Effects of Disulfide Bonds on Compactness of Protein Molecules Revealed by Volume, Compressibility, and Expansibility Changes during Reduction[†]

Kunihiko Gekko,* Akinobu Kimoto, and Tadashi Kamiyama

Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526, Japan

Received May 7, 2003

ABSTRACT: To elucidate the effects of disulfide bonds on the compactness of protein molecules, the partial specific volume ($\bar{v}°$) and coefficients of adiabatic compressibility ($\bar{\beta}_s°$) and thermal expansibility (α) of five globular proteins (ovalbumin, β-lactoglobulin, lysozyme, ribonuclease A, and bovine serum albumin) were measured in aqueous solutions with pH values of 7 and 2 at 25 °C when their disulfide bonds were totally reduced by carboxamidomethylation. Circular dichroism and fluorescence spectra show that the secondary and tertiary structures are partly disrupted by reduction, depending on the number of disulfide bonds in the proteins and the pH of the medium. The conformational changes are accompanied by decreases in $\bar{v}°$ and $\bar{\beta}_s°$ and by an increase in α, indicating that reduction decreases the internal cavity and increases surface hydration. The $\bar{\beta}_s°$ values of native or oxidized proteins decrease, and the effects of reduction on the volumetric parameters become more significant as the number of disulfide bonds increases and as they are formed over a larger distance in the primary structure. These results demonstrate that disulfide bonds play an important role, mainly via entropic forces, in the three-dimensional structure and compactness of protein molecules.

To understand the role of disulfide bonds (S−S bonds)[1] in the construction of the three-dimensional structure of proteins, a large body of experimental data has been accumulated on the stability and folding kinetics of reduced proteins (*1*, *2*). An important conclusion from these studies is that most proteins containing S−S bonds unfold when they are completely reduced even in the absence of denaturant and that the free energy of folding provides the driving force not only for correct folding but also for correct pairing of S−S bonds. However, it is unknown whether S−S bonds force the protein into its folded form or whether other forces guide the protein into a conformation in which the S−S bonds can form easily (*3*). A stabilizing force of S−S bonds is mainly attributed to the loss of conformational entropy of the unfolded polypeptide by cross-linking. This effect depends on the location of S−S bonds: the farther apart in the primary structure the cross-linked residues, the greater the decrease in conformational entropy (*4*). However, there is some evidence that cross-links affect the energetics of the folded conformation (native state) to varying extents, such as in the loss of stabilizing interactions and the possible formation of a strain associated with the introduction of S−S bonds (*5*).

The S−S cross-links restrict the ability of a protein molecule to expand, so they should affect its structural flexibility and compactness, which are decisive factors in the stability and function of proteins. However, there are limited data on this mechanism, although the changes in hydrodynamic properties due to reduction of S−S bonds have been monitored by viscosity (*6*, *7*), size-exclusion chromatography (*8*−*10*), and small-angle X-ray scattering (*11*−*13*). The flexibility and compactness of protein molecules are more sensitively reflected in the volumetric properties such as the partial specific volume, compressibility, and thermal expansibility, since these quantities involve the contributions from hydration (solvent-accessible surface area) and cavities due to imperfect atomic packing (*14*−*16*). In particular, adiabatic compressibility is linked directly to volume fluctuations (*17*) and hence is known to be a good measure of the flexibility and compactness of native and denatured proteins (*14*−*16*, *18*−*21*). It is of interest how these volumetric properties are related to the number and location of S−S bonds.

A novel approach to answering this question is to examine the volumetric properties of proteins whose S−S bonds are chemically reduced or replaced more sophisticatedly with other amino acid residues by mutation. However, it is difficult to prepare sufficient protein for volumetric measurements whose S−S bonds are site-specifically broken by chemical or mutational methods. In the present study, therefore, the partial specific volume and coefficients of adiabatic compressibility and thermal expansibility were measured in five globular proteins whose S−S bonds were totally reduced by carboxamidomethylation under neutral and acidic conditions. By comparison with the results for the nonreduced (oxidized) proteins, the role of S−S bonds in constructing the three-dimensional structure and compactness

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

* To whom correspondence should be addressed. Phone/Fax: +81-824-24-7387. E-mail: gekko@sci.hiroshima-u.ac.jp.

[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; DTT, dithiothreitol; RNase A, ribonuclease A; S−S bond, disulfide bond.

10.1021/bi030115q CCC: $25.00
Published on Web 11/01/2003

of protein molecules is discussed in terms of cavity and hydration, focusing on the effects of the composition and location of S−S bonds. To our knowledge, this is the first systematic investigation of the volumetric properties of reduced proteins, although a compressibility study has been reported only for reduced thioredoxin (*22*).

MATERIALS AND METHODS

Materials. Bovine pancreatic ribonuclease A (RNase A), bovine serum albumin (BSA), and β-lactoglobulin were purchased from Sigma. Hen egg lysozyme was obtained from Seikagaku. Ovalbumin was prepared from fresh hen egg white by an ammonium sulfate method. These proteins were selected because they have different numbers of S−S bonds and good solubility in both oxidized and reduced forms. Papain from papaya and pepsinogen from porcine (Sigma) were also used for comparison of the compressibilities of the native proteins. Monoiodoacetamide was purchased from Katayama Chemicals. Dithiothreitol (DTT) and all other chemicals were reagent-grade products from Wako Pure Chemicals.

Carboxamidomethylation of Proteins. The S−S bonds of proteins were totally reduced by DTT and then carboxamidomethylated by monoiodoacetamide according to the following procedures (*23*). A total of 0.5 g of protein was dissolved in 30 mL of distilled water containing 5 mL of 2.0 M Tris−acetate buffer (pH 8), 24 g of urea, 0.019 g of EDTA, and 0.35 g of DTT. After the mixture was stirred for 90 min at room temperature, the resulting sulfhydryl groups were carboxamidomethylated by addition of 0.685 g of monoiodoacetamide. The mixture was adjusted to pH 8.2 by the addition of concentrated aqueous 2-amino-2-(hydroxymethyl)-1,3-propanediol. After being stirred for 60 min, the mixture was adjusted to pH 3 by addition of 2 M hydrochloric acid at 4 °C. This solution was then exhaustively dialyzed against 0.05 M acetic acid. It was confirmed using mass spectrometry (JEOL SX-102A) that all of the disulfide linkages were carboxamidomethylated, based on the mass increment of 58.06 Da for an introduced acetamide residue, $-CH_2CONH_2$.

Sample Preparation. The sample solutions of pH 2 were prepared by dialyzing the stock solution of native and reduced proteins against 10 mM HCl, and those of pH 7 were prepared by slowly titrating the solutions of pH 2 with a concentrated NaOH solution. Reduced β-lactoglobulin was adjusted to pH 5.0 because its solubility is very low at pH 7. The protein concentration was determined spectrophotometrically (after density and sound-velocity measurements) using a spectrophotometer (UV-250, Shimazu). Extinction coefficients of 6.58, 26.9, 7.01, 9.60, and 7.12 dL/(g cm) at 280 nm were used for BSA, lysozyme, RNase A, β-lactoglobulin, and ovalbumin in the native state (neutral pH), respectively. The extinction coefficients of the reduced proteins at neutral pH were determined by a dry-weight method to be 6.30, 25.7, 5.49, 9.71, and 7.30 dL/(g cm) at 280 nm, respectively. The extinction coefficients of the oxidized and reduced proteins at pH 2 were determined from the relative absorbance of the respective protein solutions at pH values of 7 and 2.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were obtained at 25 °C using a spectropolarimeter (J-720W, Jasco). The protein concentrations were 0.3% and 0.03% in the near- and far-UV regions, respectively.

Fluorescence Measurements. Fluorescence emission spectra were measured at 25 °C using a spectrofluorometer (FP-750, Jasco), with excitation wavelengths of 280 nm for lysozyme, ovalbumin, and β-lactoglobulin, 275 nm for BSA, and 268 nm for RNase A. The protein concentrations were 0.002%, 0.003%, 0.03%, 0.3%, and 0.04% for lysozyme, ovalbumin, β-lactoglobulin, BSA, and RNase A, respectively.

Sound-Velocity Measurements. The velocity of sound in the protein solutions was measured (with an accuracy of 1 cm/s) by means of a "sing-around pulse method" at 5 MHz and 25 °C. The apparatus and experimental procedures were the same as those used previously (*15*, *19*, *24*). The coefficient of partial specific adiabatic compressibility of protein at infinite dilution, $\bar{\beta}_s^\circ$, was calculated with the equations (*14*, *15*):

$$\bar{\beta}_s^\circ = -(1/\bar{v}^\circ)(\delta\bar{v}^\circ/\delta P)_s = (1/\bar{v}^\circ)\lim_{c\to 0}(1/c)[\beta/\beta_o - (d-c)/d_o] \quad (1)$$

$$\bar{v}^\circ = \lim_{c\to 0}(1/c)[1-(d-c)/d_o] \quad (2)$$

where P is the pressure, β is the adiabatic compressibility of the solution, β_o is the adiabatic compressibility of the solvent, d is the density of the solution, d_o is the density of the solvent, c is the concentration of the protein (in grams per milliliter of solution), and $\bar{v}^\circ$ is the partial specific volume of the protein. The values of β and β_o were calculated from the sound velocity, u, and the density, d, of the solution or solvent (u_o and d_o) according to the Laplace equation $\beta = 1/du^2$.

Density Measurements. The densities of sample solutions and solvents were measured with an accuracy of 1×10^{-6} g mL^{-1} at 25 °C using a precision density meter (DMA-02C; Anton Paar, Austria). The partial specific volumes of the proteins were calculated from eq 2 according to standard procedures. The coefficient of thermal expansibility of proteins, $\alpha = (1/\bar{v}^\circ)(\delta\bar{v}^\circ/\delta T)_p$, was calculated from the temperature dependence of the partial specific volumes at a fixed protein concentration (0.3%), which may be regarded to be equal to those at infinite dilution of protein since the protein concentration dependence of the apparent specific volume is negligibly small. The solution density was measured in 5 deg steps over a temperature range from 10 to 45 °C using a density meter DMA5000 (Anton Paar) equipped with a Peltier temperature control system.

RESULTS

CD and Fluorescence Spectra. The effects of reduction on the protein structure were examined spectrophotometrically before volumetric measurements. Figure 1 shows CD and fluorescence spectra of the oxidized and reduced lysozyme at pH values of 7 and 2. The far-UV CD spectra indicate that the secondary structure is considerably disrupted when its four S−S bonds are reduced, with the extent being greater at lower pH. The tertiary structure was also modified at both pH values to expose the tryptophan residues to the solvent, as suggested by the peak shifts in the fluorescence spectra from 347 to 352 nm. Figure 2 shows CD and

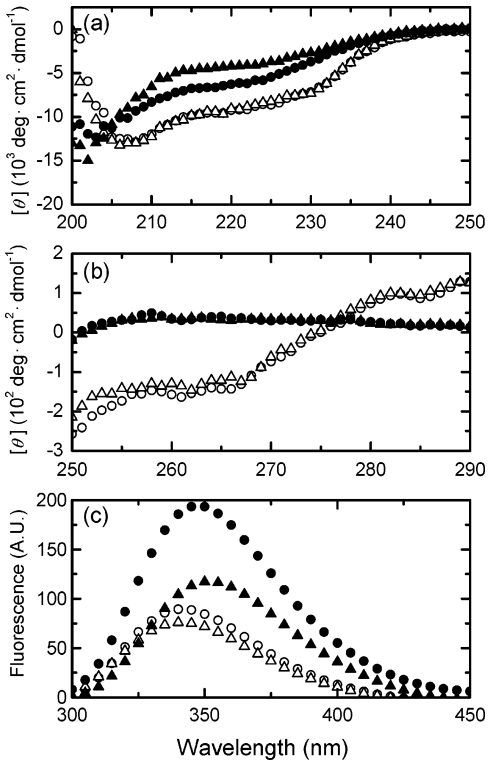


FIGURE 1: Far-UV CD (a), near-UV CD (b), and fluorescence (c) spectra of oxidized and reduced lysozyme at pH values of 7 and 2. Key: ○, oxidized at pH 7; ●, reduced at pH 7; △, oxidized at pH 2; ▲, reduced at pH 2.

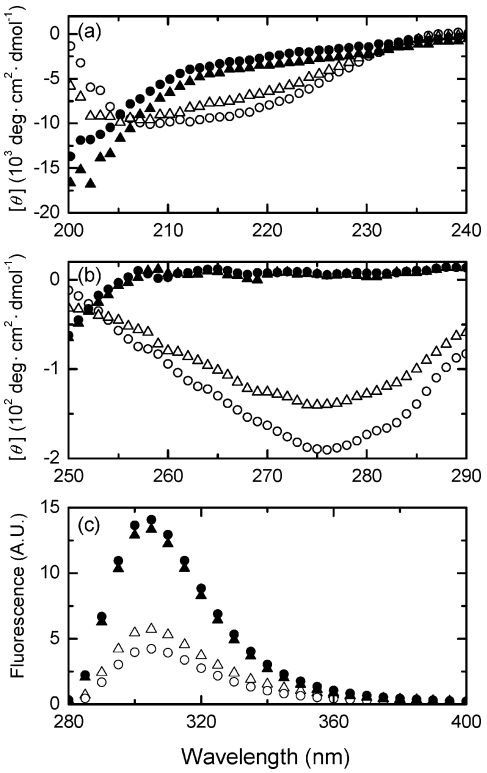


FIGURE 2: Far-UV CD (a), near-UV CD (b), and fluorescence (c) spectra of oxidized and reduced RNase A at pH values of 7 and 2. Key: ○, oxidized at pH 7; ●, reduced at pH 7; △, oxidized at pH 2; ▲, reduced at pH 2.

fluorescence spectra of oxidized and reduced RNase A at pH values of 7 and 2. It is evident that the secondary and tertiary structures of RNase A are extensively broken by reduction of its four S−S bonds at both pH values, indicating that the S−S bonds are crucial for constructing the three-dimensional structure of this protein. Similarly, a large disruption of the secondary and tertiary structures was found for BSA when its 17 S−S bonds are reduced (data not shown). In contrast, ovalbumin, having an S−S bond, shows only small changes in the CD and fluorescence spectra by reduction at the pH values tested (data not shown), suggesting minor modification of the secondary and tertiary structures. Reducing two S−S bonds of β-lactoglobulin causes only small changes in the far-UV CD spectra but large changes in the near-UV CD and fluorescence spectra at pH values of 5 and 2 (data not shown). Thus the main effects of reduction are exerted on the tertiary rather than the secondary structure of this protein.

Volume, Compressibility, and Expansibility. There was no significant protein concentration dependence of apparent specific volume for all of the oxidized and reduced proteins at the pH values tested, although the experimental error was slightly larger for reduced proteins at pH 2. The $\bar{v}°$ values at infinite dilutions of proteins were calculated by least-squares linear regression (see the fourth column of Table 1). The sound velocity in protein solutions increased in proportion to the protein concentration in the concentration range investigated (less than 0.7%). The sound-velocity increment per unit protein concentration, $\delta u/\delta c$, and the coefficient of adiabatic compressibility at infinite dilution of protein, $\bar{\beta}_s°$, are listed in the fifth and sixth columns of Table 1, respectively. It is seen that the $\bar{v}°$ and $\bar{\beta}_s°$ values are smaller at pH 2 than at neutral pH and decrease by reduction at the pH values tested for all of the proteins, except for $\bar{v}°$ of ovalbumin at pH 2.

Figure 3 shows typical plots of the partial specific volume, $\bar{v}°$, against temperature for three proteins, lysozyme, RNase A, and BSA. A highly linear relationship is evident over the temperature range examined for all of the native and reduced proteins at the pH values tested, allowing us to calculate the coefficient of thermal expansibility, α, from the slope. The α values obtained at 25 °C are listed in the last column of Table 1, which indicates that the α values of all of the proteins are larger at pH 2 than at neutral pH and are increased by reduction at the pH values tested.

DISCUSSION

The present study reveals that reduction of S−S bonds induces characteristic changes in the spectroscopic and volumetric properties of proteins, indicative of changes to the three-dimensional structure and compactness. The partial specific volume, compressibility, and expansibility, being thermodynamic quantities, sensitively reflect the modified conformation through changes in the solvent-accessible surface area and the internal atomic packing of protein molecules. The relationships between these volumetric properties and the structural changes, which depend on the composition and location of S−S bonds, provide new insight into understanding the role of S−S bonds in the compactness of protein molecules.

Contribution of Cavity and Hydration to Volumetric Parameters. The partial specific volume ($\bar{v}°$) of a protein in water consists of three contributions (*25*): the constitutive atomic volume (v_c), the cavity resulting from imperfect

Table 1: Partial Specific Volume ($\bar{v}°$), Sound-Velocity Increment ($\delta u/\delta c$), and Coefficients of Adiabatic Compressibility ($\bar{\beta}_s°$) and Thermal Expansibility (α) of Oxidized and Reduced Proteins at 25 °C

protein (ν_s)[a]	state	pH	$\bar{v}°$ (mL/g)	$\delta u/\delta c$ (m mL $g^{-1}s^{-1}$)	$\bar{\beta}_s°$ (1/Mbar)	α (10^{-4}/K)
ovalbumin (1)	oxidized	7.0	0.746 ± 0.001	234.1	9.2 ± 0.5	5.6 ± 0.1
		2.0	0.731 ± 0.003	237.7 ± 1.9	8.6 ± 0.5	7.4 ± 0.2
	reduced	7.0	0.733 ± 0.002	250.8 ± 7.5	7.9 ± 1.0	7.9 ± 0.2
		2.0	0.737 ± 0.006	253.6 ± 3.3	6.7 ± 1.0	8.2 ± 0.2
β-lactoglobin (2)	oxidized	5.0	0.751 ± 0.001	276.2	8.5 ± 0.4	6.5 ± 0.1
		2.0	0.732 ± 0.001	304.4 ± 6.1	3.4 ± 0.5	6.7 ± 0.1
	reduced	7.0	0.736 ± 0.003	303.8 ± 9.5	3.9 ± 0.9	9.6 ± 0.1
		2.0	0.689 ± 0.004	307.8 ± 2.1	−2.6 ± 0.7	12.1 ± 0.5
lysozyme (4)	oxidized	7.0	0.712 ± 0.001	257.1	4.7 ± 0.1	4.4 ± 0.3
		2.0	0.712 ± 0.002	262.0 ± 0.5	4.5 ± 0.3	5.9 ± 0.1
	reduced	7.0	0.704 ± 0.005	343.8 ± 16	−3.4 ± 1.6	10.5 ± 0.2
		2.0	0.682 ± 0.002	351.1 ± 18	−7.0 ± 1.9	11.2 ± 0.1
RNase A (4)	oxidized	7.0	0.704 ± 0.001	291	1.1 ± 0.1	6.2 ± 0.2
		2.0	0.675 ± 0.007	295.0 ± 5.1	−1.0 ± 1.2	7.9 ± 0.4
	reduced	7.0	0.667 ± 0.002	377.4 ± 17	−11.2 ± 1.6	10.7 ± 0.3
		2.0	0.661 ± 0.005	366.6 ± 5.7	−11.4 ± 1.2	11.3 ± 0.1
BSA (17)	oxidized	7.0	0.735 ± 0.001	222.4	10.5 ± 0.2	6.3 ± 0.3
		2.0	0.711 ± 0.003	250.7 ± 2.1	5.3 ± 0.6	8.2 ± 0.1
	reduced	7.0	0.726 ± 0.002	295.3 ± 5.0	3.4 ± 0.6	12.5 ± 0.6
		2.0	0.708 ± 0.006	362.5 ± 7.1	−4.4 ± 1.8	13.5 ± 0.3

[a] The value in parentheses shows the number of S−S bonds in the protein molecule.

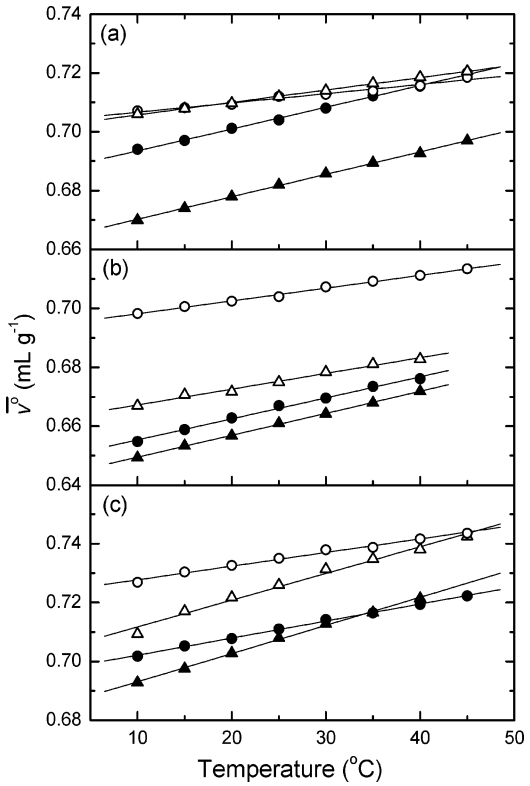


FIGURE 3: Plots of $\bar{v}°$ as a function of temperature for oxidized and reduced forms of lysozyme (a), RNase A (b), and BSA (c) at pH values of 7 and 2. Key: ○, oxidized at pH 7; ●, reduced at pH 7; △, oxidized at pH 2; ▲, reduced at pH 2. Solid lines were obtained by least-squares linear regression.

atomic packing (ν_{cav}), and the volume change due to hydration ($\Delta\nu_{sol}$):

$$\bar{v}° = \nu_c + \nu_{cav} + \Delta\nu_{sol} \quad (3)$$

Since the constitutive atomic volume may be assumed to be incompressible, the coefficient of partial specific adiabatic compressibility is mainly determined by the internal cavity and surface hydration (*14*, *15*):

$$\bar{\beta}_s° = -(1/\bar{v}°)(\delta\bar{v}°/\delta P)_s = -(1/\bar{v}°)[(\delta\nu_{cav}/\delta P) + (\delta\Delta\nu_{sol}/\delta P)] \quad (4)$$

The cavity contributes positively and hydration contributes negatively to $\bar{v}°$ and $\bar{\beta}_s°$; thus the experimentally observed $\bar{\beta}_s°$ can be positive or negative depending on the relative magnitude of each contribution. Since the cavity has a large compressibility (*14*), only a small change in the cavity significantly affects the compressibility. The coefficient of thermal expansibility, α, also involves two contributions of cavity and hydration (*16*):

$$\alpha = (1/\bar{v}°)(\delta\bar{v}°/\delta T)_p = (1/\bar{v}°)[(\delta\nu_{cav}/\delta T) + (\delta\Delta\nu_{sol}/\delta T)] \quad (5)$$

The cavity expands and hydration decreases as temperature increases, so both terms contribute positively to α.

As shown in Table 1, $\bar{v}°$ and $\bar{\beta}_s°$ decrease and α increases when the S−S bonds are reduced for all of the proteins examined at the pH values tested, except for $\bar{v}°$ of ovalbumin at pH 2, the data for which include a large experimental error. These changes in volumetric parameters can be largely attributed to modifications in hydration and the cavity because the constitutive atomic volume (ν_c) is only slightly affected by carboxamidomethylation (at most, by 0.001 mL g^{-1}). Decreases in $\bar{v}°$ and $\bar{\beta}_s°$ are possible if the constitutive atoms are packed more densely by reduction to break off the energetic strain of a protein molecule. However, this is not consistent with the results of CD and fluorescence spectra, which indicate the unfolding of protein molecules. Therefore, the decreases in $\bar{v}°$ and $\bar{\beta}_s°$ can be attributed to an increase in hydration and/or decrease in the cavity upon unfolding. This is consistent with the increase in α since α is more strongly affected by hydration than by cavity (*16*).

Figure 4 shows plots of $\bar{\beta}_s°$ against $\bar{v}°$ for the oxidized and reduced proteins at two pH values. The $\bar{\beta}_s°$ value increases with increasing $\bar{v}°$ as found statistically for other

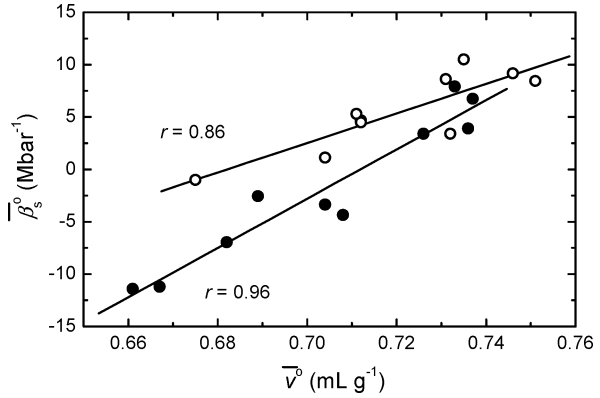


FIGURE 4: Plots of $\bar{\beta}_s^\circ$ against $\bar{v}^\circ$ of the oxidized (○) and reduced (●) forms of five proteins: ovalbumin, β-lactoglobulin, lysozyme, RNase A, and BSA. Solid lines were obtained by least-squares linear regression (r, correlation coefficient).

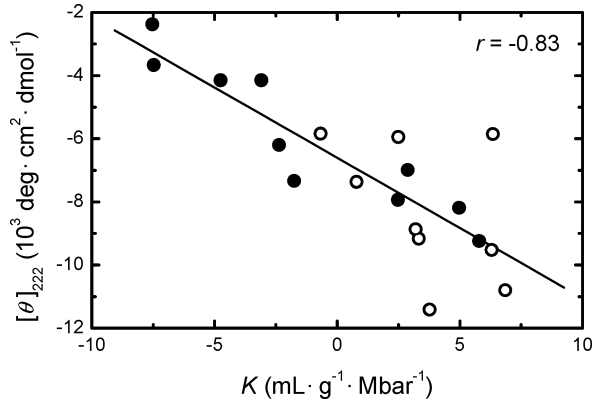
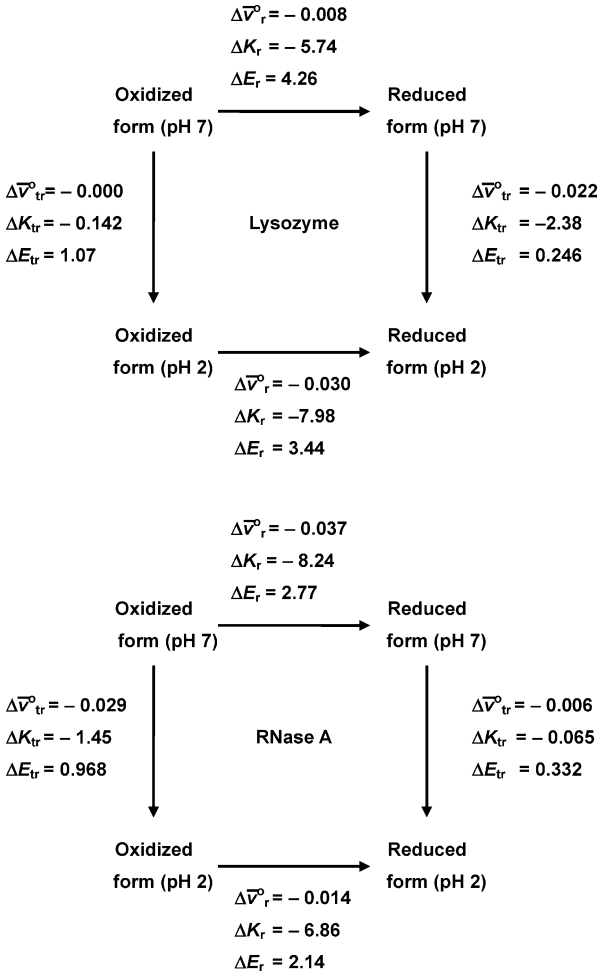


FIGURE 5: Plots of $[\theta]_{222}$ against the compressibility (K) of oxidized (○) and reduced (●) forms of the five proteins listed in the legend of Figure 4. The solid line was obtained by least-squares linear regression.

native proteins (*15*). This provides further evidence that reduction of S−S bonds concomitantly induces the increase in hydration and decrease in the cavity. It is noteworthy that the slope is greater for the reduced proteins than for the oxidized proteins, which suggests that the breaking of S−S bonds induces conformation changes more effectively in rigid proteins than in flexible ones.

Relationship between Conformation and Volumetric Properties. The effects of reduction on the volumetric properties can be quantitatively evaluated using the difference in $\bar{v}^\circ$, the compressibility K ($=\bar{v}^\circ\bar{\beta}_s^\circ$), and the expansibility E ($=\alpha\bar{v}^\circ$) between the oxidized and reduced proteins. However, reduction of S−S bonds accompanies changes in secondary and tertiary structures and expansion of polypeptide chains, and so it is difficult to attribute the changes in volumetric parameters to any one of these factors. A possible approach is to examine the correlation between the volumetric parameters and the spectroscopic data. Figure 5 shows that there is a strong correlation between $[\theta]_{222}$ and K in all of the oxidized and reduced proteins examined. A slightly positive correlation ($r = 0.51$) is also found between $[\theta]_{222}$ and E (data not shown). These results suggest that the protein structure becomes more compressible and expansible by increasing the remaining secondary structure and that the changes in volumetric properties induced by reduction are dominantly caused by unfolding of the secondary structure. More quantitative discussion based on the α helix and β sheet

Scheme 1: Volumetric Diagram of the Reduction of Lysozyme (top) and RNase A (bottom) at pH Values of 7 and 2[a]



[a] The units for $\Delta\bar{v}^\circ$, ΔK, and ΔE are mL g^{-1}, mL g^{-1} Mbar^{-1}, and mL g^{-1} K^{-1}, respectively.

contents would require detailed CD data at the shorter wavelength.

The changes in the volumetric parameters ($\Delta\bar{v}^\circ$, ΔK, and ΔE) for lysozyme and RNase A at pH values of 7 and 2 are shown in Scheme 1, in which the subscripts of each parameter ("r" and "tr") refer to the reduction and pH transfer processes, respectively. The large negative value of ΔK_r relative to $\Delta\bar{v}_r^\circ$ for lysozyme at pH 7 suggests that the reduction-induced conformational change accompanies a significant decrease in the cavity, since the adiabatic compressibility of the cavity itself is very large compared to that of water (*15*). Much higher negative values of $\Delta\bar{v}_r^\circ$ and ΔK_r are observed at pH 2, although the $\bar{v}^\circ$ and $\bar{\beta}_s^\circ$ values of the oxidized protein are very close at both pH values tested. This is possible because the reduced protein should be more extensively unfolded at a lower pH due to the electrostatic repulsion between the increased positive net charges, as confirmed by the large negative values of $\Delta\bar{v}_{tr}^\circ$ (−0.018 mL g^{-1}) and ΔK_{tr} (−2.38 mL g^{-1} Mbar^{-1}) for the transfer process of reduced protein from pH 7 to pH 2. At present, it is unknown how these volumetric parameters are affected by the high isoelectric pH of lysozyme. Although the contribution of ionization (protonation) of charged groups is also involved in $\Delta\bar{v}_{tr}^\circ$ and ΔK_{tr}, it would not be dominant

because the negligibly small values of $\Delta\bar{v}_{tr}°$ (0.0 mL g^{-1}) and ΔK_{tr} (−0.142 mL g^{-1} Mbar^{-1}) are observed for the oxidized protein whose charged groups are fully exposed to the solvent as well as for the reduced proteins. It is noteworthy that the values of $\bar{v}°$ (0.682 mL g^{-1}) and $\bar{\beta}_s°$ (−7.0 Mbar^{-1}) of reduced lysozyme at pH 2 are larger than those ($\bar{v}° = 0.579$ mL g^{-1}, $\bar{\beta}_s° = -19.9$ Mbar^{-1}) of the protein denatured by guanidine hydrochloride (*26*). This shows that lysozyme is not completely unfolded by reduction even at pH 2, as suggested by our spectroscopic data (Figure 1).

The positive value of ΔE_r indicates that the reduced lysozyme is more thermally expansible than oxidized lysozyme, mainly due to increased hydration since the cavity should decrease on reduction, as revealed by the large negative values of $\Delta\bar{v}_r°$ and ΔK_r. A slightly smaller ΔE_r value at pH 2 than at pH 7 is associated ΔE_{tr}, having a larger positive value for the oxidized protein than for the reduced protein. This suggests that the oxidized lysozyme, relative to the reduced lysozyme, expands more extensively due to the cavity effect at lower pH, since the oxidized protein is less hydrated than the reduced protein.

RNase A and lysozyme both have four S−S bonds and are of a similar molecular size. As shown in Scheme 1, however, RNase A has remarkably different volumetric properties from lysozyme: the dominant changes are found in $\Delta\bar{v}_r°$ and ΔK_r at pH 7 and in $\Delta\bar{v}_{tr}°$ and ΔK_{tr} of the oxidized protein. The large negative values of $\Delta\bar{v}_r°$ (−0.037 mL g^{-1}) and ΔK_r (−8.24 mL g^{-1} Mbar^{-1}) indicate that the S−S bonds are crucial for the compactness of RNase A relative to lysozyme. However, the values of $\bar{v}°$ (0.667 mL g^{-1}) and $\bar{\beta}_s°$ (−11.2 Mbar^{-1}) of the reduced protein at pH 7 are comparable to those at pH 2 and are still considerably larger than those ($\bar{v}° = 0.630$ mL g^{-1}, $\bar{\beta}_s° = -29.1$ Mbar^{-1} at 15 °C) for the form completely denatured by guanidine hydrochloride (*18*). Therefore, RNase A as well as lysozyme is not completely unfolded by reduction even at pH 2. The large negative values of $\Delta\bar{v}_{tr}°$ (−0.029 mL g^{-1}) and ΔK_{tr} (−1.45 mL g^{-1} Mbar^{-1}) suggest that acid denaturation accompanies an increase in hydration and a decrease in the cavity to a greater extent in the oxidized protein than in the reduced protein. However, the oxidized protein at pH 2 is still more compact than the reduced protein at pH 7, and a drastic change in compactness is brought about in the reducing process at pH 2. These results are consistent with the observation from small-angle X-ray scattering (*8*) that the reduced RNase A is more expanded and is in neither a random-coil (*27*) nor a compact denatured state (*28*).

The coupling effects of reduction and pH on the volumetric properties of the remaining three proteins can also be quantitatively discussed using Scheme 1. One conclusion is that the compactness of protein molecules is sensitively reflected in the volumetric parameters through the complicated changes in the cavity and hydration depending on the number and location of S−S bonds.

Effects of Number and Location of Disulfide Bonds. Figure 6a shows a plot of the $\bar{\beta}_s°$ values of various native proteins taken from our previous study (*15*) involving papain and pepsinogen measured in this study, as a function of S−S bond density, ν_s/N_t, which is defined as the ratio of the number of S−S bonds, ν_s, to the total number of amino acid residues, N_t, of a protein. There is evidently a highly linear

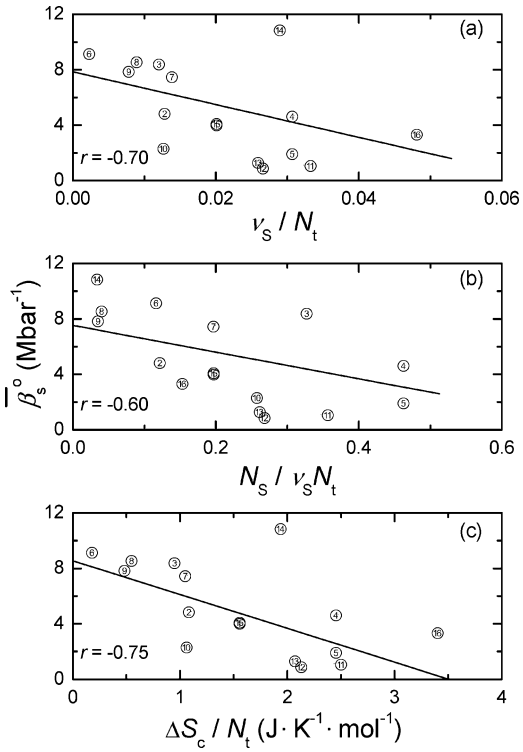


FIGURE 6: Plots of $\bar{\beta}_s°$ against ν_s/N_t (a), $N_s/\nu_s N_t$ (b), and $\Delta S_c/N_t$ (c) of native proteins at neutral pH. $\Delta S_c/N_t$ is expressed in units per mole of amino acid residue. The $\bar{\beta}_s°$ data for 14 proteins except papain and pepsinogen are taken from a previous paper (*15*). The numbers on the plots indicate the proteins: 1, α-chymotrypsinogen; 2, conalbumin; 3, β-lactoglobulin; 4, hen lysozyme; 5, turkey lysozyme; 6, ovalbumin; 7, papain; 8, pepsin; 9, pepsinogen; 10, peroxidase; 11, RNase A; 12, trypsin; 13, trypsinogen; 14, BSA; 15, α-chymotrypsin; and 16, ovomucoid. Solid lines were obtained by least-squares linear regression while excluding the data for BSA.

relationship between the two parameters ($r = -0.70$). This implies that the greater the number of S−S bonds, the larger their effects on forcing the structure to compact, and that the S−S bonds act to reduce volume fluctuations in the protein structure.

To what extent does each S−S bond contribute to the compactness or volume fluctuation of the structure of a protein? At present, it is difficult to answer this question since the S−S bonds were totally rather than selectively reduced by carboxamidomethylation in our experiments. However, we can estimate the effects of the location of S−S bonds from the average number of residues per unit S−S cross-linkage normalized by the total residue number, $N_s/\nu_s N_t$, in which N_s is the sum of the number of residues enclosed by the S−S linkages. Figure 6b shows that a plot of the $\bar{\beta}_s°$ values against $N_s/\nu_s N_t$ has a negative slope ($r = -0.60$), suggesting that the farther apart in the primary structure the cross-linked residues are, the greater their contribution to compactness of the protein structure.

The effects of the number and location of S−S bonds can be evaluated more quantitatively by the conformational entropy of the polypeptide chain. According to the statistical analysis of polymer chains (*4*), the increase in conformational entropy by the reduction of S−S bonds, ΔS_c, is

$$\Delta S_c = 0.75(2\nu_s R)(\ln N_s/2\nu_s + 3) \qquad (6)$$

where R is the gas constant. Figure 6c shows a plot of the

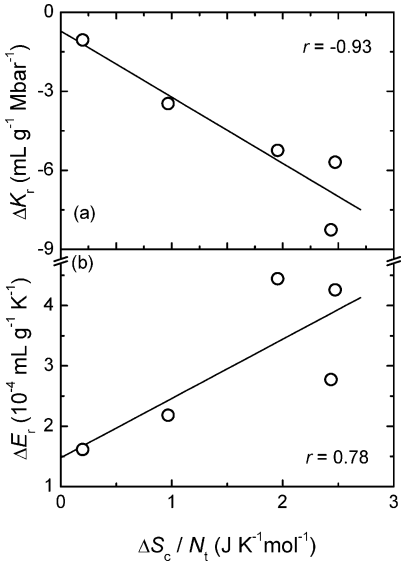


FIGURE 7: Plots of ΔK_r (a) and ΔE_r (b) of the five proteins (pH 7) as a function of $\Delta S_c/N_t$. $\Delta S_c/N_t$ is expressed in units per mole of amino acid residue. Solid lines were obtained by least-squares linear regression.

$\bar{\beta}_s°$ value against the conformational entropy change normalized by the total residue number, $\Delta S_c/N_t$, of native proteins. The observed negative slope provides evidence that the S−S bonds contribute to compactness of native proteins through entropic forces and that the number and location of S−S bonds are decisive factors in the compactness of protein structures. This can be further confirmed by comparing the effects of reduction on the conformational entropy and the volumetric parameters. Figure 7 shows plots of ΔK_r and ΔE_r against $\Delta S_c/N_t$ for the five proteins at pH 7: a similar relationship is observed at pH 2 (data not shown). As expected, there is a good correlation between these parameters, supporting the hypothesis that the effect of reduction on the compactness of the protein structure becomes more significant as the number of S−S bonds increases and as they are formed over larger distances in the primary structure.

Prediction of $\bar{\beta}_s°$ and α of Reduced Unknown Proteins. From Figure 7, we may expect that the reduction-induced changes in $\bar{\beta}_s°$ and α, $\Delta\bar{\beta}_s°$ and Δα, are also dependent on the number and location of S−S bonds although $\bar{\beta}_s°$ and α are intensive properties different from K and E. Then a multiple regression analysis was performed for the $\Delta\bar{\beta}_s°$ and Δα values of five proteins in Figure 7 using ν_s/N_t and $N_s/\nu_s N_t$ as independent variables:

$$\Delta\bar{\beta}_s° = -0.04 - 264.2\nu_s/N_t - 3.6N_s/\nu_s N_t \quad (7)$$

$$\Delta\alpha = 2.2 + 125.6\nu_s/N_t - 1.9N_s/\nu_s N_t \quad (8)$$

Despite the wide variation in the number and location of S−S bonds of these proteins, the values of $\Delta\bar{\beta}_s°$ and Δα calculated from eqs 7 and 8 are in good agreement with those obtained experimentally, as indicated by high multiple correlation coefficients of 0.92 and 0.91, respectively. This allows us to predict the effect of reduction of each S−S bond on $\bar{\beta}_s°$ and α in unknown proteins with reasonable certainty. For example, we can predict that the $\bar{\beta}_s°$ value (4.7 $Mbar^{-1}$) of lysozyme at pH 7 decreases to 3.0, 3.0, 2.4, and 2.1 $Mbar^{-1}$ by reducing one of four S−S bonds: residues 64−80, 76−94, 30−115, and 6−127, respectively. This order of $\bar{\beta}_s°$ values is consistent with that of an increasing number of residues enclosed by each S−S linkage. When four S−S bonds are reduced step by step in the order of residues 6−127, 30−115, 76−94, and 64−80, the $\bar{\beta}_s°$ value would decrease to 2.1, −0.6, −2.7, and −5.2 $Mbar^{-1}$, respectively. This suggests that reduction which induces a large change in $\bar{\beta}_s°$ and α may cause unfolding of the structure. Thus the multiple regression analyses for $\Delta\bar{\beta}_s°$ and Δα will be useful for the preliminary estimation of the contribution of each S−S bond to the compactness or flexibility of protein structures, which cannot be predicted easily from the X-ray structure.

CONCLUDING REMARKS

The present study shows that the volume, compressibility, and expansibility of proteins are sensitively influenced by reduction of S−S bonds, due partly to the loss of cavity and partly to the increase of hydration associated with the conformational changes. The effects of reduction become more significant as the number of S−S bonds increases and as they are formed over a larger distance in the primary structure. These results demonstrate that S−S bonds play an important role, mainly via the entropic force, in the three-dimensional structure and compactness of protein molecules. The volumetric study of mutant proteins whose S−S bonds are site-directedly replaced by other amino acids should lead to a more detailed understanding of the role of each S−S bond to the compactness and flexibility of protein structures.

REFERENCES

1. Anfinsen, C. B., Haber, E., Sela, M., and White, F. H., Jr. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1309−1314.
2. Creighton, T. E. (1980) *J. Mol. Biol. 144*, 521−550.
3. Gilbert, H. F. (1994) in *Mechanism of Protein Folding* (Pain, R. H., Ed.) Chapter 5, Oxford University Press, Cambridge.
4. Flory, P. J. (1956) *J. Am. Chem. Soc. 78*, 5222−5235.
5. Creighton, T. E. (1988) *BioEssays 8*, 57−63.
6. Tanford, C., Kawahara, K., and Lapanje, S. (1967) *J. Am. Chem. Soc. 89*, 729−736.
7. Kella, N. K., Kang, Y. J., and Kinsella, J. E. (1988) *J. Protein Chem. 7*, 535−548.
8. Lee, J. Y., and Hirose, M. (1992) *J. Biol. Chem. 267*, 14753−14758.
9. al-Obeidi, A. M., and Light, A. (1988) *J. Biol. Chem. 263*, 8642−8645.
10. Volles, M. J., Xu, X., and Scheraga, H. A. (1999) *Biochemistry 38*, 7284−7293.
11. Zhou, J. M., Fan, Y. X., Kihara, H., Kimura, K., and Amemiya, Y. (1998) *FEBS Lett. 430*, 275−277.
12. Chen, L., Wildegger, G., Kiefhaber, T., Hodgson, K. O., and Doniach, S. (1998) *J. Mol. Biol. 276*, 225−237.
13. Ueki, T., Hiragi, Y., Kataoka, M., Inoko, Y., Amemiya, Y., Izumi, Y., Tagawa, H., and Muroga, Y. (1985) *Biophys. Chem. 23*, 115−124.
14. Gekko, K., and Noguchi, H. (1979) *J. Phys. Chem. 83*, 2706−2714.
15. Gekko, K., and Hasegawa, Y. (1986) *Biochemistry 25*, 6563−6571.
16. Gekko, K., and Hasegawa, H. (1989) *J. Phys. Chem. 93*, 426−429.
17. Cooper, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2740−2741.
18. Tamura, Y., and Gekko, K. (1995) *Biochemistry 34*, 1878−1884.
19. Kamiyama, T., and Gekko, K. (2000) *Biochim. Biophys. Acta 1478*, 257−266.
20. Taulier, N., and Chalikian, T. V. (2002) *Biochim. Biophys. Acta 1595*, 48−70.

21. Kharakoz, D. P. (1997) *Biochemistry 36*, 10276−10285.
22. Kaminsky, S. M., and Richards, F. M. (1992) *Protein Sci. 1*, 22−30.
23. Hamaguchi, K., and Migita, S. (1964) *J. Biochem. 56*, 512−521.
24. Gekko, K., Kamiyama, T., Ohmae, E., and Katayanagi, K. (2000) *J. Biochem. 128*, 21−27.
25. Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1−63.
26. Kamiyama, T., and Gekko, K. (1997) *Chem. Lett*. 1063−1064.
27. Noppert, A., Gask, K., Muller-Frohne, M., Zirwer, D., and Damaschun, G. (1996) *FEBS Lett. 380*, 179−182.
28. Sosnick, T. R., and Trewhella, J. (1992) *Biochemistry 31*, 8329−8335.

BI030115Q