Compactness of Thermally and Chemically Denatured Ribonuclease A As Revealed by Volume and Compressibility

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ABSTRACT: The conformational changes of ribonuclease A due to thermal and guanidine hydrochloride denaturation were monitored by means of precise density and sound velocity measurements. It was found that the apparent molar volume decreased but the adiabatic compressibility increased on thermal denaturation under acidic conditions (pHs 1.60, 1.90, and 2.08). On the other hand, guanidine hydrochloride denaturation (pH 2.00) brought about large decreases in the compressibility and apparent molar volume. These results indicate that the conformation of the denatured protein is greatly different between the two types of denaturation: the thermally denatured state corresponds to the structure with enhanced thermal fluctuation having a residual secondary structure and a high local concentration of nonpolar groups exposed, but the guanidine hydrochloride denaturation leads to exposure of a large amount of amino acid residues, resulting in an increase in hydration and a decrease in the internal cavity. The compressibility changes due to both types of denaturation were not correlated to a loss of the secondary structure, as judged by means of circular dichroism. These findings suggest that the compactness and thermal fluctuation of the protein cannot be described by a two-state denaturation model and that there are some molten-globule-like intermediates in the denaturation processes.

To understand the principles of constructing a three-dimensional protein structure from an amino acid sequence, many studies have been focused on the denatured structure and unfolding−folding pathway. Since the conformation of proteins is most extensively unfolded when they are denatured by guanidine hydrochloride (GuHCl)[1] or urea, these chemically denatured states have been taken as an initial reference for considering the mechanism of folding and the stabilizing forces of the native structure. However, there is much evidence for stable conformations that are not fully folded or fully unfolded under several non-native equilibrium conditions [high ionic strength, extreme pH, organic solvent, and mild denaturant concentration (Kuwajima, 1989)]. The folding pathways for many proteins also have some intermediates which can be observed using rapid measurement techniques such as kinetic circular dichroism (Dolgikh et al., 1981; Ptitsyn, 1987; Kuwajima et al., 1987) and pulsed proton exchange nuclear magnetic resonance (Udgaonkar & Baldwin, 1988). These intermediate states under equilibrium and kinetic conditions have been called "molten globules" (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989) or "compact intermediates" (Creighton, 1990), but the definition and generality remain controversial (Baldwin, 1991). More detailed examination is required of the real features of these intermediates in comparison with the denatured states by heat and pressure, in which there remains a considerable amount of residual structure.

A key parameter for characterizing such a loose conformation is compactness or globularity, but only limited data have been reported on the direct measurement of this quantity during the unfolding or refolding processes. Small-angle X-ray scattering (SAXS), which is a useful technique for this purpose, has provided new information on the dimensions of the thermally and chemically denatured states of small globular proteins such as staphylococcal nuclease (Flanagan et al., 1992), ribonuclease A (Sosnick & Trewhella, 1992), and cytochrome *c* (Kataoka et al., 1993). Another novel technique is compressibility measurement, since it is directly linked to the volume fluctuation (Cooper, 1976) or internal cavity and surface hydration (Gekko & Noguchi, 1979; Sarvazyan, 1991). Although this type of fluctuation is a thermodynamic and macroscopic one, we have confirmed that the adiabatic compressibility sensitively reflects the structural characteristics and functional properties of native globular proteins (Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986, 1989; Gekko & Yamagami, 1991; Tamura et al., 1993). Since the hydrophobicity and helix elements are decisive factors for the compressibility (Gekko & Hasegawa, 1986), it should be a good measure of the modified compactness of globular proteins on denaturation. Compressibility studies have been preliminarily performed to characterize the thermal denaturation of chymotrypsinogen (Kharakoz & Sarvazyan, 1980) and the molten-globule state of cytochrome *c* (Nölting & Sligar, 1993).

In this study, the apparent adiabatic compressibility and specific volume of ribonuclease A (RNase A) were measured as functions of temperature and GuHCl concentration to characterize the thermally and chemically denatured states. RNase A was selected since its denaturation has been well studied and SAXS data have recently been reported (Sosnick & Trewhella, 1992). In order to examine the relationships

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[1] Abbreviations: RNase A, ribonuclease A; GuHCl, guanidine hydrochloride; CD, circular dichroism; SAXS, small-angle X-ray scattering.

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between the unfolding of the secondary structure and the compactness of the global structure, circular dichroism (CD) spectra were also measured. On the basis of the data, the protein structures in the intermediate and denatured states will be discussed in terms of the modified internal cavity and surface hydration.

MATERIALS AND METHODS

Materials. Bovine pancreatic ribonuclease A (RNase A) was purchased from Sigma (type IIA, lot 98F-8130). Ultrapure guanidine hydrochloride (GuHCl) was purchased from Schwarz/Mann Research Laboratories. Other chemicals were special reagent grade products from Wako Pure Chemicals. RNase A was completely deionized by exhaustive dialysis against distilled water at 4 °C for about 48 h. The dialyzed stock solution was diluted to a protein concentration of 6−7 mg/mL for the density and sound velocity measurements, and 0.1 mg/mL for CD measurements. Protein concentrations were determined by absorption measurements using an extinction coefficient of 7.08 dL/(g·cm) at 278 nm (Gekko & Timasheff, 1981) with a Jasco UVIDEC-610C.

For thermal denaturation studies, three sample solutions of different pHs (1.60, 1.90, and 2.08) were prepared by titrating the protein solution against HCl with a pH meter. Aqueous solutions with identical pHs were prepared as reference solvents by the same titration method. For GuHCl denaturation studies, 15−20 sample solutions with different GuHCl concentrations (0−4.5 M) and an identical protein concentration were prepared by mixing a given weight of RNase A and GuHCl solutions on a balance, both solutions being preadjusted to pH 2.00 with HCl. From the weight ratio of the two solutions, the protein and GuHCl concentrations in sample solutions were calculated using the density data for the respective solutions. According to the same procedures, aqueous solutions with various GuHCl concentrations (pH 2.00) were prepared as reference solvents. The sample solutions were incubated for at least 6 h at 25 °C for complete denaturation of the protein. Prior to the sound velocity and density measurements, the sample and reference solutions were centrifuged at 400g for 5 min to eliminate small air-bubbles and dust trapped in them.

Sound Velocity Measurements. The sound velocity in protein solutions was measured, with an accuracy of 0.5 cm/s, by means of the "sing-around pulse method" at 5 MHz. The apparatus was the same as that used in the previous study (Tamura et al., 1993). The apparent adiabatic compressibility of the solute, β_s, was calculated with the equation (Gekko & Noguchi, 1979):

$$\beta_s = -(1/v)(dv/dP)_s = (\beta_0/vc)[\beta/\beta_0 - (d - c)/d_0] \quad (1)$$

where

$$v = (1/c)[1 - (d - c)/d_0] \quad (2)$$

P is the pressure, β the adiabatic compressibility of the solution, β_0 the adiabatic compressibility of the solvent, d the density of the solution, d_0 the density of the solvent, c the concentration of the solute in grams per milliliter of solution, and v the apparent specific volume of the solute. The values of β and β_0 were calculated from the sound velocity, u, and the density, d, of the solution or solvent (u_0 and d_0) according to the Laplace equation: $\beta = 1/du^2$.

For thermal denaturation, about 1.5 mL of a sample solution was carefully introduced into the sample cell (1 cm path length), with a glass stopper to protect the solution from evaporation. The cell was immersed in a thermobath controlled with an accuracy of 0.01 °C (Neslab, RTE-110), and then the temperature was increased from 5 to 45 °C in 1−2 °C steps at 25 min intervals. The temperature of the sample solution was measured with a P_t-resistance at the bottom of the sample cell. Thus, the sound velocity in a protein solution (u) was monitored at each temperature after temperature equilibrium. In the same way, the sound velocity in the solvent (u_0) was measured at various temperatures. The excess ultrasound velocity, Δu, was obtained from the difference between u and u_0 ($\Delta u = u - u_0$), where u_0 at the temperature of the sample solution was calculated from the regression curve of the temperature dependence of u_0. The instrument constant was determined by calibration measurements at 10, 15, 20, 25, 30, and 40 °C with Na_2SO_4 solutions of known sound velocity (International Critical Tables).

The GuHCl denaturation of RNase A was monitored by measuring the sound velocity (u) in protein solutions (6−7 mg/mL) including various amounts of GuHCl (0−4.5 M) at 15 °C. The sound velocity in the solvents including various amounts of GuHCl was also measured, and from its GuHCl concentration dependence, the sound velocity of the solvent (u_0) was calculated at the same GuHCl concentration as that in the sample solution.

Density Measurements. The densities of sample solutions and solvents were measured with a precision density meter, DMA-02C (Anton Paar, Gratz). The accuracy of measurements was 1×10^{-6} g/mL. The instrument constant was determined by calibration measurements at 10, 15, 20, 25, 30, and 40 °C with NaCl solutions of known density (International Critical Tables). For thermal denaturation studies, the temperature of the sample solutions was changed in a comparable range and with comparable temperature steps to those for sound velocity measurements. The density of the solvent (d_0) was measured at various temperatures. The apparent specific volume of the protein (v) at each temperature was calculated from d and d_0 using eq 2 according to the standard procedure, in which d_0 at the temperature of the sample solution was obtained from the temperature dependence of the solvent density. In similar ways, GuHCl denaturation of the protein was monitored by density measurements of sample and solvent solutions with various GuHCl concentrations at 15 °C. The apparent adiabatic compressibility of the protein, β_s, was calculated using eq 1 with a sound velocity and density data set of the sample solutions and solvents at a given temperature or GuHCl concentration.

Circular Dichroism Measurements. The conformational changes of RNase A induced by thermal and GuHCl denaturation were monitored by means of CD measurements with a Jasco J-40A spectropolarimeter. The ellipticity at 222 nm was measured with a quartz cell of 5 mm path length in the same range and with the same temperature steps or GuHCl concentration as used for the sound velocity and density measurements. The temperature of the sample was measured, with an accuracy of 0.1 °C, by means of a digital

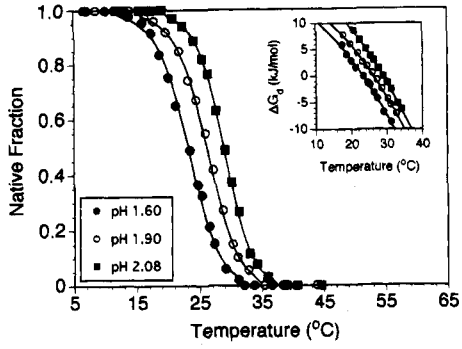

FIGURE 1: Temperature dependence of the native fraction of RNase A as determined by CD measurements at three pHs: 1.60, 1.90, and 2.08. The solid curves represent the regression lines calculated assuming a two-state model. The inset shows the temperature dependence of the free energy change of thermal denaturation, ΔG_d.

thermometer (Takara Kogyo, D221) through a sensor bathed in the sample solution.

The thermal denaturation of RNase A was examined essentially according to the procedures described in a previous paper (Gekko & Koga, 1983). The temperature of the sample was increased in 1.5−2 °C steps, and the ellipticity at 222 nm was read as the equilibrium value at 20 min after each temperature change. In order to protect the protein from damage due to ultraviolet irradiation, the slit on the polarimeter was closed during the temperature change intervals. Assuming a two-state transition for thermal denaturation, N (native) ⇌ D (denatured), the equilibrium constant, K_d, and the corresponding Gibbs's free energy change, ΔG_d, were calculated, using the equation:

$$\Delta G_d = -RT \ln K_d$$
$$= -RT \ln\{([\theta] - [\theta]_N)/([\theta]_D - [\theta])\} \quad (3)$$

where R is the gas constant, T the absolute temperature, $[\theta]_N$ and $[\theta]_D$ the ellipticities for the native and denatured states, respectively, and $[\theta]$ that observed in the transition region. $[\theta]_N$ and $[\theta]_D$ at a given temperature were estimated by assuming the same linear dependence of ellipticity in the transition region as in the native (pretransition region) and denatured (posttransition region) states. For GuHCl denaturation, the equilibrium constant, K_d, and free energy change of unfolding, ΔG_d, were calculated assuming a two-state denaturation model with the ellipticity at 222 nm, according to the procedures described in a previous paper (Gekko & Ito, 1990).

RESULTS

Thermal Denaturation. Prior to the density and sound velocity experiments, the thermal denaturation of RNase A was monitored by CD spectroscopy. Figure 1 shows the temperature dependence of the native fraction of RNase A at pHs 1.60, 1.90, and 2.08. The transition curve shifted to higher temperature with increasing pH, indicating the enhanced thermal stability. The reversibility of thermal denaturation was completely established at any pH, as revealed by the identical CD spectra before and after heating the sample solutions. The free energy change of denaturation, ΔG_d, was calculated by using eq 3 since the two-state model was acceptable, as shown by the solid curves in Figure 1. The ΔG_d values obtained are plotted as a function of temperature in the inset of Figure 1. The ΔG_d values at 15 °C were estimated by the extrapolation method, and the results are presented in Table 1(a), together with the transition temperature, T_m, defined as the temperature at $\Delta G_d = 0$.

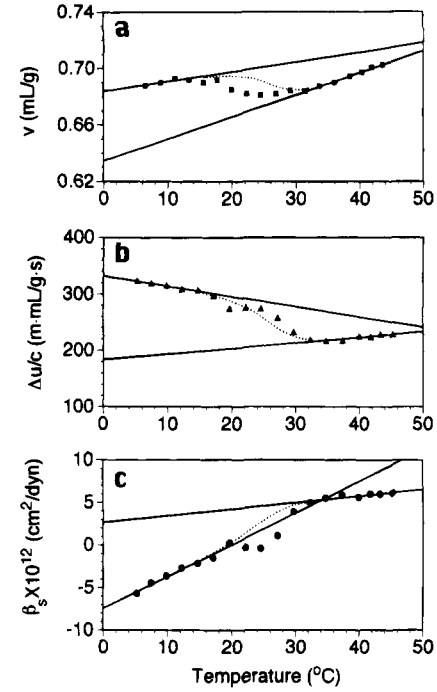

FIGURE 2: Temperature dependence of the apparent specific volume (a), the sound velocity increment per unit protein concentration (b), and the apparent adiabatic compressibility (c) of RNase A at pH 1.90. The average experimental errors involved in ν, $\Delta u/c$, and β_s were ±0.003 mL/g, ±4.1 (m·mL)/(g·s), and ±0.9 × 10^{-12} cm^2/dyn, respectively. The solid lines represent the least-squares linear regression for the pre- and posttransition regions. The regression results are listed in Table 2. The dotted lines show the transition curves calculated from the CD data assuming a two-state model.

Table 1: Thermodynamic Parameters for the Thermal and GuHCl Denaturation of Ribonuclease A at 15 °C

(a) Thermal Denaturation				
pH	T_m (°C)	ΔG_d (kJ/mol)	ΔV_d (mL/mol)	$\Delta\beta_{s,d} \times 10^{12}$ (cm^2/dyn)
1.60	23.0	7.2 ± 0.4	−320 ± 30	7.6 ± 0.8
1.90	25.8	10.4 ± 0.4	−490 ± 70	5.7 ± 1.1
2.08	28.7	13.2 ± 0.3	−670 ± 70	3.3 ± 0.3
(b) GuHCl Denaturation				
pH	C_m (M)	ΔG_d (kJ/mol)	ΔV_d (mL/mol)	$\Delta\beta_{s,d} \times 10^{12}$ (cm^2/dyn)
2.00	1.86	15.4 ± 0.8	−830 ± 610	−25.1 ± 5.6

Figure 2a shows the temperature dependence of the apparent specific volume, ν, at pH 1.90. Although the protein concentration used was considerably high (6−7 mg/mL), the reversibility of thermal denaturation was essentially complete within experimental error. Similar transition curves and good reversibility were observed at the other pHs, 1.60 and 2.08. The ν value increased with increasing temperature in the pretransition region below 15 °C, followed by a gradual decrease in the transition region down to the level in the posttransition region above 35 °C. Considering the experimental error (±0.003 mL/g), the ν value in the pre- and posttransition regions appeared to be linearly dependent on temperature as found by other groups (Cox & Schumaker, 1961; Holcomb & Van Holde, 1962). The results of least-squares linear regression analyses below 15 °C and above 35 °C are shown by the solid lines in Figure 2a and listed in

Table 2: Linear Regression Analyses for the Temperature and GuHCl Concentration Dependence of the Apparent Specific Volume (ν) and the Apparent Adiabatic Compressibility (β_s) in the Pre- and Posttransition Regions

		(a) Thermal Denaturation $\nu = a + b(T - 273.15)$		$\beta_s = a + b(T - 273.15)$	
pH	state	a (mL/g)	$b \times 10^4$ [mL/(g·K)]	$a \times 10^{12}$ (cm²/dyn)	$b \times 10^{14}$ [cm²/(dyn·K)]
1.60	native	0.688 ± 0.001	5.0 ± 4.2	−6.2 ± 0.6	27.0 ± 12.7
	denatured	0.660 ± 0.001	7.8 ± 0.4	3.4 ± 0.2	5.2 ± 4.3
1.90	native	0.684 ± 0.003	6.8 ± 2.6	−7.5 ± 0.4	37.4 ± 3.4
	denatured	0.635 ± 0.002	15.5 ± 0.5	2.7 ± 0.7	7.7 ± 1.7
2.08	native	0.692 ± 0.004	2.7 ± 7.4	−4.0 ± 0.2	27.7 ± 2.3
	denatured	0.620 ± 0.001	18.5 ± 0.1	2.6 ± 0.1	5.9 ± 2.4

		(b) GuHCl Denaturation (15 °C) $\nu = a + b$[GuHCl])		$\beta_s = a + b$[GuHCl]	
pH	state	a (mL/g)	$b \times 10^2$ [mL/(g·M)]	$a \times 10^{12}$ (cm²/dyn)	$b \times 10^{12}$ [cm²/(dyn·M)]
2.00	native	0.691 ± 0.007	3.4 ± 0.9	−4.0 ± 1.2	6.9 ± 0.9
	unfolded	0.630 ± 0.045	2.1 ± 1.3	−29.1 ± 5.5	10.5 ± 1.8

Table 2 with the results at other pHs. Assuming the same temperature derivatives of ν for the native and denatured proteins in the transition region as those in the pre- and posttransition regions, the ν values in the transition region were estimated using the native fractions obtained by CD measurements. The thus predicted transition curve (dotted line in Figure 2a) appears to be slightly larger than the ν values experimentally obtained. The apparent molar volume change due to thermal denaturation, ΔV_d, was calculated from the difference in ν between the native and denatured states at a given temperature with the molecular weight of the protein. It is evident from Figure 2a that the ΔV_d value is negative in the temperature range examined, depending on the temperature and pH. The ΔV_d values estimated at three pHs and 15 °C are listed in the fourth column of Table 1(a).

The sound velocity increment per unit protein concentration, $\Delta u/c$, and the apparent adiabatic compressibility, β_s, are plotted against temperature in Figure 2b and Figure 2c, respectively. Both parameters seem to be linearly dependent on the temperature in the pre- and posttransition regions. The results of linear regression analyses for β_s in both regions are listed in Table 2. Assuming the same temperature derivatives of the two parameters in the transition region as those in the pre- and posttransition regions, the values of $\Delta u/c$ and β_s in the transition region were estimated using the native fractions obtained by CD measurements. As shown by the dotted line in Figure 2b, the transition curve thus obtained seems to slightly deviate from the $\Delta u/c$ values experimentally observed. On the other hand, the β_s values showed a complicated temperature dependence in the transition region (Figure 2c): β_s is considerably small compared to the predicted values at the beginning of transition, followed by an increase with temperature up to the level in the posttransition state. The compressibility change due to thermal denaturation, $\Delta\beta_{s,d}$, was calculated as the difference in β_s between the native and denatured states at 15 °C. The results of calculation at three pHs are shown in the fifth column of Table 1(a).

Guanidine Hydrochloride Denaturation. Figure 3 shows plots of the apparent native fraction of RNase A estimated from the ellipticity at 222 nm as a function of the GuHCl concentration at pH 2.00 and 15 °C. The free energy change of unfolding, ΔG_d, was calculated using eq 3 and is plotted

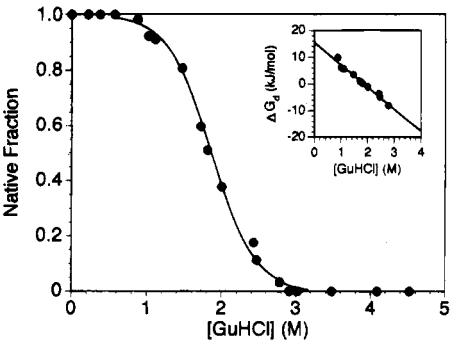

FIGURE 3: GuHCl concentration dependence of the native fraction of RNase A as determined by CD measurements at pH 2.00 and 15 °C. The inset shows the free energy change of unfolding, ΔG_d, as a function of the GuHCl concentration.

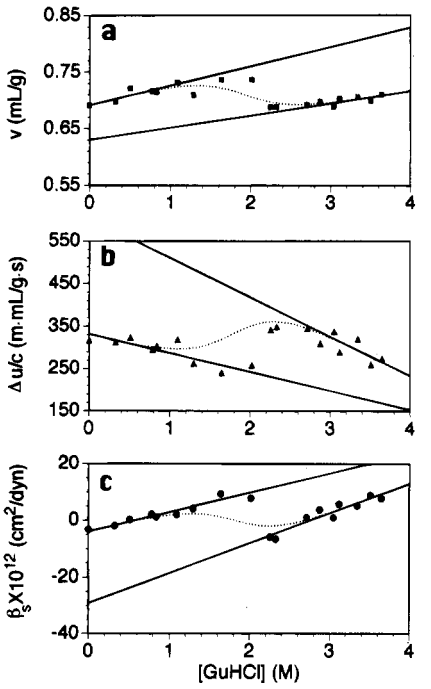

FIGURE 4: GuHCl concentration dependence of the apparent specific volume (a), the sound velocity increment per unit protein concentration (b), and the apparent adiabatic compressibility (c) of RNase A at pH 2.00 and 15 °C. The average experimental errors involved in ν, $\Delta u/c$, and β_s were ±0.004 mL/g, ±7.0 (m·mL)/(g·s), and ±1.1 × 10^{-12} cm²/dyn, respectively. The solid lines represent the least-squares linear regression for the pre- and posttransition regions. The regression results are listed in Table 2. The dotted lines show the transition curves calculated from the CD data assuming a two-state model.

against the GuHCl concentration in the inset of Figure 3. Since a good linear relationship was observed, it fitted the equation (Pace, 1985):

$$\Delta G_d = \Delta G_d^0 - m[\text{GuHCl}] \quad (4)$$

where ΔG_d^0 is the ΔG_d value in the absence of GuHCl and the slope, m, is a parameter reflecting the cooperativity of the transition. The values of ΔG_d^0 and C_m (=$\Delta G_d^0/m$), the GuHCl concentration at the midpoint of the transition, were calculated by the least-squares method, and the results are listed in Table 1(b).

Figure 4 shows plots of the apparent specific volume, ν, the sound velocity increment per unit protein concentration, $\Delta u/c$, and the apparent adiabatic compressibility, β_s, as a

function of the GuHCl concentration at pH 2.00 and 15 °C. There is a considerably large dispersion of the experimental data, as compared with thermal denaturation, due to the separate preparation of the reference and sample solutions with high GuHCl concentration. Nevertheless, it can be seen that the values of ν, $\Delta u/c$, and β_s are linearly dependent on the GuHCl concentration in the pre- and posttransition regions. The results of least-squares regression analyses for ν and β_s in both regions are listed in Table 2 and shown by the solid lines in Figure 4. The dotted lines in this figure show the transition curves predicted from the CD data assuming a two-state transition. As shown in Figure 4a, the ν value of the native protein is larger than that of the denatured one at any GuHCl concentration examined, indicating the negative volume change on GuHCl denaturation. Since the apparent specific volumes observed involve the contribution of GuHCl bound to the protein at a given concentration of GuHCl, the volume change due to GuHCl denaturation was estimated from the extrapolated values of the two linear lines for the pre- and posttransition regions to infinite GuHCl dilution. The thus obtained molar volume change of unfolding in water, ΔV_d, is listed in Table 1(b). The ν values in the transition region appear to deviate positively from the transition curve at the initial stage of denaturation, although the deviation may be negligible around the latter half of the transition.

The values of $\Delta u/c$ and β_s more definitely deviate from the transition curves, as shown in Figure 4b,c: they seem to remain on the extrapolated lines of native values at the beginning of the transition, but drastic changes occur in the latter half of the transition (over 2 M GuHCl). At any GuHCl concentration, the β_s value of the native protein is larger than that of the denatured one, indicating the decrease in compressibility on denaturation. The compressibility change due to GuHCl denaturation, $\Delta\beta_{s,d}$, was estimated, as for the volume change, by extrapolating the two linear lines for the pre- and posttransition regions to infinite GuHCl dilution. The $\Delta\beta_{s,d}$ values thus obtained is listed in the fifth column of Table 1(b).

DISCUSSION

Thermal Denaturation. As shown in Figure 2 and Table 1, the volume change due to thermal denaturation of RNase A is $-(320 - 670)$ mL/mol, the extent being larger at lower temperature and higher pH. Similar values were found on dilatometric measurements (Holcomb & Van Holde, 1962), and also can be calculated from the densimetric data of Privalov et al. (1989). Since the constitutive atomic volume of a protein does not change on denaturation, the observed volume changes could be ascribed to the modification of the internal cavity and hydration on the protein surface (Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986; Sarvazyan, 1991). It is generally known that a considerable amount of nonpolar groups is exposed to the solvent on thermal denaturation, as revealed by the large increase in heat capacity (Privalov et al., 1989). Disruption of the secondary structure is also accompanied by replacement of the peptide−peptide hydrogen bonds by peptide−water hydrogen bonds. The resulting increase in hydration around the polar and nonpolar groups should contribute to the decrease in the apparent molar volume of the denatured protein since the volume changes due to hydration are negative (Gekko & Noguchi, 1979). The contribution of electrostricted hydration to the observed volume changes would be small since most ionic groups are located on the protein surface.

The change in the internal cavity on thermal denaturation is not so easily rationalized. If a protein is completely unfolded into a random-coil state, the cavity would disappear, leading to a large decrease in the apparent molar volume. However, many experimental data have demonstrated that thermally denatured RNase A remains in a compact conformation with some residual secondary structure (Privalov et al., 1989; Sosnick & Trewhella, 1992; Seshadri et al., 1994). In our CD experiments, the ellipticity change at 222 nm due to thermal denaturation was about 65% of that due to GuHCl denaturation. The recent SAXS analysis of Sosnick and Trewhella (1992) showed that the radius of gyration, R_g, increases from 15.0 Å (native state) to 19.3 Å (thermally denatured state). When the protein is reduced, these values increase to 28 Å, but it is still smaller than that (41 Å) of the random-coil conformation. These results suggest that a considerable amount of cavity remains in the interior of the thermally denatured protein, although the hydration effects would overcome the cavity effects, leading to a decrease in the apparent molar volume of the protein.

The characteristic differences between the native and denatured structures more sensitively manifest themselves in the temperature and pressure derivatives of the specific volume. As shown in Figure 2a and the fourth column of Table 2(a), the thermal expansion coefficient, dv/dT, of the denatured protein is larger than that of the native one at any pH examined, the former decreasing and the latter increasing with an increase in pH. These values are close to the expansion coefficients obtained on pycnometric and dilatometric analyses (Cox & Schumaker, 1961; Holcomb & Van Holde, 1962). Assuming negligibly small expansion of the van der Waals volume of the constitutive atoms, dv/dT could be ascribed to the temperature derivatives of the cavity, V_{cav}, and the volume change due to hydration, ΔV_{sol} (Gekko & Hasegawa, 1989):

$$dv/dT = dV_{cav}/dT + d\Delta V_{sol}/dT \quad (5)$$

Although it is difficult to estimate the two terms separately, the temperature effects on X-ray crystallographic data may represent a quantitative measure of the expansion of the cavity. Tilton et al. (1992) found that in the temperature range of 98−300 K, a RNase A molecule expands by 0.4% per 100 K, which corresponds to 2.8×10^{-4} mL/(g·K). This expansion is primarily due to a decrease in the local atomic packing density and the larger motions of the secondary structure or exposed surface loops. If the same level of expansion is assumed for the cavity in the native protein in solution, more than half of dv/dT can be ascribed to the cavity effect. Since the atomic packing density would decrease on thermal denaturation, as expected from the increased R_g (Sosnick & Trewhella, 1992), the thermally denatured protein would have a larger positive dV_{cav}/dT value than the native one. In this respect, it is noteworthy that myoglobin, a protein with a significant internal cavity resulting from the packing of α-helices about the prosthetic heme group, has a 2−3-fold larger thermal expansion coefficient than RNase A, a tightly packed protein (Frauenfelder et al., 1987; Tilton et al., 1992). The second term, $d\Delta V_{sol}/dT$, on the right-hand side of eq 5 should also be positive since the amount of hydration decreases to make

ΔV_{sol} less negative at higher temperature. The hydration around polar and nonpolar groups is more sensitive to temperature variation than electrostricted hydration (Iqbal & Verrall, 1987); therefore, the increases in these two types of hydration should contribute to make $d\Delta V_{sol}/dT$ more positive. Thus, both the cavity and hydration would positively contribute to the increased expansion coefficient of the thermally denatured protein.

The apparent adiabatic compressibility of RNase A increases on thermal denaturation, as shown in Figure 2c and Table 1. This means that the volume fluctuation of the protein is enhanced by thermal denaturation since it is directly linked to isothermal or adiabatic compressibility (Cooper, 1976; Gekko & Hasegawa, 1986). The relaxational contribution may be involved in these volume fluctuations due to increased conformational freedom or the proton transfer process (Sarvazyan & Hemmes, 1979). The amount of relaxational contribution is unknown for RNase A, but from the sound absorption measurements, it has been estimated for other proteins to be at most 10% in the total compressibility which corresponds to a few percent in the sound velocity change at the frequence used (Sarvazyan & Hemmes, 1979). Proton exchange relaxation, which may exist at low pH, would not significantly affect the compressibility change, since the pH of the solution is held constant and the pK_a of ionic groups, most of them being located on the protein surface, would not change during denaturation. Thus, the observed compressibility changes for RNase A might be dominantly ascribed to the modified hydration and cavity on denaturation.

Evidently, the finding that the compressibility increases despite the decrease in specific volume cannot be explained by a simple hydrophobic model for denaturation, in which the compressibility should decrease with decreasing cavity and increasing hydration. A possible explanation for this contradiction might be given if the local concentration of nonpolar groups exposed is quite high in protein systems and the volume change for the rupture of hydrophobic bonds is partly positive (Boje & Hvidt, 1972; Gekko & Noguchi, 1979). In fact, the volume changes for the transfer of water into organic solvents are known to be positive (Masterton & Seiler, 1968), although the transfer of nonpolar groups into water produces negative volume changes (Kauzmann, 1959). Since the apparent adiabatic compressibility of the cavity is more than 10-fold that of pure water (e.g., 550×10^{-12} cm^2/dyn for chymotrypsinogen) (Gekko & Noguchi, 1979), only a small amount of the local cavity in a cluster of nonpolar groups exposed would overcome the negative contribution of the increased hydration, leading to a larger compression of the thermally denatured protein ($\Delta\beta_{s,d} > 0$).

A positive compressibility change and a negative volume change have also been observed for pressure denaturation of RNase A (Brandts et al., 1970), chymotrypsinogen (Hawley, 1971), and myoglobin (Zipp & Kauzmann, 1973). The volume change of RNase A varies from −45 mL/mol at pH 2 and 23.7 °C to −4.5 mL/mol at pH 4 and 55.1 °C, while the isothermal compressibility increases by about 1.5×10^{-12} cm^2/dyn (Brandts et al., 1970). These results suggest that when denatured by pressure, RNase A still has a loosely packed and more flexible structure, as compared with the native state. However, the thermal fluctuation of the protein would not be so greatly enhanced by pressure denaturation compared with thermal denaturation, since the volume and compressibility changes due to pressure denaturation are considerably small.

Another noticeable result is that the specific volume and compressibility in the transition region deviate from the transition curves expected for a two-state denaturation model (Figure 2). This indicates that the global structure or compactness may be disrupted in a different manner from the secondary structure: the conformation still remains compact relative to the breaking of the secondary structure. Similar deviation from a two-state model was also observed in the SAXS data of Sosnick and Trewhella (1992): R_g slightly decreases at the beginning of denaturation, and then after passing the minimum, it increases greatly to the maximum value with increasing temperature, and then decreases to the level in the denatured state. The combination of these SAXS data and our compressibility data leads us to speculate that the partially denatured protein (at the initial stage of denaturation) may have a more compact conformation than the native state and the nonpolar groups exposed on unfolding would form a cluster in the latter half or around the final stage of the transition. It is probable that the breaking of a small amount of helix contributes to the increase in the compactness of the global structure (or decrease in the thermal fluctuation), since the helix elements, being rigid and bulky, would play the role of a dynamic domain in the protein structure (Gekko & Hasegawa, 1986). Thus, these compressibility data would be positive evidence that partially denatured proteins during thermal denaturation may exist as compact intermediates or molten-globules.

Guanidine Hydrochloride Denaturation. As a reference state of the denatured protein, GuHCl-induced denaturation was examined. As shown in Figure 4 and Table 1, the apparent molar volume and adiabatic compressibility of RNase A decreased on GuHCl denaturation. The observed volume change, −830 mL/mol, is larger (more negative) than that obtained for thermal denaturation at any pH. The apparent adiabatic compressibility of the denatured protein in water (15 °C and pH 2.00), -29.1×10^{-12} cm^2/dyn, is comparable with those of amino acids so far reported (Millero et al., 1978; Cabani et al., 1981). These results support the general acceptance that the protein structure is almost completely unfolded by GuHCl to expose many amino acid residues, accompanied by a decrease in the cavity and an increase in hydration. However, it will be difficult to estimate the compressibility of a denatured protein using the compressibility data for the constitutive amino acid residues because the extent of unfolding would be restricted by disulfide bonds and/or residual secondary structure which may still remain. For the pure theoretical case of a fully unfolded protein, the compressibility has been calculated to be about -80×10^{-12} cm^2/dyn (Iqbal & Verrall, 1988).

The interpretation of the volume and compressibility changes at a given concentration of GuHCl may be complicated by the preferential binding of GuHCl to the protein. Nevertheless, it is interesting that the β_s value largely deviates from the transition curve calculated assuming a two-state model. At the initial stage of GuHCl denaturation, the protein structure seems to remain compact despite the breaking of the secondary structure, and the main exposure of amino acid residues would occur in the latter half (over 2 M GuHCl) of the transition. Similar noncooperative disruption of the global structure and the sencondary structure has been found on the urea and GuHCl denaturation of some

small globular proteins (Kuwajima, 1989; Sanz & Fersht, 1993). So far as indicated by our compressibility data, RNase A partially denatured by GuHCl seems to have a compact intermediate or molten-globule-like structure, exhibiting compressibility comparable with that of the native structure. However, this does not necessarily mean that the intermediate is a unique species. The intermediate should rather be regarded as an ensemble of conformations that progressively unfold with increasing GuHCl concentration, as proposed by Palleros et al. (1993). Recently, Nölting and Sligar (1993) found that the molten-globule state of cytochrome *c* at pH 2.2 and 200 mM NaCl has slightly larger compressibility (a softer structure) than the native structure at neutral pH, and an expanded form at pH 2.2 and 10 mM NaCl. The high ionic strength of GuHCl used as a denaturant may be partially responsible for the compact intermediate structure of RNase A in the transition region.

As demonstrated in the present study, the apparent molar volume and adiabatic compressibility sensitively reflect the conformational changes of RNase A on thermal and GuHCl denaturation. Since these parameters involve the complicated contributions of hydration and the internal cavity, there are limitations to the quantitative discussion of the results. Nevertheless, these data could give new insights into the compactness and thermal fluctuation of denatured proteins which cannot be characterized by other spectroscopic techniques such as CD and fluorescence. A combination of compressibility and SAXS data would be fruitful for elucidating the real features of the denatured and intermediate structures of proteins.

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