*), and the Rap1A complex of RalGDS-RBD (RalCom) (*18*). Examples of nonlinear pressure shifts are shown in panels B and C of Figure 1, for which the plots of amide ^{1}H chemical shifts of DHFR and RalGDS-RBD against pressure are in sharp contrast to those in Figure 1A. It is clear that the degree of nonlinearity varies greatly from protein to protein, and appears to be characteristic for each protein. To explore the generality of the nonlinearity in proteins and possible origins, we quantitatively analyze the nonlinearity and compare the nonlinearities among different proteins. Although experimental conditions such as temperature and pH differ slightly for proteins (see the legend of Figure 3), most of them lie within a pH range between 3.8 and 7.4 (except for blgb at pH 2.0), at a fixed temperature of either 25 or 36 °C.

Analysis of Pressure Shifts

Estimation of linearity and nonlinearity of chemical shift changes with pressure is derived from least-squares fits of experimental data for individual ^{1}H and ^{15}N signals to the following equation:

$$\delta_i = a_i + b_i p + c_i p^2 \quad (1)$$

where p is the pressure (bars), δ_i is the chemical shift (parts per million) for the ith residue, a_i (parts per million) is the chemical shift at 1 bar, and b_i (parts per million per bar) and c_i (parts per million per square bars) are the linear and

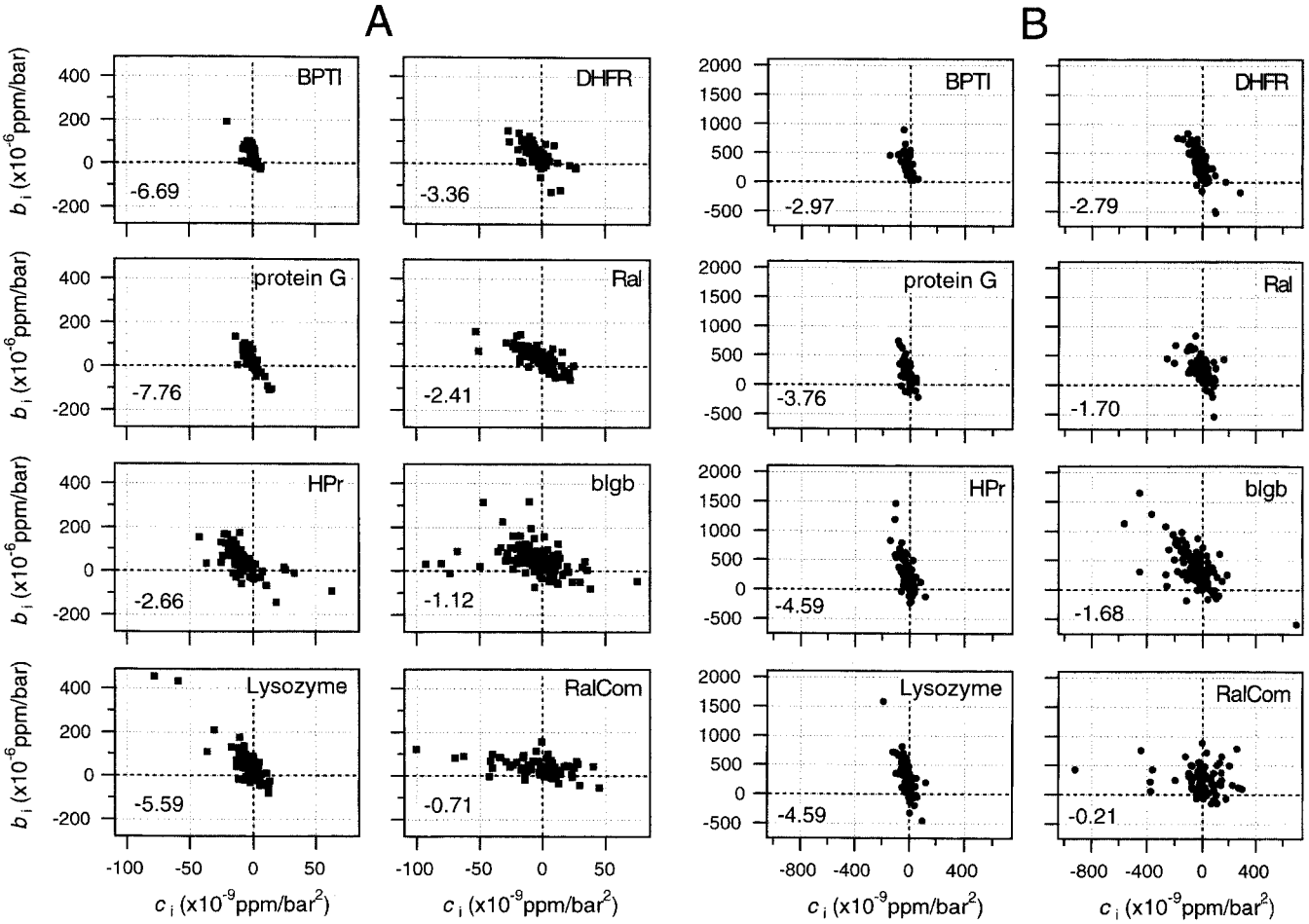

FIGURE 2: Correlation between the second-order coefficient c_i and the first-order coefficient b_i of eq 1 for individual ^{1}HN (A) and ^{15}N (B) signals in eight globular proteins. The experimental conditions were as follows: BPTI (5.8 kDa), 2 mM ^{15}N-labeled BPTI, 100 mM acetic acid buffer, pH 4.6, 36 °C, and 30−2000 bar; protein G (5.6 kDa), 10 mM [^{13}C,^{15}N]immunoglobulin-binding domain of protein G, 100 mM MES buffer, pH 5.6, 25 °C, and 30−2000 bar; lysozyme (12.9 kDa), 1.7 mM ^{15}N-labeled hen lysozyme, 100 mM formic acid buffer, pH 3.8, 25 °C, and 30−2000 bar; HPr (8.8 kDa), 1.5 mM ^{15}N-labeled HPr, 10 mM Tris-HCl buffer, 0.5 mM NaN_3, 0.5 mM *p*-PMSF, 0.5 mM EDTA, pH 7.1, 25 °C, and 30−2000 bar; DHFR (16.2 kDa), 1.2 mM ^{15}N-labeled DHFR, 20 mM Tris-HCl buffer, pH 7.0, 15 °C, and 30−2000 bar; RalFree (8.7 kDa), 1.2 mM ^{15}N-labeled RalGDS-RBD, 15 mM Tris-HCl buffer, 150 mM NaCl, 10 mM DTE, pH 7.3, 25 °C, and 30−2000 bar; β-lactoglobulin (16.1 kDa), 2 mM ^{15}N-labeled β-lactoglobulin, 100 mM maleic acid buffer, pH 2.0, 36 °C, and 30−2000 bar; and RalCom (8.7 + 16.7 = 25.4 kDa), the complex of 1.2 mM ^{15}N-labeled RalGDS-RBD with nonlabaled Rap1A, 15 mM Tris-HCl, 150 mM NaCl, 10 mM DTE, pH 7.3, 25 °C, and 30−2000 bar.

nonlinear (second-order) coefficients, respectively (*17*). The pressure dependence of the shift in the low-pressure range is primarily determined by the b_ip term, while the pressure dependence in the high-pressure range is determined more by the c_ip^2 term. The equation may be compared with the thermodynamic equation showing the dependence of ΔG on the volume change ΔV_0 and the compressibility change Δk (*5*)

$$\Delta G = \Delta G_0 + (\Delta V_0)p - (\Delta k/2)p^2 \qquad (2)$$

where ΔG_0 is the Gibbs energy difference at 1 bar. In the following, individual ^{15}N and ^{1}H shifts of eight uniformly ^{15}N-labeled globular proteins ranging from a small protease inhibitor (BPTI, 58 kDa) to a large molecular complex (RalGDS-RBD−Rap1A complex, 25.4 kDa) are analyzed by eq 1.

First, we find that both the b_i and c_i values vary considerably from site to site within the same protein. The distributions of values of the linear coefficient b_i and nonlinear coefficient c_i for ^{1}H are shown in the vertical scale and in the horizontal scale, respectively, in Figure 2A. In all eight proteins, both the b_i (vertical) and c_i (horizontal) values are distributed over positive and negative ranges, although b_i tends to be positive. The distribution of b_i values does not vary greatly from protein to protein (for example, BPTI and RalCom showing similar distributions of b_i values), but the distribution of c_i values (horizontal) shows a large variation from protein to protein. Also, there is a negative correlation between b_i and c_i values for each protein that was investigated. The number in each figure represents the best-fit slope for the correlation. The slopes vary enormously, from −7.76 for protein G to −0.71 for RalCom, increasing in the following order: protein G < BPTI < lysozyme < DHFR < HPr < RalFree < blgb < RalCom. Similar correlations were found for ^{15}N (Figure 2B). The variation of the slope for ^{15}N is less pronounced, but increases in a similar order: HPr and lysozyme < protein G < BPTI < DHFR < RalFree < blgb < RalCom. The variation of the slopes apparently reflects the fact that the nonlinearity (the distribution in c_i values) varies greatly from protein to protein.

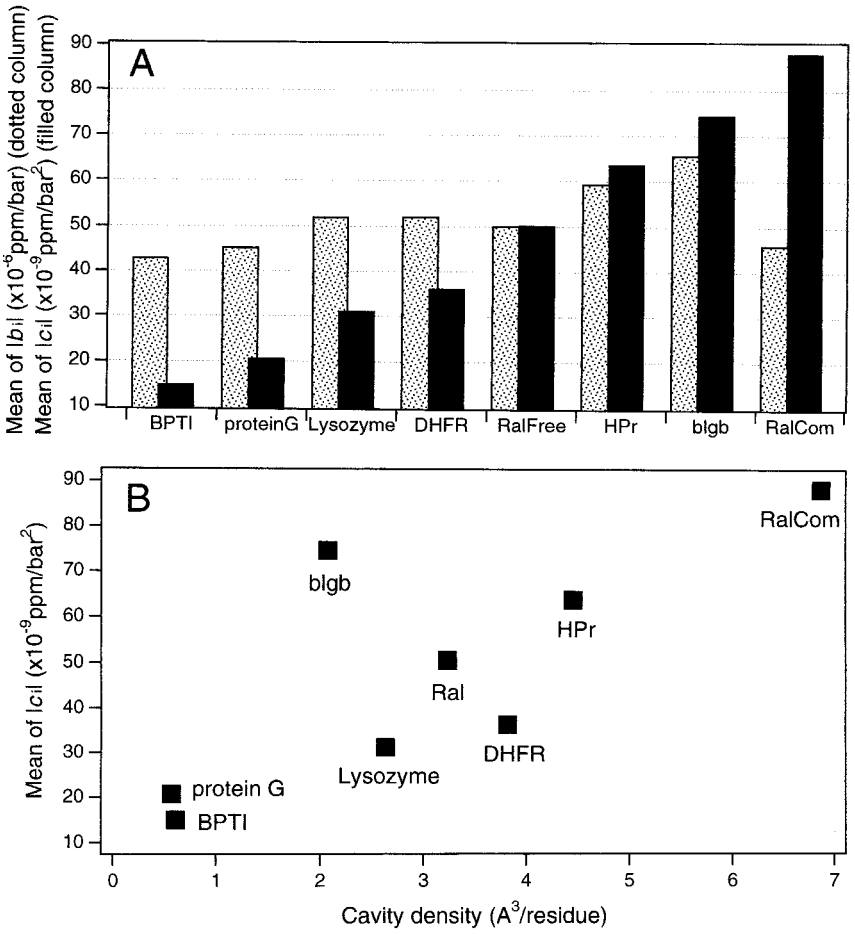

FIGURE 3: (A) Histograms of the mean of the absolute values of the second-order coefficient c_i (filled columns) and the first-order coefficient b_i (dotted columns) for ^{1}HN in eight globular proteins. (B) Plot of the mean of the absolute values of the second-order coefficient c_i of ^{1}HN pressure shifts vs the density of cavities (the total cavity volume divided by the number of amino acid residues) for the eight proteins. Cavity volumes of >20 Å^3 are employed (calculated by GRASP using PDB coordinates).

To compare the magnitude of the distribution of the linear and nonlinear coefficients among different proteins in a more quantitative way, we take the mean *absolute value* of b_i and the mean *absolute value* of c_i to represent the distribution of b_i and c_i values for each protein, respectively, and compare them among different proteins (Figure 3, filled columns and dotted columns, respectively). Interestingly, for both ^{1}H and ^{15}N, the mean absolute value of the nonlinear c_i coefficients varies from one protein to another (increasing in the order BPTI < protein G < lysozyme < DHFR < RalFree < HPr < blgb < RalCom for ^{1}H; Figure 3), whereas the mean absolute value of the linear b_i coefficients varies little for different proteins. A less distinct but similar trend is observed for ^{15}N. Therefore, the expectation from the variation of the slope of the b_i-c_i correlation for different proteins (Figure 2) is confirmed. These results indicate that *the nonlinear pressure response, rather than the linear pressure response*, more sensitively represents the character of each protein. While the linear coefficient b_i represents the response of a protein structure extrapolated to 1 bar, the nonlinear coefficient c_i represents the response of a protein structure at elevated pressures (eq 1). For some proteins such as BPTI and protein G, the response is the same at low and high pressures, but for other proteins such as β-lactoglobulin and RalCom, the response varies enormously at low and high pressures. The phenomenon is new, but general. The fact that the structural response of a protein is so different in the low- and high-pressure ranges and between different proteins in the high-pressure range seems to be a feature of the basic design of protein structure.

Nature of the Linear Pressure Response

When hydrostatic pressure is applied to a protein solution, compression of the protein molecule takes place along with compression of solvent (*19*−*21*). The degree of compression of the protein may be expressed macroscopically by a partial molar quantity known as the compressibility coefficient defined by

$$\beta_T = -(1/V)(\delta V/\delta p)_T \qquad (3)$$

From the adiabatic compressibility obtained from sound velocity measurements, β_T is estimated to be on the order of $\sim 10^{-5}$ bar^{-1} for various globular proteins (*19*). Although β_T contains a contribution from a hydrated layer ($\beta_{T,h}$) in addition to the contribution from the protein molecule itself ($\beta_{T,P}$), an X-ray study (*13*) and an NMR (*4*) investigation of lysozyme under pressure show that the average three-dimensional structure of a folded protein is compressed in a major part of the molecule, meaning $\beta_{T,P}$ is positive.

Before considering the origin of the nonlinear pressure dependence of chemical shifts, we must first understand what the linear pressure dependence or a constant slope in Figure 1A implies. Any change in chemical shift must be a result

of a change in *structure or conformation* that is averaged over the NMR time scale (<1 ms). In the limit where the chemical shift is a linear function of internuclear distance and torsion angles, the linear chemical shift change with pressure is understood to result from a linear change in averaged internuclear distances and torsion angles, the combination of which determines the compressibility coefficient $\beta_{T,P}$ of the protein. The linear pressure shift, therefore, shows that the compressibility coefficient $\beta_{T,P}$ is independent of pressure within the range that was studied (1−2000 bar), and arises from a small shift of population *within* the basic native ensemble (*9*). The fluctuation of the protein volume (δV) in the native ensemble is statistically related to the isothermal compressibility coefficient $\beta_{T,P}$ by the relation

$$\langle(\delta V)^2\rangle = kTV\beta_{T,P} \quad (4)$$

where $\langle(\delta V)^2\rangle$ is the ensemble average of squared volume fluctuation, k the Boltzmann constant, T the absolute temperature, and V the volume of the protein (*22*). The constant compressibility coefficient also means that the volume fluctuation resulting from the fluctuation in internuclear distances and torsion angles is constant versus the variation in pressure.

The observation that the average value of the b_i coefficients varies little for the eight proteins that were investigated (Figure 2 and Figure 3, dotted columns) would, therefore, mean that the magnitude of structural fluctuation (internuclear distances and torsion angles of the main chain) is similar for all these proteins at 1 bar and represents an intrinsic and common property of the basic native ensemble of globular proteins at 1 bar. The native ensemble average is represented by the X-ray crystal structure or the NMR solution structure determined at 1 bar. The result leads to the notion that *the fluctuation of the native ensemble as monitored at the main chain amides is of comparable magnitude for globular proteins, despite their greatly different tertiary folds*.

Nature of the Nonlinear Pressure Response

Pressure-induced shifts for many other proteins are slightly (e.g., lysozyme and DHFR) or markedly (e.g., blgb and RalCom) nonlinear (Figure 1B). The nonlinearity simply means that for these proteins the compressibility coefficient $\beta_{T,P}$ varies with pressure. The remarkable nonlinearity of several of the proteins considered here is not explained by a shift of population within the basic native ensemble (N), but only by the involvement of an ensemble (N′) different from the basic native ensemble.Ensemble N′ not only has a compressibility different from that of basic native ensemble N, giving different slopes of pressure shifts, but also has a partial molar volume smaller than that of N so that its relative population ([N′]/[N]) increases with increasing pressure p according to the relation

$$[N']/[N] = \exp(-\Delta G/RT) \quad (5)$$

and eq 2. Here ΔG denotes the Gibbs energy difference between N and N′. N′ would generally have a slightly more open and hydrated structure than N, as implied by a smaller partial volume, i.e., negative ΔV_0 (*23*−*26*).

The distinct nonlinear pressure shifts over a relatively low-pressure range (500−1000 bar) as seen in panels B and C of Figure 1 indicate that the population of N′ increases significantly with pressure, even at relatively low pressures. This means from eq 2 that the Gibbs energy difference between N and N′ (ΔG_0) is not very large (probably on the order of 1 kcal/mol) or, in other words, that N′ is *a low-lying* excited state of a protein. Furthermore, in general it is likely that N′ involves a family of different conformers, or subensembles, with different ΔG_0 and ΔV_0 values. The finding of nonlinear pressure shifts in most of the proteins studied here suggests that *low-lying excited states are common in many globular proteins, but that their fractional populations vary significantly from protein to protein.*

We may comment on the relationship between the low-lying excited states revealed here from nonlinear pressure shifts and the local unfolding states reported previously for RalFree (*15*) and blgb (*17*) which are revealed from selective loss of signals characteristic of the folded states. In these proteins, the pressure range for nonlinear shifts and that for intensity changes partly overlap. In such a case, one should be aware that the chemical shift analysis may contain contributions from the locally unfolded protein, because the locally unfolded species has a folded part of the polypeptide chain whose signals are indistinguishable (in rapid exchange on the NMR time scale) from those of N′ and N. Actually, however, the energetic distinction between a "low-lying excited state" and a "locally unfolded state" is often obscure. In fact, some of the local unfolding states are extremely low-lying [e.g., ∼1.4 kcal/mol above the native state in RalFree (*15*)]. Thus, even when the nonlinear chemical shift has mixed contributions from the folded proteins as well as from the locally unfolded ones, the nonlinearity should still be taken to represent properties of low-lying excited states.

Recently, Kharakoz (*27*) discussed mechanisms for nonlinearity in the mechanical response of protein structure, but did not consider low-lying excited states as its origin. Up to the present, the existence of equilibrium low-lying excited states in proteins has seldom been recognized experimentally, though often assumed in explaining biological functions of proteins, or inferred indirectly. This is because most physicochemical studies of proteins are carried out at 1 bar, where the population of an excited conformer is below the detection level of most spectroscopic methods ([N′]/[N] < 0.1 in eq 5). However, when [N′]/[N] < 0.1 in eq 5, ΔG_0 > 1.4 kcal/mol at 300 K, a very small energy difference. So far, only the time accumulation techniques such as hydrogen exchange experiments have generally been used to overcome this low-intensity barrier. Previously, Baxter et al. (*28*) observed the nonlinear dependence of ^{1}HN chemical shifts on temperature in BPTI, hen lysozyme, and the N-terminal domain of phosphoglycerate kinase, and attributed it to the improving access to low-lying excited conformational states at higher temperatures. Unlike the pressure dependence reported in the work presented here, however, they found strong nonlinearity in many of the ^{1}HN chemical shifts of BPTI, clearly showing that the origin of nonlinearity is different between the two cases.

Significance and Origin of Low-Lying Excited States in Protein Function

The large variation in the occurrence of excited conformers among different proteins suggests that their roles may vary

according to protein function. Some proteins have very low excited states, while others (such as BPTI and protein G) have much higher excited states. This poses an interesting question with respect to their significance in protein function and to their common origin in protein structure.

The increasing order of nonlinearity of proton shifts (BPTI < protein G < lysozyme < DHFR < RalFree < HPr < blgb < RalCom) likely indicates the trend of decreasing ΔG_0. It is intriguing in this respect that BPTI, a serine protease inhibitor, shows the smallest nonlinear response to pressure, implying the practical absence of low-lying excited states or very high ΔG_0. A serine protease inhibitor is commonly designed to be highly resistant to protease digestion by suppressing conformational flexibility to an extremely low level. Low conformational mobility of the folded state is consistent with the very slow folded state hydrogen exchange rates observed for BPTI (*29*) and for the *Streptomyces* subtilisin inhibitor (*30*). Thus, the absence of a significant population of low-lying excited states is a prerequisite for a potent proteinaceous protease inhibitor.

In contrast, significant nonlinearity is detected in an enzyme lysozyme which hydrolyzes polysaccharides, in the interdomain region where the substrate binds (*31*), suggesting that N′ is involved in the enzymatic reaction. The nonlinearity increases further in enzymes DHFR and HPr. The former is thought to undergo a series of conformational changes for performing reduction of dihydrofolate with the aid of a cofactor (*32*), thereby requiring low-lying N′ for function, and the latter must undergo conformational changes in accepting and releasing a phosphate during its functional cycle (*33*). A further increase in nonlinearity is found in RalGDS-RBD, which transfers signals from Ras-like proteins to Ral (*34*). β-Lactoglobulin has an ability to bind and transport various hydrophobic compounds such as retinol in milk, which would be facilitated by a conformational change from N to N′ (*17*). In short, in the examples shown in Figure 3, it appears that proteins with different functions require low-lying excited state at different ΔG_0 levels.

From the above consideration, it is probable that the levels of low-lying excited state conformers are evolutionarily controlled for each protein. The structure of N′ is illustrated qualitatively, on an individual residue basis, by mapping the nonlinear pressure shifts onto the three-dimensional structure of the native structure N (*17*). This representation indicates where the difference in structure (hydrogen bond distances and backbone torsion angles) takes place in the transition from N to N′. The localized nature of the nonlinear shifts is easily recognized by the small number of spots showing highly deviated c_i values in Figure 2. The location and the extent of such conformational changes are quite characteristic for each protein, and represent different functional roles in a variety of proteins (*15*−*17*, *31*).

Origin of Nonlinearity and Low-Lying Excited States

Examination of Figure 2 reveals that a negative correlation commonly exists between c_i and b_i values for each protein. The negative correlation arises because increasing pressure *suppresses* the pressure-dependent shift, giving positive curvatures for initially (at 1 bar) decreasing chemical shifts and negative curvatures for initially (at 1 bar) increasing chemical shifts. The generality of this trend suggests a common mechanism for the nonlinear pressure shifts of most proteins. Suppression of the pressure-dependent shift with increasing pressure means that the efficiency of pressure decreases with pressure or is even reversed for some signals as seen in Figure 1C.

What could be the common mechanism causing the loss of compression efficiency with pressure for all the proteins that were examined? The fact that the N′ state has a lower partial volume than the N state suggests that the N′ state is likely to be more hydrated than N. In support of this view, the nonlinear shift is observed for residues close to water-accessible cavities within a folded protein structure in hen lysozyme (*31*), RalGDS-RBD (*15*), and β-lactoglobulin (*17*). These findings suggest that the nonlinear shift is related to the hydration of water-accessible cavities. The native structures of globular proteins usually have some internal water molecules even at 1 bar. They go rapidly in and out of the protein interior on the NMR time scale (*35*). Mobile defects and water penetration have been discussed for many years (*36*, *37*), but at 1 bar, the effect has been too small to be detected directly by spectroscopic methods. The number of internal water molecules in equilibrium with the bulk water is expected to increase at high pressures (*38*). It is likely that at a relatively low pressure this would occur first for water-accessible cavities and then extend into other regions of the protein at higher pressures. This would increase the number of conformers around cavities and work against a characteristic dispersion of chemical shifts, resulting in the suppression of the positive and negative pressure shifts.

In support of this hypothesis, a good correlation is found between the mean of the absolute c_i values for amide protons (given by the filled columns of Figure 3A) and the density of cavities (the total volume of cavities divided by the number of amino acid residues) in the native ensemble of these proteins (Figure 3B). Though the nonlinearity seems to be exceptionally high for a β-barrel protein (blgb), a clear tendency is that the larger the cavity density, the larger the nonlinearity. It is likely that the low-lying excited state does not correspond to a single structure but spans a continuous range of structures with different hydration numbers and locations. When the hydration becomes substantial, it will collapse into local or total unfolding, making a part of or the whole the polypeptide segment hydrated and exposed, as is the case for RalGDS-RBD (*15*) and β-lactoglobulin (*17*).

Conclusion

The pressure dependence of NMR chemical shifts (^{1}H, ^{15}N, and ^{13}C) is a sensitive measure of the nonlinear pressure response of a protein structure, which is highly characteristic for each protein. The nonlinearity arises because low-lying excited states are mixed into the native ensemble with increasing pressure. The low-lying excited states are usually hidden at 1 bar because their populations are low. However, they are important in determining the dynamic nature of a protein, which can be essential for its function. The analysis of the nonlinear pressure shifts in a multidimensional NMR spectrum reveals site-specific conformational changes in going from the native state to the low-lying excited states.

ACKNOWLEDGMENT

We express our hearty gratitude to all our collaborators who have contributed to the experimental data on which this

work is based. Our special thanks go to Professors H.-R. Kalbitzer and K. Gekko for their stimulating discussions.

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BI010312U