Compressibility–Structure Relationship of Globular Proteins†

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ABSTRACT: The adiabatic compressibility, $\bar{\beta}_s$, of 11 globular proteins in water was determined by means of sound velocity measurements at 25 °C. All the proteins studied except for subtilisin showed positive $\bar{\beta}_s$ values, indicating the large internal compressibility of the protein molecules. The intrinsic compressibility of proteins free from the hydration effect appeared to be comparable to that of normal ice. The compressibility data for 25 proteins, including 14 reported previously [Gekko, K., & Noguchi, H. (1979) *J. Phys. Chem. 83*, 2706–2714], were statistically analyzed to examine the correlation of the compressibility with some structural parameters and the amino acid compositions of proteins. It was found that $\bar{\beta}_s$ increases with increasing partial specific volume and hydrophobicity of proteins. The helix element also seemed to be a dynamic domain to increase $\bar{\beta}_s$. Four amino acid residues (Leu, Glu, Phe, and His) greatly increased $\bar{\beta}_s$, and another four (Asn, Gly, Ser, and Thr) decreased it. Some empirical equations were derived for the estimation of the $\bar{\beta}_s$ values of unknown proteins on the basis of their amino acid compositions. The volume fluctuations of proteins revealed by the compressibility data were in the range of 30–200 mL/mol, which corresponded to about 0.3% of the total protein volume. The conformational fluctuation seemed to enhance the thermal stability of proteins.

X-ray diffraction data for globular proteins have revealed that they have a precisely defined equilibrium structure in the native state, and the packing densities of atoms or groups within the molecules are as high as those found for crystalline amino acids and small organic compounds (Chothia & Janin, 1975; Richards, 1977). These data suggest a compact, rigid, and static nature of the structure of globular proteins. Nevertheless, there is a considerable body of experimental evidence showing that some packing defects or cavities may exist that permit sizable internal motions and flexibility in response to thermal or mechanical forces (Frauenfelder et al., 1979; Artymiuk et al., 1979). Hydrogen-exchange experiments as well as ones involving other relaxational techniques demonstrated that a protein molecule actually undergoes substantial fluctuations as to the relative positions of its constituent atoms. [Some dynamic aspects and models of protein molecules have been recently reviewed (Careri et al., 1975, 1979; Woodward & Hilton, 1979; Karplus & McCammon, 1981; Käiväräinen, 1985)]. However, a full understanding of the role of the fluctuation in protein functions and biochemical phenomena will require further detailed information on the magnitude of the flexibility or rigidity of protein molecules in solution and on the flexibility–structure relationships.

The flexibility of proteins should be reflected in their compressibility since it is directly related to the volume fluctuation (Cooper, 1976). During the last 10 years, a considerable amount of data has accumulated on the adiabatic compressibility of proteins since the accurate measurement of sound velocity became possible in dilute solutions (Millero et al., 1976; Sarvazyan & Hemmes, 1979; Gekko & Noguchi, 1979; Eden et al., 1982; Gavish et al., 1983; Gekko, 1984). An important finding in these studies as well as earlier ones (Jacobson, 1950; Miyahara, 1956; Anderson, 1963) was that globular proteins exhibit positive compressibility, indicating the large internal compressibility of their molecules. However, the relationship between this compressibility and structure has hardly been discussed at a molecular level, probably because of the complicated effects of hydration on the compressibility. In an earlier paper (Gekko & Noguchi, 1979), we proposed a method to estimate hydration term and found that the compressibility is a function of the hydrophobicity of proteins. Eden et al. (1982) found that the apparent compressibility of cytochrome *c* increases upon oxidation. Gavish et al. (1983) recently predicted that the secondary structure elements may be associated with the dynamic domains of proteins. These findings suggest that the compressibility may rather sensitively depend on the structural characteristics of globular proteins.

In this paper, we report the results of adiabatic compressibility measurements of 11 proteins and discuss the compressibility–structure relationships of globular proteins in terms of some molecular parameters on the basis of the results of statistical analysis of the compressibility data for 25 proteins, including 14 reported previously (Gekko & Noguchi, 1979). Furthermore, the magnitude of the volume fluctuations of these proteins was estimated by using the isothermal compressibility derived from the adiabatic compressibility.

Experimental Procedures

Materials. All the proteins used in this study were commercial products: peroxidase from horseradish (Wako, lot WKE 7791), insulin from bovine pancreas (Fluka, lot CH-9470), carbonic anhydrase from bovine erythrocytes (Biozyme, lot 12X), trypsinogen from bovine pancreas (Millipore Co., lot 18N712), α-chymotrypsin from bovine pancreas (Miles Lab., lot 7049), and α-amylase from *Bacillus subtilis* (Seikagaku Kogyo, lot 1460A). Other proteins were purchased from Sigma: cytochrome *c* from horse heart (lot 62F-7415), catalase from bovine liver (lot 32F-7985), subtilisin BPN′ from *B. subtilis* (lot 22F-0084), soybean trypsin inhibitor (lot 91F-8110), and gelatin from calf skin (lot 122F-0288). These products were used without further purification. The structural characteristics of these proteins are listed in Table I together with those of 14 proteins investigated in a previous study (Gekko & Noguchi, 1979). All other chemicals were special

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Table I: Molecular Parameters of the Proteins Used

sample no.	protein	$10^{-3}M$[a]	H_ϕ[b]	P[c]	fraction (%) of[d] α helix	β sheet	S–S[e]
1	peroxidase	40.0	940	0.94	40[f]	0	4
2	cytochrome *c*	12.4	1049	1.53	27	6	0
3	catalase	232.0 (4)	1029	1.12	50[g]		
4	subtilisin BPN′	27.5	859	0.93	21	31	0
5	insulin	36.0 (6)	996	0.84	31	18	3
6	carbonic anhydrase	30.0	1029	1.01	20[h]		0
7	trypsinogen	23.0	893	1.00	8[i]		6
8	α-chymotrypsin	25.7	908	0.85	8	10	5
9	soybean trypsin inhibitor	21.5	1031	0.93	0[j]		2
10	α-amylase	45.5	970	1.25	20[k]		0
11	gelatin	100					0
12	ribonuclease A	13.7	777	1.73	18	44	4
13	α-chymotrypsinogen A	25.7	908	0.85	9	36	5
14	trypsin	23.0	884	1.01	8[i]		6
15	bovine serum albumin	68.0	990	1.23	70[l]	15	17
16	lysozyme	14.3	893	1.04	29	16	4
17	β-lactoglobulin	18.4	1080	0.97	10[m]	43	2
18	myoglobin	17.0	1038	1.09	77	2	0
19	α-lactalbumin	14.3	1022	1.15	33[n]	17	4
20	hemoglobin	68.0 (4)	985	0.84	70	0	0
21	pepsin	35.5	929	0.87			3
22	α_s-casein	23.6	1054	1.25	6[o]		0
23	ovomucoid	28.0	807	1.26	26[p]	46	9
24	ovalbumin	46.0	992	0.90	33[q]	30	1
25	conalbumin	75.5	995	1.40	11[r]	32	9

[a] Molecular weight. The number of subunits is given in parentheses. [b] The total hydrophobicity divided by the number of residues (cal/residue) (Nozaki & Tanford, 1971; Bigelow & Channon, 1976). [c] Polarity parameter of Bigelow (1967). [d] Determined by circular dichroism or optical rotation analysis. From Chen et al. (1972) and Chou and Fasman (1974), unless otherwise noted. [e] Number of disulfide bonds per molecule (subunit). [f] Strickland (1968). [g] Samejima and Kita (1969). [h] Pocker and Sarkanen (1978). [i] Based on the X-ray analysis by Stroud et al. (1974) and Bode and Schwager (1975). [j] Ikeda et al. (1968). [k] Mitchell et al. (1973). [l] Era et al. (1983). [m] Townend et al. (1967). [n] Nitta et al. (1984). [o] Herskovits (1966). [p] Watanabe et al. (1981). [q] Watanabe et al. (1980). [r] Yeh et al. (1979).

reagent grade products from Wako Pure Chemicals.

Preparation of Sample Solutions. Soybean trypsin inhibitor, catalase, and amylase were dissolved in 0.01, 0.02, and 0.02 M phosphate buffer (pH 7.0), respectively. Insulin was dissolved in 0.03 M phosphate buffer (pH 8.0) after zinc atoms were removed by dialysis against 0.01 M HCl. Carbonic anhydrase was dissolved in 0.04 M Tris buffer (pH 7.6). Other proteins were dissolved in distilled water. All these solutions (protein concentrations 0.5–1.0%) were exhaustively dialyzed against the respective solvents at 4 °C. Five sample solutions of different protein concentrations were prepared by diluting the dialyzed stock solution with the dialysate after purification through a glass filter, taking care to avoid vaporization of the solvent (water). In this work, the Donnan equilibrium effect was not taken into account since the net charge of proteins is small at around neutral pH and the preferential binding of solvent components to proteins is substantially negligible with the buffer compositions used.

Sound Velocity Measurements. The sound velocity in protein solutions was measured by means of a "sing-around pulse" method, of high stability and precision, developed by Greenspan and Tschiegg (1956). The apparatus and procedures were essentially the same as used in the previous studies (Gekko & Noguchi, 1971, 1974, 1979; Gekko et al., 1985). About 15 mL of a sample solution was introduced into an inverted T-type glass cell equipped with two ceramic transducers (3 MHz), one at each end of the horizontal glass tube. The sound path length between the two transducers and the electric delay time of the equipment were predetermined by calibration measurement of the sound velocity in distilled water at various temperatures between 20 and 30 °C. The sample cell was immersed in a water bath at 25 ± 0.001 °C, and the sound velocity in a solution was determined by measuring the pulse repetition with an electronic frequency counter (Hewlett-Packard Model 5221B). The accuracy of the measurements corresponded to a sensitivity of 1 part in 10^6, i.e. ±1 cm/s.

The partial specific adiabatic compressibility of the solute, $\bar{\beta}_s$, is defined as (Shiio, 1958)

$$\bar{\beta}_s = -(1/\bar{v}^\circ)(\partial\bar{v}^\circ/\partial P) = (\beta_0/\bar{v}^\circ)\lim_{c\to 0}[(\beta/\beta_0 - V_0)/c] \quad (1)$$

where

$$V_0 = (d - c)/d_0 \quad (2)$$

$$\bar{v}^\circ = \lim_{c\to 0}[(1 - V_0)/c] \quad (3)$$

β is 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; V_0, the apparent volume fraction of the solvent in solution; and $\bar{v}^\circ$, the partial specific volume of the solute. The values of β and β_0 can be calculated from the sound velocity, u, and the density, d, of the solution or solvent, by the Laplace equation:

$$\beta = 1/du^2 \quad (4)$$

Density Measurements. The densities of the solvents and solutions were measured at 25 ± 0.01 °C with a precision density meter, DMA-02C (Anton Paar, Gratz). The instrument constant was determined by calibration measurement with NaCl solutions of known density (International Critical Tables). The partial specific volumes of proteins, $\bar{v}^\circ$, were calculated with eq 2 and 3 using the density and concentration data.

Protein Concentration Determination. The concentrations of two proteins, insulin and carbonic anhydrase, in solution were determined by absorption measurements with extinction coefficients of 10.52 dL/(g·cm) at 276 nm (Frank & Veros, 1968) and 16.08 dL/(g·cm) at 280 nm (Biozyme analytical

Table II: Partial Specific Volume and Adiabatic Compressibility of Proteins in Water at 25 °C[a]

sample no.	protein	$\bar{v}°$ (mL/g)	du/dc [m·mL/(g·s)]	$\lim_{c\to 0}$ $[(\beta/\beta_0 - V_0)/c]$	$10^{12}\bar{\beta}_s$ (cm²/dyn)
1	peroxidase	0.702	263.9	0.036	2.36
2	cytochrome *c*	0.725	309.3	0.001	0.066
3	catalase	0.733	245.4	0.090	5.45
4	subtilisin BPN′	0.703	275.9	−0.018	−1.11
5	insulin	0.742	206.0	0.155	9.25
6	carbonic anhydrase	0.742	267.6	0.106	6.37
7	trypsinogen	0.718	297.3	0.022	1.34
8	α-chymotrypsin	0.717	281.9	0.067	4.15
9	soybean trypsin inhibitor	0.713	311.3	0.0027	0.17
10	α-amylase	0.725	246.8	0.084	5.12
11	gelatin	0.689	316.4	−0.038	−2.50
12	ribonuclease A	0.704	291.0	0.018	1.12
13	α-chymotrypsinogen A	0.717	302.0	0.066	4.05
14	trypsin	0.719	371.6	0.015	0.92
15	bovine serum albumin	0.735	222.4	0.173	10.5
16	lysozyme	0.712	257.1	0.074	4.67
17	β-lactoglobulin	0.751	276.2	0.144	8.45
18	myoglobin	0.747	254.9	0.149	8.98
19	α-lactalbumin	0.736	250.1	0.138	8.27
20	hemoglobin	0.745	245.0	0.183	10.9
21	pepsin	0.743	251.9	0.145	8.60
22	α_s-casein	0.732	270.0	0.094	5.68
23	ovomucoid	0.696	268.7	0.053	3.38
24	ovalbumin	0.746	234.1	0.153	9.18
25	conalbumin	0.728	272.6	0.080	4.89

[a] The results for 14 proteins (12–25) were taken from the previous paper (Gekko & Noguchi, 1979).

data), respectively. The absorption measurements were carried out with a Jasco UVIDEC-610C spectrophotometer after sound velocity and density measurements. The concentrations of other proteins were determined by the dry-weight method. In most cases, the results were in good agreement with the concentrations estimated by means of absorption measurements with the extinction coefficients given in the literature.

Statistical Analysis. Statistical analysis of the compressibility data was performed with a PC-8801 personal computer (NEC).

Results and Discussion

In all cases, there was a good linear relationship between the apparent partial specific volume and the protein concentration. This allowed easy extrapolation of $(1 - V_0)/c$ to infinite dilution of a protein by means of the least-squares method, leading to the determination of the partial specific volume, $\bar{v}°$, with an accuracy of ±0.002 mL/g. The results are presented in Table II. The $\bar{v}°$ values for most proteins may be regarded as identical with (or close to) the values in the literature, considering the experimental uncertainty and the different experimental conditions. For all the proteins studied, the sound velocity of the solutions increased in proportion to the protein concentration in the concentration range investigated (less than 1%). The concentration dependence of the sound velocity, du/dc, calculated by means of the least-squares method is shown in the fourth column of Table II. The du/dc values for most proteins were in the range of 200–300 m·mL/(g·s), while the value was for trypsin exceptionally high. There was a linear relationship between $(\beta/\beta_0 - V_0)/c$ and c for all the proteins, as shown in Figure 1, in which some proteins were omitted to avoid complication of the figure. The values of $\lim_{c\to 0} (\beta/\beta_0 - V_0)/c$ obtained by an extrapolation procedure and of $\bar{\beta}_s$ calculated with eq 1 are listed in the fifth and last columns of Table II, respectively. The results for 14 proteins reported in a previous paper (Gekko & Noguchi, 1979) are also included in this table since they were used for statistical analysis of the compressibility–structure relationship of proteins. In most cases, the $\bar{\beta}_s$ values were determined with an experimental error of less than 5%.

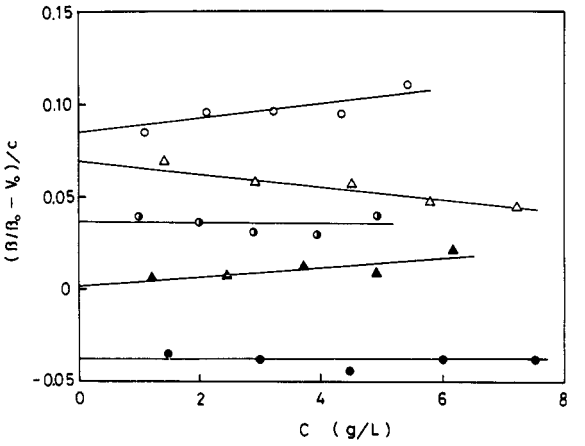

FIGURE 1: Typical plots of $(\beta/\beta_0 - V_0)/c$ against protein concentration, c: (○) α-amylase; (△) α-chymotrypsin; (◑) peroxidase; (▲) cytochrome *c*; (●) gelatin.

Contribution of Hydration and Cavity to the Compressibility. It can be seen in Table II that the $\bar{\beta}_s$ value is positive for all the globular proteins examined except subtilisin. This demonstrates that the interior of globular proteins is highly compressible in contrast to in the case of fibrous proteins (e.g., gelatin) or polypeptides whose $\bar{\beta}_s$ values are negative (Sarvazyan & Hemmes, 1979). Since $\bar{\beta}_s$ of any amino acid in water is negative (Millero et al., 1978; Cabani et al., 1981), we cannot easily deduce the $\bar{\beta}_s$ value of a globular protein from the compressibility data for its constituent amino acids. This situation is completely different from the case of the partial specific volume, which can be estimated with good approximation as the sum of the constitutive atomic or group volumes (Edsall, 1953; Zamyatnin, 1972). The partial specific volume of a protein in water is expressed as the sum of three contributions (Kauzmann, 1959): (1) the constitutive volume estimated as the sum of the constitutive atomic or group volumes (V_c); (2) the volume of the cavity or void in the molecule due to imperfect atomic packing (V_{cav}); (3) the volume change due to solvation or hydration (ΔV_{sol}).

$$\bar{v}° = V_c + V_{cav} + \Delta V_{sol} \quad (5)$$

Table III: Supposed Intrinsic Adiabatic Compressibility of Some Proteins[a]

protein	$10^{12}\bar{\beta}_p$ (cm^2/dyn)
lysozyme	16–23 (5.6–10)[b]
α-chymotrypsinogen A	15–23
ovalbumin	23–28
bovine serum albumin	23–30
β-lactoglobulin	12–22
α_s-casein	9–19
myoglobin (met)	6.1–12[b]
myoglobin (apo)	2.4–8.2[b]
cytochrome *c* (ferri)	14.2[c]
cytochrome *c* (ferro)	8.5[c]
	av 12–20

[a] For details of the estimation, see the text and the previous papers (Gekko & Noguchi, 1979; Gekko, 1984). [b] Gavish et al. (1983). [c] Eden et al. (1982).

Here, V_{cav} involves not only the incompressible cavity formed on the closest packing of atoms but also the compressible void space generated on the random close packing of them. This volume has been estimated to be 0.02–0.05 mL/g, which corresponds to 3–6% of $\bar{v}°$ (Zamyatnin, 1972; Gekko & Noguchi, 1979). ΔV_{sol} is ascribed to three types of hydration, electrostriction around the ionic groups, hydrogen-bonded hydration around the polar groups and hydrophobic hydration. Each of them produces a negative volume change, and the resulting negative ΔV_{sol} has been shown to tend to cancel out almost completely the positive value of V_{cav}.

Differentiation of eq 5 with pressure, P, under the isoentropic (adiabatic) conditions yields the equation for $\bar{\beta}_s$:

$$\bar{\beta}_s = -(1/\bar{v}°)(\partial\bar{v}°/\partial P) = -(1/\bar{v}°)(\partial V_c/\partial P + \partial V_{cav}/\partial P + \partial\Delta V_{sol}/\partial P) \quad (6)$$

The first term on the right-hand side of eq 6 would be negligibly small since the constitutive volume, V_c, may be substantially regarded as the sum of the van der Waals volumes of the constitutive atoms. Thus, the experimentally determined adiabatic compressibility of a protein would be mainly due to the contributions of cavity and hydration

$$\bar{\beta}_s = -(1/\bar{v}°)(\partial V_{cav}/\partial P + \partial\Delta V_{sol}/\partial P) \quad (7)$$

The first term on the right-hand side contributes positively and the second term negatively to $\bar{\beta}_s$, and both terms are canceled out at absolute compression of the order of 10×10^{-12} mL·cm^2/(g·dyn) (Gekko & Noguchi, 1979). Thus, the positive $\bar{\beta}_s$ observed for globular proteins could be ascribed to a large cavity effect overcoming the hydration effect.

It is noteworthy that the $\bar{\beta}_s$ values of globular proteins vary over a considerably wide range whereas their heat capacities remain almost constant, 0.3–0.4 cal/(g·deg) [for example, Privalov (1974)]. This suggests that although the compressibility is a bulk thermodynamic property, it may rather sensitively depend on the structural characteristics of individual proteins. For example, subtilisin, which exceptionally shows a negative $\bar{\beta}_s$ value, is known to have a core of almost entirely packed hydrophobic side chains (Wright et al., 1969). The $\bar{\beta}_s$ values of lysozyme and α-lactalbumin are very different in spite of the high similarity of their primary and secondary structures. Therefore, it is interesting to examine the relationship between the compressibility and the structures of globular proteins. Essentially, such a correlation study might not be performed with $\bar{\beta}_s$ but with the intrinsic compressibility, β_p, of the protein molecule itself, free from the hydration effect, since this allows direct comparison with the results of X-ray studies. The apparent β_p value could be estimated with the equation $\beta_p = -(\partial V_{cav}/\partial P)/(V_c + V_{cav})$, by subtracting the hydration terms from eq 5 and 7. Table III shows the probable

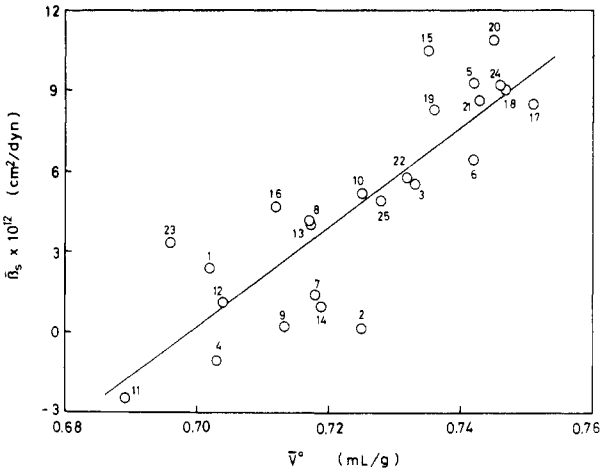

FIGURE 2: Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the partial specific volume, $\bar{v}°$, of proteins. The numbers of the points correspond to the sample numbers of the respective proteins in Table II. The solid line represents the least-squares linear regression, eq 8.

β_p values for some proteins, which were calculated from the analytical data for the hydration terms, ΔV_{sol} and $(\partial\Delta V_{sol}/\partial P)$ (Gekko & Noguchi, 1979; Gekko, 1984). As can be seen in this table, such calculation involves too great variations in β_p for comparison of the values for differnt proteins because of the uncertainty in estimation of the hydration terms. Hereafter, therefore, the statistical analyses were performed with experimentally determined $\bar{\beta}_s$, taking into consideration the effects of both cavity and hydration. Nevertheless, the results in Table III lead to an important suggestion that the intrinsic compressibility of globular proteins is in the order of (10–20) $\times 10^{-12}$ cm^2/dyn, i.e., comparable to that of normal ice.

Correlation between $\bar{\beta}_s$ and Some Molecular Parameters. First, the effect of the molecular weight of proteins on $\bar{\beta}_s$ was examined. According to the results of X-ray analysis, the ratio of the accessible surface area (A_s) to the volume (V) of a protein can be statistically related to its molecular weight (M) with the equation, $A_s/V = 8.77M^{-1/3}$ (Richards, 1977). This equation suggests that an increase in the molecular weight of a protein would diminish the hydration effect (surface effect) relative to the packing effect in the interior of the molecule (volume effect), resulting in an increase in $\bar{\beta}_s$. However, the plots of $\bar{\beta}_s$ against M (molecular weight of the subunit for oligomeric proteins) did not necessarily show a definite correlation. Since most proteins with large molecular weight consist of some subunits or structural domains, the relative effect of the accessible surface area or hydration may not decrease with increasing molecular weight so much as that in the case of a spherical protein with an identical molecular weight. In fact, there is good compensation between V_{cav} and ΔV_{sol} independent of the molecular weight of proteins. Thus, the idea that a protein with a large molecular weight may contain a large amount of cavity and have a large $\bar{\beta}_s$ does not necessarily hold true.

However, it was found that $\bar{\beta}_s$ is highly correlative with the partial specific volume of a protein. As shown in Figure 2, $\bar{\beta}_s$ increases with increasing $\bar{v}°$, as expected from the positive contribution of cavity and the negative contribution of hydration. The least-squares linear regression of these plots yields the following relationship with the high correlation coefficient of 0.85:

$$\bar{\beta}_s = (185.0\bar{v}° - 129.3) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (8)$$

This relationship was also found to cover the $\bar{\beta}_s - \bar{v}°$ data for some other proteins reported, indicating that eq 8 is useful for

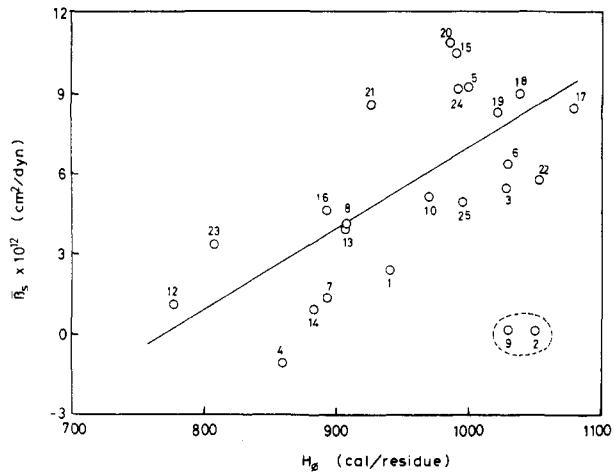

FIGURE 3: Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the average hydrophobic energy, H_ϕ, of proteins. The numbers of the plots correspond to the sample numbers of the respective proteins in Table II. The solid line represents the least-squares linear regression, eq 9, for the 22 proteins other than the two proteins within the dotted line.

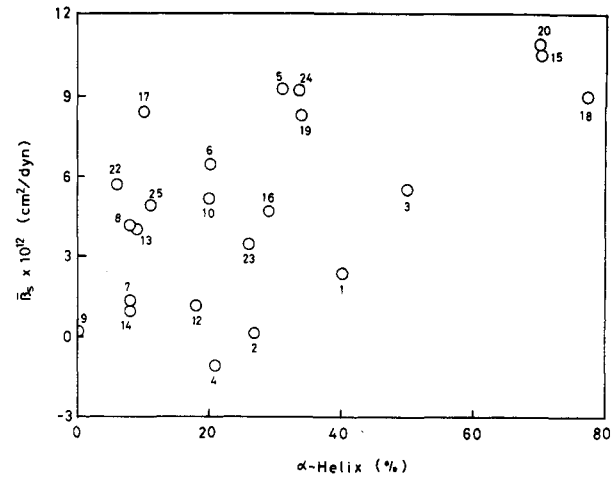

FIGURE 4: Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the α-helix content (%) of proteins. The numbers of the plots are the same as the sample numbers of the respective proteins in Table II.

predicting the $\bar{\beta}_s$ value of an unknown protein from its $\bar{v}°$ value. This equation also suggests that a protein with a $\bar{v}°$ less than 0.70 mL/g would have a negative $\bar{\beta}_s$ on account of the predominant hydration effect. The deviation of some proteins from the regression line is not due to experimental error but would be ascribed to the characteristics of the individual proteins, especially to the difference in the V_c term. If the quantity ($V_{cav} + \Delta V_{sol}$) or ($\bar{v}° - V_c$) is taken instead of $\bar{v}°$, better correlation might be obtained. However, it seems difficult to determine realistic values for this quantity except for some typical cases, because of the almost complete compensation of the two terms V_{cav} and ΔV_{sol} [usually $|V_{cav} + \Delta V_{sol}| < 0.01$ mL/g (Gekko & Noguchi, 1979)].

A property directly related to cavity and hydration is the hydrophobicity of proteins: the cavity would be mainly generated by imperfect packing of hydrophobic amino acid residues localized in the interior of the protein molecules, and the nonpolar surface would cause the decrease in hydration. As shown in Figure 3, there is a good correlation between $\bar{\beta}_s$ and H_ϕ, the average hydrophobic energy per constitutive amino acid residue (Nozaki & Tanford, 1971; Bigelow & Channon, 1976), clearly demonstrating that the more hydrophobic a protein is the more compressible it is. This is also derived from the correlation between $\bar{\beta}_s$ and Bigelow's polarity parameter, P (Bigelow, 1967), which is defined as the volume ratio of polar amino acids to nonpolar ones (Gekko & Noguchi, 1979). The $\bar{\beta}_s$ values of 22 proteins, other than cytochrome c and soybean trypsin inhibitor, can be expressed by the following linear regression with a correlation coefficient of 0.70:

$$\bar{\beta}_s = (0.0293H_\phi - 22.37) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (9)$$

Although the reason for the deviation of the above two proteins is not clear, the small $\bar{\beta}_s$ of soybean trypsin inhibitor may be related to its low helix content as discussed below. For cytochrome c, the oxidation state of its heme iron may be an important factor: the $\bar{\beta}_s$ of this protein has been reported to be -2.56×10^{-12} and 3.19×10^{-12} cm²/dyn for the ferro and ferri states, respectively (Eden et al., 1982). The $\bar{\beta}_s$ obtained in this work lies between these two limiting values probably because our sample is a mixture of the two states.

It is of interest what influences the secondary structures have upon the compressibility of proteins. In Figure 4, the $\bar{\beta}_s$ values of 23 proteins are plotted against their α-helix contents estimated by circular dichroism or optical rotation analysis (Table I). Although the correlation is not satisfactory, the helix-rich proteins seem to be highly compressible: the $\bar{\beta}_s$ values of typical helix proteins, myoglobin, hemoglobin, and bovine serum albumin, are very large compared with those of essentially nonhelix proteins, trypsin, trypsinogen, and soybean trypsin inhibitor. This is positive evidence for the prediction of Gavish et al. (1983) that the secondary-structure elements may be responsible for a dynamic domain of a protein molecule even if the intrinsic compressibility of the helix itself is negligibly small (Noguchi & Yang, 1971; Makino & Noguchi, 1971; Sarvazyan and Hemmes, 1979). Clearly, some proteins with large $\bar{\beta}_s$, despite low helix contents (e.g., β-lactoglobulin and α_s-casein), are highly hydrophobic ($H_\phi > 990$, $P < 1.0$). Thus, the data in Figures 3 and 4 allow us to propose the hypothesis that both the hydrophobicity and helicity contribute in increasing the $\bar{\beta}_s$ of proteins. The multiple correlation coefficient for $\bar{\beta}_s$ of the 23 proteins in Figure 4 was 0.71 when the helix content and H_ϕ were used as independent variables. A similar discussion may be possible for the effect of the β structure on $\bar{\beta}_s$. However, no correlation was found between $\bar{\beta}_s$ and the β-sheet content, suggesting that the β structure may affect the compressibility in a more complicated or negligible manner.

The compressibility of globular proteins would be affected by many other structural factors such as disulfide bonds, prosthetic groups (metal, carbohydrate, and lipid), coenzymes, and domain structures. The latter factor may be particularly important since in many cases clefts or cavities are located between the structural domains (Janin & Wodak, 1983). In the present study, however, these factors were not considered further since they seem to be dependent on the specific individuality of proteins and are thus outside the scope of statistical analysis.

Relationship between $\bar{\beta}_s$ and the Amino Acid Composition. In the previous section, we found that $\bar{\beta}_s$ is correlative with molecular parameters of proteins such as $\bar{v}°$, H_ϕ, and P. These findings suggest that $\bar{\beta}_s$ would be a function of the amino acid composition of proteins since these parameters are closely related to the composition. Then, we examined the relationship between $\bar{\beta}_s$ and the amino acid compositions of a set of proteins (the 23 proteins listed in Table II other than gelatin and amylase) by means of single-residue, group, and multiple correlation analyses. For single-residue correlation analysis, first the volume fraction of each of the 20 different amino acid residues in each protein was computed by using the molar volume of each residue listed in Table IV (Zamyatnin, 1972).

Table IV: Single Residue Correlations of Amino Acids with $\bar{\beta}_s$ of Proteins

amino acid	residue vol (mL/mol)[a]	correlation coeff (r)		P_α[b]	r_{T_m}[c]
Leu	101.9	0.70	group 1	1.21 (H)	0.4627
Glu	85.2	0.46	group 1	1.51 (H)	0.8469
Phe	113.3	0.46	group 1	1.13 (h)	0.2827
His	91.9	0.41	group 1	1.00 (I)	0.2607
Cys	65.0	0.02		0.70 (i)	0.0516
Trp	135.9	−0.01		1.08 (h)	−0.1180
Ala	52.6	−0.02		1.42 (H)	−0.1753
Asp	69.1	−0.03		1.01 (I)	0.1427
Lys	105.1	−0.03		1.16 (h)	0.5776
Arg	109.3	−0.04		0.98 (i)	−0.0267
Val	85.2	−0.06		1.06 (h)	−0.5766
Met	98.4	−0.08		1.45 (H)	0.0452
Pro	73.8	−0.10		0.57 (B)	−0.1797
Tyr	115.9	−0.14		0.69 (b)	−0.1159
Gln	85.8	−0.24		1.11 (h)	−0.1864
Ile	101.9	−0.25		1.08 (h)	0.1543
Thr	70.8	−0.29	group 2	0.83 (i)	−0.3742
Ser	54.9	−0.38	group 2	0.77 (i)	−0.8687
Gly	36.5	−0.51	group 2	0.57 (B)	−0.3418
Asn	68.5	−0.65	group 2	0.67 (b)	−0.0874

[a] Molal volumes of amino acid residues used for the calculation of their volume fractions (Zamyatin, 1972). [b] Helical conformational parameter determined by Chou and Fasman (1978). The symbols in parentheses represent the helical assignments: H, strong helix former; h, former; I, weak former; i, indifferent; b, helix breaker; B, strong breaker. [c] The single residue correlation coefficient for the thermal denaturation temperature (Ponnuswamy et al., 1982).

The volume fraction was adopted as the residue composition since the compressibility is a function of the volume or packing density, and better correlation to $\bar{\beta}_s$ was actually observed in any correlation mode as compared with the residue mole fraction. The influence of each type of residue on $\bar{\beta}_s$ of a protein molecule was determined by calculating the correlation coefficient between them:

$$r = \frac{N\sum XY - (\sum X\sum Y)}{\{[N\sum X^2 - (\sum X)^2][N\sum Y^2 - (\sum Y)^2]\}^{1/2}} \quad (10)$$

where r is the residue correlation coefficient, and N, X, and Y represent the number of proteins, the independent variable (volume fraction of a residue), and the dependent variable ($\bar{\beta}_s$), respectively. The single-residue correlation coefficients, r, thus obtained for the 20 types of residues are given in Table IV together with the helix conformational parameters, P_α, determined by Chou and Fasman (1978). It can be seen that the r values show a wide distribution, from positive to negative values: four residues (Leu, Glu, Phe, and His) show a strong ability to raise $\bar{\beta}_s$; on the contrary, another four (Asn, Gly, Ser, and Thr) lower it. The other 12 residues do not have a noticeable effect on $\bar{\beta}_s$, although Ile and Gln show a slight lowering ability. The small r value for Cys suggests that the disulfide linkage is not a dominant factor that determines the compressibility of proteins. According to the previous section, it may be expected that r is generally positive for nonpolar residues and negative for polar or ionic residues. However, such regularity is not necessarily the case. The r values of Trp, Ala, Val, and Ile are negative despite their nonpolar nature. Most polar or ionic residues show negative r values, but Glu and His have exceptionally large positive ones. These results suggest that the compressibility of proteins is not only affected by the polar or nonpolar nature of the residues but also by other factors such as higher ordered structurs of the polypeptide chain. In this respect, it is interesting to note that all four residues having large positive r values (Leu, Glu, Phe, and His) are helix-structure formers and the two residues having large negative r values (Asn and Gly) are helix-structure breakers (see Table IV). These results support the idea mentioned above that the helix element could be a dynamic domain for volume fluctuation and thus increase the compressibility of proteins. In the cases of Glu and His, the helix-forming effect would overcome the charge effect (hydration effect), resulting in an increase in the compressibility of proteins. The level of r of other residues may be interpreted, in part, as a result of compensation of the two counteracting effects on $\bar{\beta}_s$: helix-forming (or -breaking) ability and polarity (or hydrophobicity).

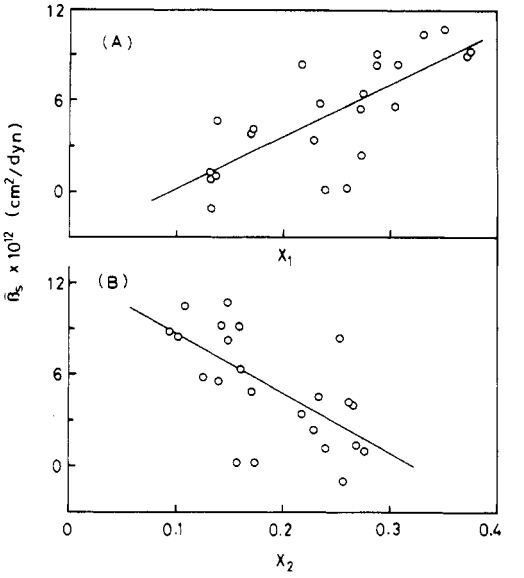

FIGURE 5: (A) Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the total volume fraction, X_1, of the four residues Leu, Glu, Phe, and His. The solid line represents the least-squares linear regression, eq 11. (B) Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the total volume fraction, X_2, of the four residues Asn, Gly, Ser, and Thr. The solid line represents the least-squares linear regression, eq 12.

For group correlation, sets of residues that raised and lowered $\bar{\beta}_s$ were selected on the basis of the r values given in Table IV. The four residues at the top of the table (Leu, Glu, Phe, and His) were taken as the compressibility-raising set (group 1) and the four residues at the bottom of the table (Asn, Gly, Ser, and Thr) as the compressibility-lowering set (group 2). Although Ile and Gln show a slight lowering ability, they were not included in group 2 since the group correlation was highest when the above two sets of four residues were selected respectively for each group. The sum of the volume fractions of respective residues in each group (X_1 or X_2) was computed for each protein and plotted against the $\bar{\beta}_s$ of the protein (Figure 5). Clearly, there is a significant correlation between the two quantities, as indicated by the correlation coefficients, $r_1 = 0.74$ (group 1) and $r_2 = -0.62$ (group 2). The least-squares linear regression of these plots for the two groups was

$$\bar{\beta}_s = (34.15X_1 - 3.26) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (11)$$

$$\bar{\beta}_s = (-37.89X_2 - 12.27) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (12)$$

Thus, since $\bar{\beta}_s$ can be regarded as a function of these two groups of residues, multiple correlation analysis was performed by using both X_1 and X_2 as independent variables following the standard procedures. The multiple correlation coefficient was determined to be 0.74, and the regressed equation was

$$\bar{\beta}_s = (40.9X_1 + 10.1X_2 - 6.83) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (13)$$

The $\bar{\beta}_s$ values predicted with this equation are plotted against the experimentally observed ones in Figure 6, in which the line with a 45° slope represents the perfect agreement between the

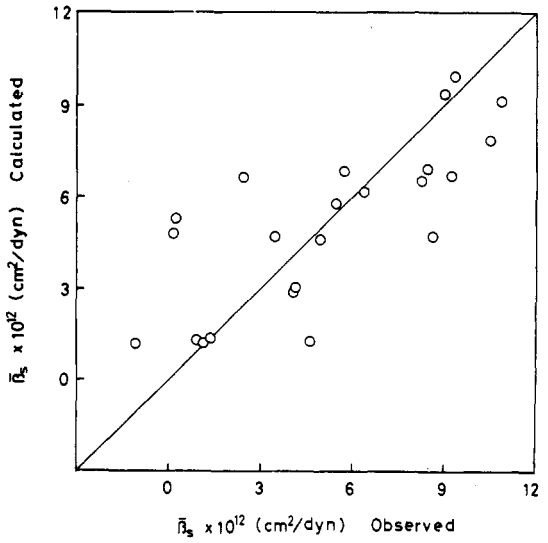

FIGURE 6: Plots of the adiabatic compressibility, $\bar{\beta}_s$, calculated with eq 13 against the experimentally observed values. The line with a 45° slope corresponds to the perfect agreement between the theoretical and experimental values.

two $\bar{\beta}_s$ values. Considering the wide variation in nature of the proteins used, this correlation should be regarded as rather excellent. If appropriate independent variables are further added, the correlation may become much better. With the polarity parameter, P, in addition to X_1 and X_2, the multiple correlation coefficient was found to be 0.76 for the regressed equation

$$\bar{\beta}_s = (30.7X_1 - 3.47X_2 - 3.12P + 1.58) \times 10^{-12} \text{ cm}^2/\text{dyn} \quad (14)$$

As discussed above, the compressibility of globular proteins is highly correlative with their amino acid compositions. This suggests that the flexibility of protein molecules is predominantly determined at the level of the amino acid composition and that it will be possible, to a fair certainty, to predict the compressibility of an unknown protein if its amino acid composition is known by using some equations derived (e.g., eq 13 and 14).

Volume Fluctuation. We have so far discussed the compressibility–structure relationship on the basis of a rigid and static nature of protein structures. As mentioned in the introduction, however, there is much evidence showing that globular proteins undergo considerable fluctuations. The positive $\bar{\beta}_s$ values observed clearly demonstrate that the interior of protein molecules is really flexible and dynamic. According to statistical thermodynamics, the volume fluctuation of a protein, δV, can be related to the isothermal compressibility, $\bar{\beta}_T$, with the equation (Cooper, 1976)

$$\overline{\delta V^2} = kTV\bar{\beta}_T \quad (15)$$

where k is the Boltzmann constant, T the absolute temperature, and V the total volume of the protein. This relation allows us to estimate the volume fluctuation of a protein when its $\bar{\beta}_T$ value is known. Experimental data on $\bar{\beta}_T$ of proteins are very scarce since it is technically difficult to measure the partial specific volume as a function of the hydrostatic pressure on densimetric or ultracentrifugal analysis. However, $\bar{\beta}_T$ values can be derived from the adiabatic compressibility, $\bar{\beta}_s$, with the equations

$$\bar{\beta}_T = \bar{\beta}_s + \alpha^2 T\bar{v}^\circ/c_p \quad (16)$$

$$\alpha = (1/\bar{v}^\circ)(\partial\bar{v}^\circ/\partial T) \quad (17)$$

Table V: Isothermal Compressibility and Volume Fluctuation of Proteins at 25 °C[a]

protein	$10^{12}\bar{\beta}_T$ (cm²/dyn)	δV_{rms} (mL/mol)	$100 \times (\delta V_{rms}/V)$ (%)
peroxidase	6.70	68.3	0.24
cytochrome *c*	4.27	30.8	0.34
catalase	9.59	201	0.12
subtilisin BPN′	3.22	39.3	0.20
insulin	13.4	94.0	0.35
carbonic anhydrase	10.5	76.0	0.34
trypsinogen	5.58	47.8	0.28
α-chymotrypsin	8.32	62.2	0.33
soybean trypsin inhibitor	4.44	41.1	0.20
α-amylase	9.32	87.0	0.26
gelatin	1.92	57.3	0.08
ribonuclease A	5.48[b]	36.2	0.38
α-chymotrypsinogen A	6.95[b]	56.9	0.30
trypsin	5.16	46.0	0.28
bovine serum albumin	14.6[b]	135	0.27
lysozyme	7.73[b]	44.2	0.43
β-lactoglobulin	11.8[b]	63.6	0.46
myoglobin	13.1	64.1	0.51
α-lactalbumin	12.4	56.9	0.54
hemoglobin	15.0	137	0.20
pepsin	12.7	91.1	0.35
α_s-casein	9.84	64.9	0.37
ovomucoid	7.76	61.2	0.31
ovalbumin	12.1[b]	101	0.30
conalbumin	9.08	111	0.20
			av 0.30

[a] Estimated with eq 15 and 16. See the test for details. [b] Gekko and Noguchi (1979).

where α and c_p are the thermal expansion coefficient and the heat capacity of a protein at constant pressure, respectively. The values of $(\partial\bar{v}^\circ/\partial T)$ and c_p have been determined for some proteins [see the references in Gekko and Noguchi (1979)], and the mean values, $(\partial\bar{v}^\circ/\partial T) = 3.7 \times 10^{-4}$ mL/(g·deg) and $c_p = 0.32$ cal/(g·deg), could be satisfactorily deduced for other unknown proteins. The $\bar{\beta}_T$ values thus calculated from our $\bar{\beta}_s$ values are listed in the second column of Table V. It can be seen that $\bar{\beta}_T$ is greater by (3–4) $\times 10^{-12}$ cm²/dyn than $\bar{\beta}_s$, independent of the nature of the proteins. This difference in the two compressibilities is comparable to that observed for amino acids in water (Cabani et al., 1981). The rms fluctuation of the partial molal volume of a protein, δV_{rms}, was calculated with eq 15 using these $\bar{\beta}_T$ values, and the results are presented in Table V together with the ratio of it to the molal volume, $\delta V_{rms}/V$. The δV_{rms} values fall within the range of 30–200 mL/mol, being dependent on the nature of the proteins (especially the molal volume). These fluctuations correspond to a volume of 2–11 mol of water, which if concentrated in one area, would produce sufficient cavities or channels in the protein molecules to allow the entry of solvent or probe molecules. This interpretation is essentially consistent with the mobile defect model (Lumry & Rosenberg, 1975) or the solvent penetration model (Woodward & Hilton, 1979). Of course, we cannot infer the microscopic characteristics of the fluctuations from the compressibility data as well as other thermodynamic quantities. According to the results of statistical analyses reported in the previous sections, however, such volume fluctuations appear to result predominantly from the imperfect atomic packing of hydrophobic regions and the dynamic-domain character of the α helix.

The order of the volume fluctuation observed is very small relative to the overall dimensions of proteins: the $\delta V_{rms}/V$ values of most proteins fall within the range of 0.2–0.4% (the mean value is 0.3%). If the volume fluctuation comprises uniform expansion and contraction of a molecule, the corre-

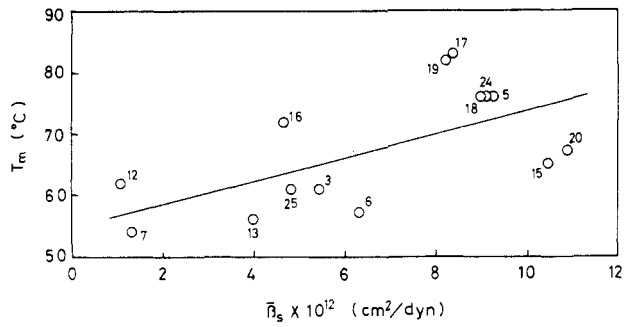

FIGURE 7: Plots of the adiabatic compressibility, $\bar{\beta}_s$, against the thermal denaturation temperature, T_m, of proteins. The numbers of the points are the same as the sample numbers of the respective proteins in Table II. The solid line represents the least-squares linear regression (the correlation coefficient is 0.61).

sponding fluctuation in the radius of the protein molecule would be about 0.1%, e.g., 0.02 Å, for an average radius of 20 Å. This value seems to be somewhat smaller than the magnitude of the fluctuation detected by X-ray analysis (Karplus & MaCammon, 1981). However, it should be noted that the δV_{rms} values estimated here are the lower limit of the probable fluctuation since the solvation factor is still included in the $\bar{\beta}_T$ values used. If the intrinsic isothermal compressibility of protein molecules (although its determination is not easy as mentioned above) is used instead of $\bar{\beta}_T$, then a greater volume fluctuation (at the most, a 50% increase in δV_{rms}) might be expected for the protein molecules in the absence of solvent, water. The $\delta V_{rms}/V$ values obtained (about 0.3%) are considerably small compared with the ratios of the cavity space to the total volume, V_{cav}/V, which were estimated to be 3–6% by densimetric analysis (Gekko & Noguchi, 1979). This suggests that such a void space is essentially incompressible and 10–20% of the space is responsible for the volume fluctuation of protein molecules.

It was found that the isothermal compressibilities of ribonuclease A and chymotrypsinogen increase on pressure denaturation by about 1.5×10^{-12} and 1.57×10^{-12} cm²/dyn, respectively, although the absolute values of $\bar{\beta}_T$ in the native and denatured states remain unknown (Brandts et al., 1970; Hawley, 1971). Using the $\bar{\beta}_T$ values in Table V for the native state, we can deduce the $\bar{\beta}_T$ values of pressure-denatured ribonuclease A and chymotrypsinogen to be 7.0×10^{-12} and 8.5×10^{-12} cm²/dyn, respectively. These results lead to an important suggestion that the volume fluctuation of the two proteins increases by about 10% on pressure denaturation and the enhanced internal flexibility overcomes the effect of increased hydration. Table V as well as Table II may contain some other information on the flexibility–structure relationship of globular proteins, but quantitative comparison of δV_{rms} or $\delta V_{rms}/V$ for different proteins must be made with caution because the $\bar{\beta}_T$ values used involve the hydration effect. At the present stage, it should be emphasized that the volume fluctuation of globular proteins, as revealed by compressibility, is in the order of 0.3% of the total volume, which corresponds to 0.1% of the average radius.

Relationship between Fluctuation and Structural Stability. A matter of interest is what role the conformational fluctuation plays in the structural stability of proteins, but at present no general principle is known. This problem may be solved by examining whether or not there is a primary correlation between $\bar{\beta}_s$ and the thermal stability of proteins (the relevant stability is one against pressure denaturation, but the available data are very few). Then, the $\bar{\beta}_s$ values were plotted against denaturation temperature, T_m, for 14 proteins (Figure 7), whose T_m values are well characterized and are available in the literature (Bull & Breese, 1973; Stellwagen & Wilgus, 1978; Donovan et al., 1975). Although the correlation is not satisfactory, the data in this figure suggest that a compressible protein is more thermostable. The thermal stability of proteins has been discussed in terms of the amino acid composition, the aliphatic or hydrophobic index, the average residue volume, the accessible surface area, and their complex roles (Bull & Breese, 1973; Stellwagen & Wilgus, 1978; Argos et al., 1979; Ikai, 1980; Ponnuswamy et al., 1982). Argos et al. (1979) found that the thermal stability of a protein is primarily enhanced by increased internal hydrophobicity and helix-stabilizing residues, which are also compressibility-raising factors, as discussed in the previous section. Ponnuswamy et al. (1982) have recently examined the correlation between the amino acid composition and T_m for 15 proteins including 10 of the proteins in Figure 7. The single correlation coefficient, r_{T_m}, determined by them, is listed in the last column of Table IV. It can be seen that r_{T_m} depends on the residue almost in parallel with the correlation coefficient, r, observed for $\bar{\beta}_s$ (the correlation coefficient between the two coefficients, r_{T_m} and r, is 0.67); that is, a compressibility-raising residue tends to increase the thermal stability of a protein. On the basis of this evidence, we propose the hypothesis that the conformational fluctuation plays an important role in the structural stability of globular proteins. However, further detailed investigation is required to confirm this hypothesis and for its reasonable interpretation, since the present correlation analyses were based on limited numbers and kinds of protein molecules.

In the present study, it was found that the compressibility is considerably correlated with the structural characteristics and amino acid compositions of proteins. Some empirical equations obtained may allow us to determine the compressibilities or volume fluctuations of unknown proteins from their amino acid compositions. Although the compressibility is a bulk thermodynamic property, it could provide some important information on the microscopic states of the interior and/or the surface of protein molecules in combination with the data obtained from X-ray and nuclear magnetic resonance analyses. A further advanced compressibility study is required to elucidate the relationship between the compressibilities and functions of proteins. In this respect, it is interesting that γ-immunoglobulin shows exceptionally large isothermal compressibility (Sharp et al., 1978), which is probably due to the flexible domains for antibody interaction.

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