Compressibility and Volume Changes of Lysozyme Due to Guanidine Hydrochloride Denaturation

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The apparent specific volume and adiabatic compressibility of lysozyme largely decreased upon guanidine hydrochloride denaturation. These results demonstrate that a large amount of amino acid residues exposes accompanying an increase in hydration and a decrease in the internal cavity. Both parameters deviated from the two-state transition curves, suggesting noncooperative disruption of the compactness and the secondary structure.

The conformation of protein is extensively unfolded by guanidine hydrochloride (GuHCl) or urea and the unfolded state has been taken as an initial reference for protein folding. However, there is much evidence for stable conformations that are not fully folded or fully unfolded under several conditions, such as high ionic strength, extreme pH, organic solvent, and mild denaturant concentration.[1] Detailed characterization of these non-native conformations is required for understanding the principles of constructing a three dimensional structure and the folding mechanism of proteins. The partial specific volume and compressibility are important physical quantities directly related to the compactness or globularity of the protein molecule because they involve the contributions of surface hydration and internal cavity.[2,3] However, only limited data have been reported on the volume and compressibility changes due to protein denaturation, some of them being not necessarily consistent probably because of the technical difficulties.[4-8] Recently, we found that the GuHCl denaturation of ribonuclease A brings about large decrease in the molar volume and adiabatic compressibility compared with thermal denaturation.[7] To confirm this, in this communication, we have studied the volume and adiabatic compressibility changes associated with GuHCl denaturation of lysozyme.

Hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Ltd. (six times crystallized, lot No. E83Y03). This protein was completely deionized by exhaustive dialysis against distilled water at 4 ℃. Ultrapure guanidine hydrochloride (GuHCl) was purchased from Schwarz/Mann Research Laboratories. All other chemicals were special reagent grade products from Wako Pure Chemicals. For GuHCl denaturation studies, 20-30 sample solutions with different GuHCl concentrations (0-7 M) and an identical protein concentration (6 mg/cm^3) were prepared as follows. A given amount of GuHCl was dissolved into protein solution containing HCl in a 5 cm^3-flask and it was filled up with distilled water. The protein and GuHCl concentrations in the sample solutions (0.1 mM HCl) were calculated using their density data from the weight ratio of GuHCl and the protein solution whose concentration was photometrically predetermined with an extinction coefficient of 2690 cm^3/(g cm) at 280 nm. According to the same procedures, aqueous solutions with various GuHCl concentrations (0.1 mM HCl) were prepared as reference solvents. The sample solutions were incubated for at least 6 h at 25 ℃ to complete denaturation of the protein.

The GuHCl denaturation of lysozyme was monitored by measuring the sound velocity (u) and density (d) in protein solutions including various amounts of GuHCl at 25 ℃. The sound velocity was measured with an accuracy of 1 cm/sec by means of a "sing-around pulse method" at 6 MHz. The density was measured with an accuracy of 10^{-6} g/cm^3 using a precision density meter, DMA-02C (Anton Paar, Gratz). The apparatus and experimental procedures were essentially the same as those used in the previous study.[7] The apparent specific volume (v) and adiabatic compressibility (β_s) of the protein were calculated, using Eqs. 1 and 2 with a sound velocity and density data set of the sample solutions (u and d) and solvents (u_0 and d_0) at a given

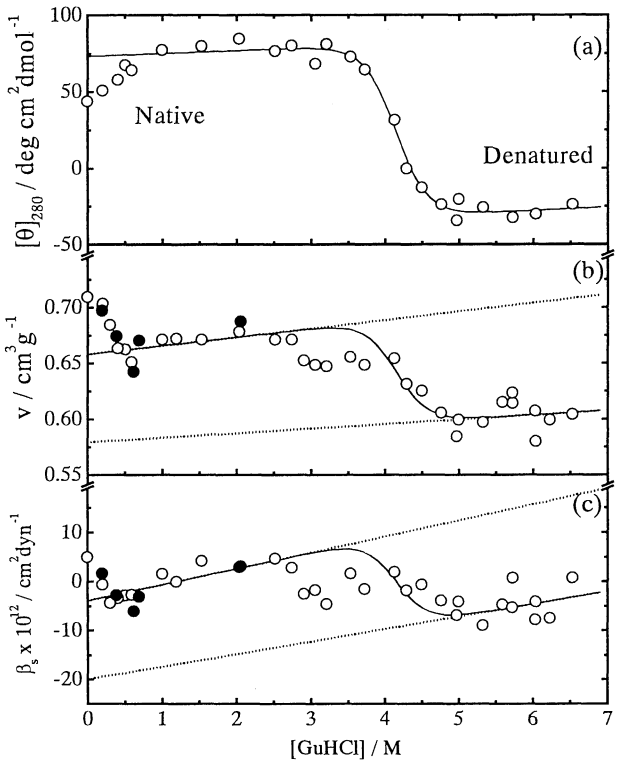

Figure 1. GuHCl concentration dependence of the molar ellipticity at 280 nm (a), the apparent specific volume (b), and the apparent adiabatic compressibility (c) of lysozyme at pH 4 and 25 ℃. The closed circle represents the data when NaCl was added instead of GuHCl. The experimental errors were within the size of each symbol. The solid lines show the transition curves calculated from the CD data at 222 nm assuming a two-state denaturation model. The dotted lines represent the least-squares linear regression of the data in the pre- and posttransition regions. The values of v and β_s extrapolated to infinite dilution of GuHCl are listed in Table 1. (1dyn=10^{-5}N)

GuHCl concentration,

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

$$\beta_s = -(1/v)(\partial v/\partial P) = (\beta_0/vc)\left[(\beta/\beta_0)-(d-c)/d_0\right] \quad (2)$$

where P is the pressure, c the concentration of the solute in grams per milliliter of solution, β and β_0 the adiabatic compressibilities of solution and solvent, respectively, which were calculated with the Laplace equation, $\beta = 1/u^2d$. The values of u_0 and d_0 were accurately interpolated by the 5th order regression analysis of the GuHCl concentration. The conformational change of lysozyme due to GuHCl denaturation was also monitored by means of circular dichroism (CD) measurements at 222 and 280 nm with a Jasco J-720W spectropolarimeter.

Figure 1 shows plots of the molar ellipticity at 280 nm, the apparent specific volume, and the apparent adiabatic compressibility as a function of the GuHCl concentration at pH 4 and 25 °C. The solid lines in this figure show the transition curves predicted from the ellipticity at 222 nm assuming a two-state denaturation model. The ellipticity at 280 nm increased considerably by adding a small amount of GuHCl (less than 0.5M) but that at 222 nm did not show any change (Figure 1a). In this GuHCl concentration region, the values of v and β_s largely decreased. Addition of NaCl instead of GuHCl also brought about the comparable decreases in v and β_s (Figure 1b and c). These results suggest that the secondary structure is not modified but the tertiary structure becomes more compact by binding GuHCl or Cl^- ions prior to unfolding. The actual binding of Cl^- ions was qualitatively confirmed from the titration curves.[9] The volume and compressibility changes in this process were estimated to be -710 cm^3/mol and -8.8 × 10^{-12} cm^2/dyn, respectively, by comparing the v and β_s values observed in water (0.1mM HCl) with those at infinite dilution of GuHCl, which were obtained by the linear extrapolation of the data in the pretransition region, 0.5-2.5M GuHCl (dotted lines in Figure 1b and c).

As revealed by the coincident ellipticity changes at 222 and 280 nm (Figure 1a), the secondary and tertiary structures cooperatively unfold in 3-5M GuHCl, accompanying the large decreases in v and β_s values (Figure 1b and c). This means that the volume and compressibility changes upon GuHCl denaturation, ΔV and $\Delta\beta_s$, are negative. Since the ΔV and $\Delta\beta_s$ values are dependent on the GuHCl concentration, they were estimated from the extrapolated values of the two linear dotted lines assumed for the pre- and posttransition regions to infinite GuHCl dilution (Table 1). The thus obtained ΔV and $\Delta\beta_s$ were -1200 cm^3/mol and -16×10^{-12} cm^2/dyn, respectively. Although these values involve large experimental errors and may still refer to the protein bound Cl^- ions, they are comparable with the results observed for ribonuclease A.[7] The apparent adiabatic compressibility of the denatured protein, -19.9×10^{-12} cm^2/dyn, is close to those of amino acids so far reported.[10,11] These results support the general acceptance that a protein structure is almost completely unfolded by GuHCl and a large amount of amino acid residues is exposed to solvent, 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 acids residues because the extent of unfolding would be restricted by disulfide bonds and/or the residual secondary structure. For the pure theoretical case, the adiabatic compressibility of a fully unfolded protein has been calculated to be about -80×10^{-12} cm^2/dyn.[11]

Table 1. Apparent specific volume and adiabatic compressibility of lysozyme at infinite dilution of GuHCl (pH 4 and 25 °C)

State	v (cm^3/g)	$\beta_s\times10^{12}$ (cm^2/dyn)
Native (in water)	0.709±0.002	4.6±1.6
Native	0.659±0.005	-3.9±1.3
Unfolded	0.579±0.040	-19.9±15

The changes in v and β_s in the transition region are not easily rationalized since they involve the complicated contributions of the preferential binding of GuHCl besides the conformational change of the protein. Nevertheless, it is interesting that the v and β_s values do not correlate to a loss of the secondary structure. At the initial stage of unfolding, the protein structure seems to become more compact or rigid and the main exposure of amino acid residues would occur in the latter half of the transition (over 4M GuHCl). This result is consistent with those of the small angle X-ray scattering study that the radius of gyration increases prior to unfolding of the secondary structure.[12] Similar noncooperative disruption of the global structure and the secondary structure has been found for the urea and GuHCl denaturation of some other small globular proteins.[1,13] These evidences as well as our previous study[7] implicate that the compactness of protein does not necessarily follow a two-state denaturation model and that there are some intermediates not fully unfolded in the denaturation process. These intermediates have been called "molten globules" but the definition and generality remain controversial.[14] The volume and compressibility data, involving the complicated contributions of hydration and the internal cavity, could give new insights into the compactness of denatured or molten globule states of proteins which cannot be characterized by other spectroscopic techniques.

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