Pressure Denaturation of Proteins: Evaluation of Compressibility Effects†

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ABSTRACT: One of the key pieces of information from pressure denaturation experiments is the standard volume change for unfolding ($\Delta V°$). The pressure dependence of the volume change, the standard compressibility change ($\Delta K°_T$), is typically assumed to be zero in the analysis of these experiments. We show here that this assumption can be incorrect and that the neglect of compressibility differences can skew the interpretation of experimental results. Analysis of experimental, variable-pressure NMR data for bovine pancreatic ribonuclease A in ^2H_2O at pH* 2.0 and 295 K yielded the following statistically significant, non-zero values: $\Delta K°_T = 0.015 \pm 0.002$ mL mol^{-1} bar^{-1}, $\Delta V° = -21 \pm 2$ mL mol^{-1}, and $\Delta G° = 2.8 \pm 0.3$ kcal mol^{-1}. The experimental protein stability is in good agreement with one ($\Delta G° = 2.5$ kcal mol^{-1}) determined independently for the same protein by calorimetry at atmospheric pressure under equivalent conditions [Makhatadze, G. I., Clore, G. M., and Gronenborn, A. M. (1995) *Nat. Struct. Biol. 2*, 852−855]. The positive value for $\Delta K°_T$ indicates that the denatured form of ribonuclease A is more compressible than the native form; this is explained in terms of an interplay between the intrinsic compressibility of the protein and solvation effects. When the same data were fitted to a model that assumes a zero compressibility change, the $\Delta G°$ value of 4.0 ± 0.1 kcal mol^{-1} returned by the model no longer agreed with the independent measurement, and the $\Delta V°$ returned by the model was a very different -59 ± 1 mL mol^{-1}. By contrast, it was not possible to carry out a similar thermodynamic analysis of fluorescence spectroscopic data for the denaturation of staphylococcal nuclease to yield well-defined values of $\Delta G°$, $\Delta V°$, and $\Delta K°_T$. This limitation was shown by evaluation of synthetic data to be intrinsic to spectroscopic data whose analysis requires fitting of the plateaus at either side of the transition. Because NMR data do not have this requirement, they can be analyzed more rigorously.

Spectroscopy at variable pressure is a powerful approach for studying the volumetric characteristics of proteins in different states. For example, the observation that proteins unfold at high pressures demonstrates that the partial molar volume of the unfolded protein is smaller than that of the folded protein. The magnitude of the standard volume change for unfolding provides unique insight into packing and hydration differences between folded and unfolded proteins. Recent empirical studies suggest that the volume change results from compensation between packing and hydration effects (*1*, *2*). It is known that folded proteins, although well packed, are compressible (*3*). Thus, higher-order pressure effects, such as the standard compressibility change for unfolding ($\Delta K°_T$)[1] may be needed to characterize the unfolding reaction. Typically, however, pressure denaturation experiments are analyzed under the assumption that the partial compressibilities of the folded and unfolded states are equivalent (*4*−*8*). Because pressure is becoming more widely used as a variable for studying protein denaturation, it is important that the validity and consequences of this assumption be thoroughly assessed.

We used complementary approaches to investigate compressibility changes associated with protein denaturation. To examine the effect of $\Delta K°_T$ on the analysis of pressure data, synthetic spectroscopic data sets were generated for a two-state process with spectroscopic changes and $\Delta G°$ and $\Delta V°$ values typical for those observed in pressure denaturation of proteins, but with $\Delta K°_T$ at various non-zero values. These data sets were fitted to a typical model that assumes a compressibility change of zero. In this manner, it was possible to determine the sensitivity of component parameters to the compressibility change and hence the plausibility of evaluating $\Delta K°_T$ from experimentally determined data. To explore the evaluation of $\Delta K°_T$ from experimental data, the pressure denaturation of staphylococcal nuclease was monitored by fluorescence spectroscopy, and the pressure denaturation of ribonuclease A was monitored by NMR spectroscopy. The resulting data from each were fitted to two models, one that assumes a compressibility change of zero and a second that includes the compressibility change as an adjustable parameter. The results from these two approaches indicated that high-quality spectroscopic data are required for evaluating whether a compressibility change exists and

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[1] Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; pH*, pH uncorrected for the deuterium isotope effect; K_{obs}, observed equilibrium constant; I, signal intensity; I_N, native protein signal intensity; I_D, denatured protein signal intensity; K_S, adiabatic (isentropic compressibility); K_T, isothermal compressibility; K_P, intrinsic protein compressibility; K_W, contribution to K_T from protein−water interactions.

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that the neglect of even small compressibility changes can lead to large errors in the free energy and volume changes derived from a pressure denaturation experiment.

EXPERIMENTAL PROCEDURES

Production and Purification of Protein Samples. Recombinant staphylococcal nuclease (SNase) and bovine pancreatic ribonuclease A (RNase A) were overproduced in *Escherichia coli* BL21(DE3) cells and purified as described previously (*9*, *10*). Staphylococcal nuclease samples for fluorescence spectroscopy were dissolved to a concentration of approximately 50 μM in 20 mM bis-Tris at pH 6.0. RNase A samples for NMR spectroscopy were dissolved in ^2H_2O, and the pH was set to 3.0 with a small amount of deuterium chloride. The samples were incubated at 37 °C for 3 h to exchange labile protons and then lyophilized. After lyophilization, the sample was dissolved in deuterium oxide containing 20 mM maleic acid to a concentration of 1 mM. DSS was added to a concentration of 0.1 mM as a chemical shift reference. Finally, the pH* was set to 2.0 by adding small aliquots of deuterium chloride.

The ionization reaction volumes of both buffers used in this study are relatively small, and hence, their pK_a's are insensitive to pressure. For example, $\Delta V°_{\text{ioniz}}$ for maleic acid is -5.1 mL mol^{-1} (*11*). Assuming that the ionization reaction volume is independent of pressure, the pH changes only by -0.27 unit over 4000 bar. However, it is important to note that, as K_w is pressure-dependent (*11*), holding pH constant by using buffers that have small ionization volumes necessarily causes the pOH to become a function of pressure. Under the conditions used here, the pOH changes by $+1.6$ units from 1 to 4000 bar.

High-Pressure Fluorescence. Fluorescence experiments from 1 to 2500 bar were performed as described previously (*7*).

High-Pressure NMR Spectroscopy. ^{1}H NMR spectra of ribonuclease A were collected as a function of pressure between 1 and 4200 bar using a custom probe operating in a wide bore Bruker DMX400 instrument. The design and construction of this probe will be described elsewhere (E. S. Mooberry, K. E. Prehoda, and J. L. Markley, manuscript in preparation). The protein sample (700 μL) was loaded into a 5.9 mm outside diameter NMR tube with medium wall thickness (Wilmad), and the sample was covered by a movable Vespel piston. Pressure was generated and maintained by a computer-controlled pressurizing system (model APP APCS-60K-1, Advanced Pressure Products, Ithaca, NY). The pressure was transmitted to the sample using Fluorinert (FC-40, 3M Specialty Chemicals, Minneapolis, MN), an electronics cleaning fluid consisting primarily of fluorocarbons, as the pressurizing fluid. The temperature of the sample was maintained within ± 0.1 °C by circulating water from a thermostated bath through copper tubing surrounding the high-pressure vessel. The temperature of the sample was monitored using a thermocouple mounted in the high-pressure vessel. At each pressure, 32−1024 transients were collected with a 3 s relaxation recovery period. The program FELIX95 (Biosym, San Diego, CA) was used for processing the NMR data and for fitting the signals to Lorentzian line shapes for quantification. An exponential window function of 3 Hz was applied to the data before Fourier transformation. At each pressure, the total peak area from the histidine signals was summed, and the individual signals were normalized to this value. The partially overlapping signals from histidines 105 and 119 were summed and treated as a single peak.

Pressure Denaturation Data Analysis. The program PRO FIT (Cherwell Scientific) was employed in fitting the fluorescence and NMR data to the equations given below. Confidence intervals of 68.3% were estimated from a Monte Carlo method that simulates a large number of fits with a series of synthetic data sets when multiple data sets were not taken. The value of the ideal gas constant, R, used was 83.1441 mL bar mol^{-1} K^{-1} (*12*); the difference between this value and a rounded value for the gas constant used previously (*8*) may account for the slightly different parameters for protein denaturation reported here. Equations for fitting pressure denaturation data were derived from eq 1, which describes the response of the standard free energy of the system to pressure:

$$\int_{P_0}^{P_1} \Delta V° \, dP = \int_{P_0}^{P_1} \left(\frac{\partial \Delta G°}{\partial P} \right)_{T,\text{pH}} dP \qquad (1)$$

For the model assuming a $\Delta K°$ of 0, the data were fitted to eq 2:

$$\Delta V°P_{P_1} - \Delta V°P_{P_0} = \Delta G°_{P_1} - \Delta G°_{P_0} \qquad (2)$$

To investigate the dependence of the volume change on pressure, the isothermal compressibility change, $\Delta K°_T$ [$K° = -(\partial V°/\partial P)_T$], is introduced into the model:

$$\Delta V°P_{P_1} + {}^1/_2\Delta K°P_{P_1}{}^2 - \Delta V°P_{P_0} - {}^1/_2\Delta K°P_{P_0}{}^2 = \Delta G°_{P_1} - \Delta G°_{P_0} \qquad (3)$$

It is important to note that the isothermal compressibility used here is not the same as the isentropic compressibility evaluated from techniques that measure the sound velocity in solution. The relationship between the compressibilities discussed here and those measured by acoustic techniques is discussed below.

The pressure dependence of the standard free energy change was determined from the observed equilibrium constant, K_{obs}, calculated from the relationship

$$K_{\text{obs}} = \frac{I_N - I}{I - I_D} \qquad (4)$$

where I_N and I_D are the intrinsic signal intensities of native and denatured protein, respectively, and I is the signal intensity at a discrete pressure. When the intrinsic signal intensities (plateaus) are not known (as with fluorescence, UV/vis, IR, etc.), the two parameters must be included as adjustable parameters in the fit. Therefore, the analysis of pressure data in which the plateau values are adjustable requires a four-parameter fit when assuming a compressibility change of zero and a five-parameter fit when $\Delta K°_T$ is included in the analysis. For a process that is slow on the NMR chemical shift time scale, the quantities I_N and I_D, corresponding to signals from one or more nuclei in a particular state, have well-defined values. For example, at any stage of the pressure denaturation for the native state,

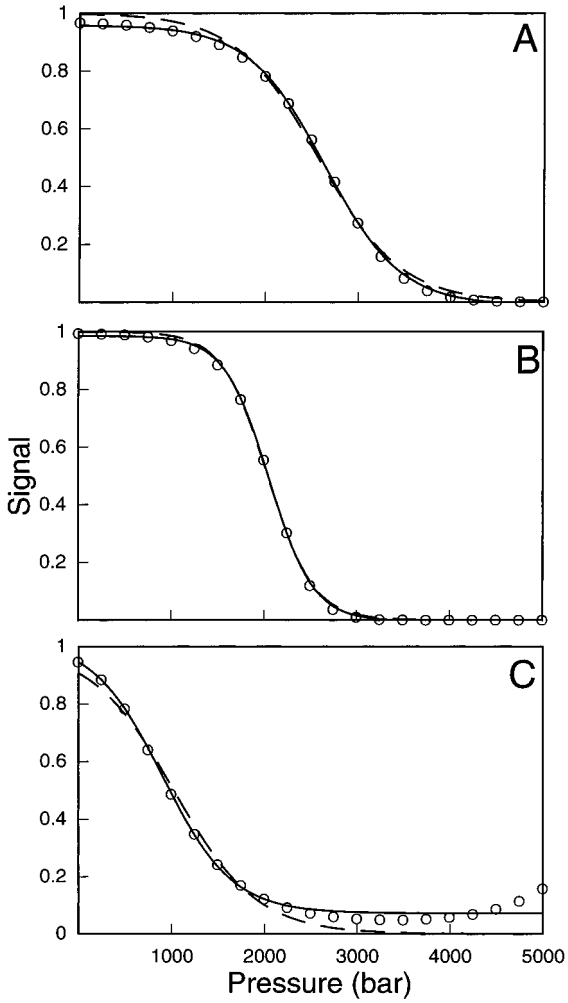

FIGURE 1: Synthetic data sets (○) incorporating different sets of thermodynamic parameters with non-zero compressibilities (listed below; all at $T = 298$ K) were fitted to models that assumed a zero compressibility change to determine if the oversimplification could be detected. Solid lines (—) represent results for four-parameter fits (as needed when spectral plateaus are fitted) with values returned by the model listed below; dashed lines (– – –) represent results for two-parameter fits (as possible with NMR data). (A) Synthetic data: $\Delta G^\circ_{1\,bar} = 2$ kcal mol^{-1}, $\Delta V^\circ_{1\,bar} = -6$ mL mol^{-1}, and $\Delta K^\circ_T = 0.02$ mL mol^{-1} bar^{-1}. Four-parameter results: $\Delta G^\circ_{1\,bar} = 3.8$ kcal mol^{-1}, and $\Delta V^\circ_{1\,bar} = -60$ mL mol^{-1}. (B) Synthetic data: $\Delta G^\circ_{1\,bar} = 3$ kcal mol^{-1}, $\Delta V^\circ_{1\,bar} = -20$ mL mol^{-1}, and $\Delta K^\circ_T = 0.04$ mL mol^{-1} bar^{-1}. Four-parameter results: $\Delta G^\circ_{1\,bar} = 5$ kcal mol^{-1}, and $\Delta V^\circ_{1\,bar} = -103$ mL mol^{-1}. (C) Synthetic data: $\Delta G^\circ_{1\,bar} = 1.7$ kcal mol^{-1}, $\Delta V^\circ_{1\,bar} = -85$ mL mol^{-1}, and $\Delta K^\circ_T = -0.025$ mL mol^{-1} bar^{-1}. Four-parameter results: $\Delta G^\circ_{1\,bar} = 1.4$ kcal mol^{-1}, and $\Delta V^\circ_{1\,bar} = -66$ mL mol^{-1}.

I_D for the native state is zero, whereas I_N for the native state is the sum of intensities of the signals of interest from the native and denatured states. Thus, NMR data have the distinct advantage of requiring fewer adjustable parameters in the fit: a two-parameter fit, when ΔK° is assumed to be negligible, and a three-parameter fit, when ΔK°_T is included in the analysis.

RESULTS

Synthetic Data. To assess the feasibility of evaluating the compressibility change from pressure denaturation data with known properties, we generated synthetic data sets with non-zero compressibility changes and fitted them assuming a zero compressibility change. Some representative examples are shown in Figure 1. In this analysis, a good fit indicates that the model is insensitive to the compressibility change. The four-parameter model (I_N, I_D, $\Delta G^\circ_{1\,bar}$, and $\Delta V^\circ_{1\,bar}$) was found to fit the synthetic data very well (Figure 1, solid lines), despite its neglect of non-zero compressibility inherent in the data; this suggests that attempts to determine the compressibility change for a reaction from data that require a five-parameter fit may be futile. Furthermore, the fitted values for the free energy and volume changes contained very large systematic errors when the compressibility change was neglected. For example, the synthetic data in Figure 1A were derived by assuming values for $\Delta G^\circ_{1\,bar}$ of 4 kcal mol^{-1} and for $\Delta V^\circ_{1\,bar}$ of -60 mL mol^{-1}, whereas fitting to the model gave values for $\Delta G^\circ_{1\,bar}$ of 5 kcal mol^{-1} and for $\Delta V^\circ_{1\,bar}$ of -102 mL mol^{-1}. Only in the case of negative compressibility changes, where renaturation at high pressure would occur (Figure 1C), did the fit from the four-parameter model diverge appreciably from the synthetic data.

When the two-parameter model ($\Delta G^\circ_{1\,bar}$ and $\Delta V^\circ_{1\,bar}$) was used, as is possible with NMR data, the inadequacy of the neglect of ΔK°_T became more apparent, although the magnitude of the discrepancy between the data and the model was found to depend on the specific values of $\Delta G^\circ_{1\,bar}$ and $\Delta V^\circ_{1\,bar}$ used in generating the data (e.g., the data in Figure 1A are fitted less well than those in Figure 1B).

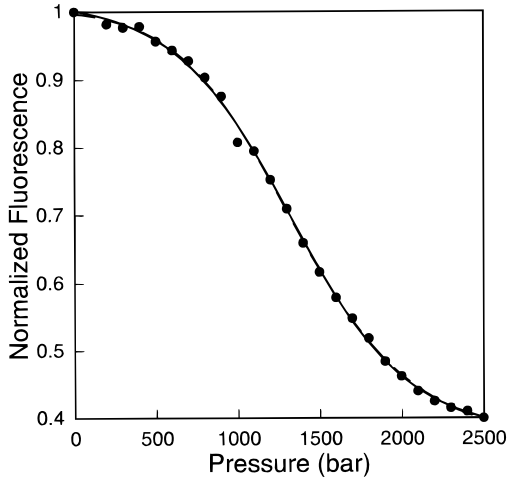

FIGURE 2: Filled circles (●) indicating the experimental intrinsic fluorescence of staphylococcal nuclease measured as a function of pressure. The curves show the best fits to the data by models that (—) assume a zero compressibility change and that (– – –) fit the change in compressibility. Because the two curves coincide within experimental error, it is concluded that the two models cannot be distinguished on the basis of the experimental data.

High-Pressure Fluorescence. Staphylococcal nuclease (SNase) was the protein used for the collection of fluorescence data for this analysis, because SNase is denatured reversibly by high pressure and undergoes a large decrease in intrinsic fluorescence when it unfolds. At 311 K and pH 5.3, SNase denatures over a pressure range of greater than 2500 bar (Figure 2). The pressure dependence of the intrinsic tryptophan fluorescence of SNase was modeled according to eqs 2 and 3 (Table 1). Both models provided fits to the experimental data of roughly equal quality, but the two models returned very different values for $\Delta G^\circ_{1\,bar}$ and $\Delta V^\circ_{1\,bar}$ and slightly different values for the intrinsic signal intensities. Furthermore, the model that includes the compressibility

Table 1: Analysis of Fluorescence Data for the Pressure Denaturation of Staphylococcal Nuclease by Models That Assume a Zero Compressibility Change (eq 2) or Fit the Compressibility Change (eq 3)[a]

parameter	eq 2	eq 3
I_N	1.016 ± 0.006	1.006 ± 0.009
I_D	0.377 ± 0.006	0.35 ± 0.03
$\Delta G°_{1\ bar}$ (kcal mol^{-1})	2.28 ± 0.08	2.6 ± 0.3
$\Delta V°_{1\ bar}$ (mL mol^{-1})	-72 ± 2	-94 ± 19
$\Delta K°$ (mL mol^{-1} bar^{-1})	0	0.02 ± 0.02
χ^2	0.00107	0.00100

[a] $T = 310.9$ K; pH 6.0. Errors are one standard deviation determined from the deviation of the best-fit curve from the experimental data.

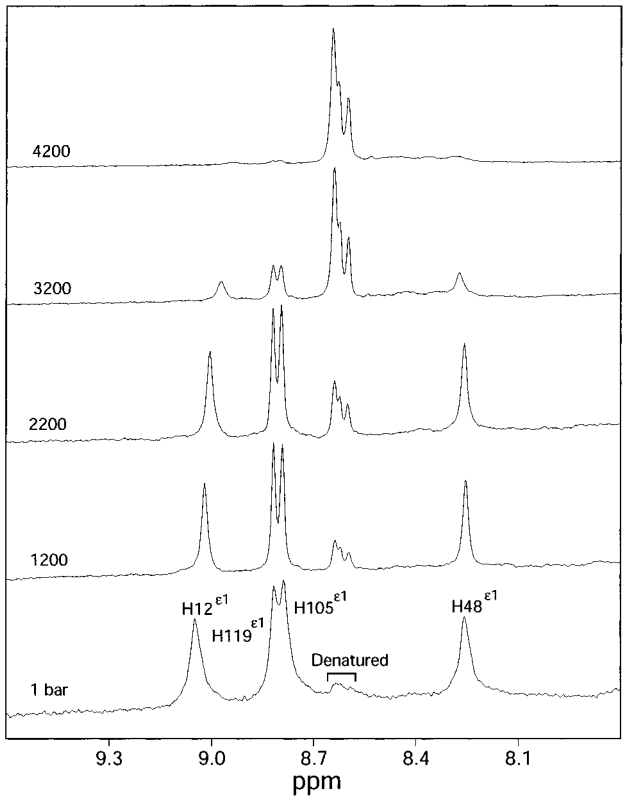

FIGURE 3: Pressure dependence (from 1 to 4200 bar) of the histidine $^1H^{\epsilon 1}$ region of the 400 MHz ^1H NMR spectrum of bovine pancreatic ribonuclease A at pH* 2.0 and 295 K.

change returned parameters with poorer precision. For example, the volume change determined when the compressibility change was assumed to be zero was -72 ± 1 mL mol^{-1}, whereas that determined when the compressibility was fitted was -94 ± 11 mL mol^{-1}. The lower precision results because a wider range of volume changes fit the data with roughly equal values of χ^2. Thus, the analysis of experimental data confirmed what had been concluded from the synthetic data, namely, that a four-parameter model is insensitive to compressibility changes and may give erroneous results if $\Delta K°_T$ is non-zero.

High-Pressure NMR. RNase A was chosen as the source of NMR data on pressure denaturation, because it unfolds reversibly and yields variable-pressure ^1H NMR data of high quality. In addition, the pressure denaturation of RNase A has been investigated by ultraviolet absorption (*4*, *13*, *14*), and a detailed study has been performed on the apparent isentropic compressibilities of native and denatured RNase A (*15*). Figure 3 shows the histidine $^1H^{\epsilon 1}$ region of

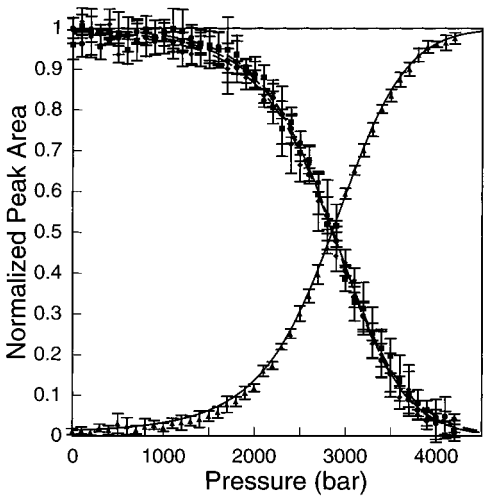

FIGURE 4: Normalized peak areas of the histidine $^1H^{\epsilon 1}$ NMR signals of folded (■) H12, (●) H105 and H119, and (◆) H48 and unfolded (▲) bovine pancreatic ribonuclease A as a function of pressure from 1 to 4200 bar (derived from spectra of the kind shown in Figure 3). The theoretical curves include fitting of the free energy, partial molar volume, and compressibility changes for the reaction. Fitted parameters are shown in Table 2.

Table 2: Analysis of NMR Data for the Pressure Denaturation of Bovine Pancreatic Ribonuclease A by Models That Assume a Zero Compressibility Change (eq 2) or Fit the Compressibility Change (eq 3)[a]

histidine $^1H^{\epsilon 1}$ signal	$\Delta G°_{1\ bar}$ (kcal mol^{-1})	$\Delta V°_{1\ bar}$ (mL mol^{-1})	$\Delta K°_T$ (mL mol^{-1} bar^{-1})	χ^2
$\Delta K°_T$ Fixed at Zero (eq 2)				
H12	4.0 ± 0.1	-58 ± 2	0	0.019
H48	3.9 ± 0.1	-58 ± 2	0	0.027
H119 and H105	4.04 ± 0.08	-59 ± 1	0	0.012
denatured	4.01 ± 0.06	-59 ± 1	0	0.0076
$\Delta K°_T$ Included in the Fit (eq 3)				
H12	3.3 ± 0.3	-37 ± 9	0.008 ± 0.003	0.017
H48	2.5 ± 0.2	-13 ± 6	0.016 ± 0.002	0.015
H119 and H105	2.8 ± 0.1	-20 ± 5	0.014 ± 0.002	0.0058
denatured	2.78 ± 0.07	-21 ± 2	0.014 ± 0.001	0.0014

[a] $T = 295$ K; pH* 2.0. Errors are standard deviations from four separate experiments.

representative ^1H NMR spectra of RNase A at pressures between 1 bar and 4.2 kbar. Although the pressure denaturation of RNase A was proposed to be a multistep process on the basis of a similar analysis of its histidine signals (*4*), our data, which have been obtained at higher sensitivity and NMR field strength and are from four separate experiments, are fully consistent with a two-state process. Specifically, the denaturation profiles derived from individual histidine peaks in the folded state (His12, His48, and His105 and His119) coincide with one another within experimental error (Figure 4). Our analysis thus was carried out under the assumption of a two-state reaction.

The results of fitting the denaturation profiles (Figure 4) to eqs 2 and 3 are shown in Table 2. In these fits, the signal intensities from each histidine in the native and denatured forms of the protein were constrained to their theoretical values. For the native protein, I_N for an individual peak was constrained to the normalized sum of the histidine signals (total peak area divided by 4) and I_D was constrained to zero. For the denatured protein, I_N was constrained to zero and I_D was constrained to the normalized sum of all histidine signals.

In each case, the fit was better (as measured by χ^2) when the compressibility change was included as an adjustable parameter than when it was not (Table 2). Data fitting for all four separate signals yielded positive values for the compressibility change, with the following most probable values for the thermodynamic parameters: $\Delta K°_T = 0.015 \pm 0.002$ mL mol^{-1} bar^{-1}, $\Delta V° = -21 \pm 2$ mL mol^{-1}, and $\Delta G° = 2.8 \pm 0.3$ kcal mol^{-1}.

The validity of fitting the compressibility is reinforced by comparison with an independently measured value for stability of RNase. The range of $\Delta G°_{1\,bar}$ values returned from analyses with a $\Delta K°_T$ of 0 was 3.9−4.0 kcal mol^{-1}, whereas the range with fitted compressibility was 2.5−3.3 kcal mol^{-1} (Table 2). The latter range, but not the former, includes a value for the protein stability determined independently by differential scanning calorimetry under the conditions used here [$\Delta G°_{1\,bar} = 2.5$ kcal mol^{-1} (*16*)].

DISCUSSION

In 1973, Zipp and Kauzmann (*17*) stated that the volume change for protein folding is positive at atmospheric pressure but becomes negative at higher pressures. Their conclusion that proteins exhibit positive compressibility changes upon unfolding was based on results from the pressure denaturation of metmyoglobin. Subsequently, however, the pressure dependence of the volume change for protein denaturation has been ignored, largely because the quality of available data did not support its evaluation. It is of interest to revisit this question now, because pressure is becoming increasingly popular as a tool for investigating protein denaturation, and because in many studies, attempts are being made to measure small differences in volume changes very accurately and to interpret these in terms of structural differences (*7*, *18*, *19*). We have shown here that the neglect of compressibility in reactions with even small compressibility changes leads to significant errors in $\Delta G°_{1\,bar}$ and $\Delta V°_{1\,bar}$. Thus, it is important to evaluate compressibility changes whenever feasible.

Whereas acoustic techniques, which measure the velocity of sound in solution, have been used extensively in recent years to measure the adiabatic (isentropic) compressibility of proteins (*20*−*27*), practical approaches to the measurement of the more relevant and intuitive isothermal compressibility have not been forthcoming. It must be stressed that the isothermal and isentropic compressibilities cannot be used interchangeably and that it is not practical to interconvert the two quantities, because doing so requires accurate values for other thermodynamic terms that are not easily measured (*28*). Consequently, predictions of pressure denaturation experimental results (which are performed at constant temperature) cannot be made from isentropic compressibilities.

As we have shown here, the experimental determination of $\Delta K°_T$ is challenging. The feasibility of determining the compressibility change from data that require the inclusion of spectral plateaus as adjustable parameters appears to be doubtful. Even when the plateaus are not included in the analysis (Figure 4), very high quality data are required. Furthermore, the pressure dependence of $\Delta K°_T$ itself is unknown, although it has been asserted that the isentropic compressibilities are pressure-dependent (*29*).[2] A higher-order effect of this kind would further complicate the evaluation of accurate values of $\Delta G°_{1\,bar}$ and $\Delta V°_{1\,bar}$ from pressure denaturation data.

The physical basis for the partial compressibility change of a solute lies in its intrinsic compressibility and in the interaction of the solute with the solvent (*28*). For the protein denaturation reaction, this is expressed by eq 5

$$\Delta K°_T = \Delta K_P + \Delta K_W \quad (5)$$

where ΔK_P is the change in intrinsic compressibility of the protein upon denaturation and ΔK_W is the change in solvent compressibility for the reaction. On the basis of known properties of proteins and model compounds at high pressure, each of these components can be evaluated at a qualitative level.

Analysis of NMR data for the pressure denaturation of RNase A yielded a statistically significant compressibility change of approximately 0.015 mL mol^{-1} bar^{-1}. This positive value for $\Delta K°_T$ indicates that the change in the partial molar volume ($\Delta V° = V°_u - V°_f$) becomes more negative with increasing pressure. Thus, with increasing pressure, the partial molar volume of the denatured protein system ($V°_u$) must decrease relative to that of the native protein ($V°_f$).

Structural information obtained at variable pressure has provided useful insights into the intrinsic compressibility of proteins. Comparison of the X-ray structure of hen egg white lysozyme determined at 1 kbar with that determined at atmospheric pressure showed that the volume decreased by 44 mL mol^{-1} (*30*). This volume decrease gives a K_T value for the protein of 0.04 ± 0.01 mL mol^{-1} bar^{-1}. Interestingly, the protein did not undergo uniform compression. In fact, certain secondary structure elements were expanded when deformed by high pressure. Because covalent bonds are highly incompressible, a denatured protein is expected to have a very low intrinsic compressibility. Consequently, ΔK_P is expected to make a negative contribution to the observed compressibility change for denaturation.

NMR spectroscopic results lend support to the concept that the folded form of the RNase A molecule is compressible whereas the unfolded form is not. Pressure has a noticeable effect on the chemical shifts of the histidines of native RNase A referenced to DSS but not on those of denatured RNase A (*4*). It is unlikely that these changes result from pressure effects on pK_a values, because the pH of the sample is far from the pK_a values of the histidines themselves, and because only one of the histidines is close to an acidic group. Akasaka and co-workers have found significant pressure effects on ^{1}H NMR chemical shifts of signals from folded hen egg white lysozyme but not from denatured lysozyme (*31*). Their results are consistent with positive compressibility for the folded protein, in that increasing pressure leads to upfield shifts of signals from hydrophobic groups (consistent with closer packing).

The other contribution to the observed compressibility change comes from ΔK_W, the compressibility change of the solvent. Because the experimentally observed compressibility difference determined here is positive, whereas that

[2] It is entirely possible that the isentropic compressibilities of native and denatured proteins are pressure-dependent whereas the isothermal compressibilities are not.

from the protein itself is negative, the compressibility change from solvent must be large and positive (i.e., $|\Delta K_W| > |\Delta K_P|$). Measurements of the pressure dependence of the solubility of liquid hydrocarbons in water (*32*) showed that the partial molar isothermal compressibility of benzene in water is approximately 0.0015 mL mol^{-1} bar^{-1}. As the intrinsic compressibility of benzene is expected to be very small, a large portion of its partial molar compressibility must be due to the solute−solvent interaction. Since the unfolded protein is much more highly solvated than the folded protein, the positive compressibility of the solvation is expected to make a positive contribution to the observed compressibility change (enough to overcome the negative contribution from the protein molecule itself). The backbone amide protons of bovine pancreatic trypsin inhibitor have been found to exhibit chemical shift changes consistent with pressure-induced shortening of hydrogen bonds (*33*). Interestingly, those amides showing the largest changes are those exposed to solvent; thus, these shift changes may reflect compression of the solvation layer.

We conclude that the compressibility change for protein denaturation cannot be automatically assumed to be zero. From an analysis of synthetic data sets, we have found it impractical to quantify $\Delta K°_T$ from pressure denaturation data (such as fluorescence) whose analysis requires the fitting of additional terms from the plateaus of the denaturation profile. In addition, the free energy and volume changes returned from the analysis of such data are extremely sensitive to changes in compressibility, yet their accuracy and precision are compromised whenever the compressibility change is fitted. High-pressure NMR offers a practical approach for the measurements of compressibility changes for the protein denaturation reaction because the plateaus have known, fixed theoretical values. Using this technique, we have shown that $\Delta K°_T$ for the denaturation of RNase A is positive. This result is interpreted in terms of a negative compressibility change for the protein molecule itself coupled with a larger compensating positive compressibility change arising from the associated solvent layer, which becomes more extensive when the protein is denatured and which has an intrinsic positive compressibility.

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