Partial Volumes and Compressibilities of Extended Polypeptide Chains in Aqueous Solution: Additivity Scheme and Implication of Protein Unfolding at Normal and High Pressure

D. P. Kharakoz

Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, 142292 Pushchino, Moscow, Russia

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ABSTRACT: An empirical additivity method for calculation of the partial volumes and adiabatic compressibilities of extended oligo- and polypeptides having arbitrary amino acid compositions has been developed and tested by comparison with available experimental data. Its accuracy is the best among the known empirical approaches. Comparison of experimental data on protein denaturation with the results of calculation allows one to discriminate between the unfolded and molten globule states of globular proteins and to estimate the extent of unfolding. For the first time, experimental nonlinear data for the volume−pressure relationship in proteins and model compounds have been used to interpret the high-pressure denaturation of proteins. It has been shown that the two denatured states, molten globule and unfolded ones, can be attained by a pressure rise: the molten globule state by moderate pressure and the unfolded one by high pressure. The relationship between volumetric properties and hydration is briefly discussed.

The state and structure of proteins under high pressure is a matter of rapidly growing interest (Robinson & Sligar, 1995; Royer, 1995; Yamagushi et al., 1995). Partial volume and its pressure derivatives (e.g., partial compressibility) are the basic parameters determining the protein stability under elevated pressure at constant temperature. A large body of empirical data on volume and compressibility of proteins and their low-molecular mass constituents has been accumulated. These data can contribute to our understanding of the volumetric properties of native proteins [for review, see Sarvazyan (1991) and Chalikian et al. (1994)]. It seems to be the right time to further develop the empirical basis for interpreting the properties of proteins in the denatured state and to discuss the expectations for protein stability under high pressure on the basis of the normal-pressure volumetric data available.

Other important aspects relating to the volumetric properties of proteins are the hydration−volume and hydration−compressibility relationships. For instance, hydropathy indices for amino acid residues depend on their partial volumes (Kyte & Doolitle, 1982), and consequently, accurate partial volume values are needed for calculation of the indices. Partial compressibility is sensitive to hydration. The major part of the change in compressibility upon global transformations of proteins is due to hydration processes [for review, see Chalikian et al. (1994) and see also recent works by Chalikian et al. (1996b), Priev et al. (1996), and Kharakoz and Bychkova (1997) and references therein]. The greater the hydration, the smaller the partial compressibility; this is a rule for protein solutions at normal temperature and pressure.

The values of partial volume and compressibility of completely unfolded polypeptide chains are essential reference points in the empirical analysis of volume−compressibility−structure relationships. Comparison of the volumetric properties of a protein in a particular conformation with the properties for the completely unfolded state is helpful in the analysis of protein hydration [see, e.g., Kharakoz and Bychkova (1997)]. Unfortunately, these reference points cannot be obtained experimentally for natural proteins in aqueous solutions free of strong chemical denaturants. The only way to estimate these values is by empirical calculation from experimental data on model compounds, whose conformation in solution is extended and fully accessible to the solvent. Small molecules (amino acids and short peptides) and some synthetic polypeptides can be used as model compounds.

The development of empirical additivity schemes for calculation of partial volumes has a long history (Traube, 1899; Cohn & Edsall, 1943). The accuracy of the calculations is rather high; a deviation from experimental data of about 1% was reported for small molecules (Cabani et al., 1981; Zamyatnin, 1984). Compared to the partial volume, the partial compressibility is more sensitive to the intramolecular group−group interactions, and thus, additivity calculations are less precise but still satisfactory; the accuracy is several percent for small molecules, like amino acids and sugars (Kharakoz, 1991), and about 25% for such complex structures as the hydrated surface of native globular proteins (Kharakoz & Sarvazyan, 1993). All of the known additivity schemes, developed for both partial volume (Zamyatnin, 1972, 1984; Iqbal & Verrall, 1988) and partial compressibility (Iqbal & Verrall, 1988) of polypeptides, had a common disadvantage: ionization of atomic groups was not taken into consideration explicitly when the contributions of amino acid residues were evaluated. Besides, none of the additivity methods existing so far has been tested with the use of experimental data on unfolded polypeptides.

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In this report, a new, more accurate, empirical scheme for calculation of the partial volumes and partial adiabatic compressibilities of polypeptides at 25 °C is presented. In this scheme, the effects of ionization are taken explicitly into account. Ionization contributes about 3% to the partial volume and 20% to the partial compressibility. The scheme was verified by a comparison of the calculations with experimental data reported for a large number of short peptides and for several unfolded synthetic polypeptides. For the first time, the nonlinear pressure dependence of partial volumes was included in the analysis of pressure-induced denaturation of proteins. New information on unfolding under normal and high pressures in the absence of strong denaturants was obtained from a comparison of the calculated and experimental values. In particular, it was concluded that (i) the extent of unfolding of proteins under normal conditions is only 30−40% as shown for metmyoglobin and ferricytochrome *c*, (ii) there is a range of moderate pressure values (near 1−2 kbar) within which the pressure-induced unfolding is not possible, and (iii) the pressure-induced unfolding is possible (if at all) only at higher pressures (above 2 kbar), while moderate pressures instead stabilize the molten globule state.

METHODS

Additivity Scheme Formulation

Basic Points. The additivity scheme is based on an approach similar to that used by Iqbal and Verrall (1988). The scheme, however, differs from the latter by an explicit inclusion of ionization effects which were neglected in all earlier approaches. A partial molar quantity $\bar{X}_2$ [volume, $\bar{V}_2$, or adiabatic compressibility, $\bar{K}_2 \equiv -(\partial \bar{V}_2/\partial P)_S$, where P is the pressure and S is the entropy] is calculated in the belief that amino acid residue contributions are additive:

$$\bar{X}_2 = \sum_{i=1}^{N} \tilde{X}_i^\circ + X_{\text{term}}^\circ + \Delta_{\text{ion}}X + \delta X_{\text{dipeptides}} \qquad (1)$$

where N is the number of residues in a polypeptide chain, $\tilde{X}_i^\circ$ is the increment for the ith residue with the side chain being in the non-ionized state; X_{term}° is a constant which accounts for the contribution of terminal groups in the nonionized state, $\Delta_{\text{ion}}X$ is the effect of ionization of atomic groups at given ionic conditions, and $\delta X_{\text{dipeptides}}$ is a specific correction applied only to dipeptides, i.e., the correction for the interaction between the close charged terminal groups in dipeptides.

The basic parameters in eq 1 were obtained from the data on oligoglycines and from comparison of free amino acids with glycine assuming that *the increments of volume and compressibility per side group do not depend on whether the group is attached to the free amino acid or to a completely extended peptide*. At first glance, an alternative way suggested by Reading and Hedwig (1992) seems to be more pertinent. The authors have suggested that tripeptides having different compositions in which the side groups are placed in the middle position should be used. This way is rejected here for two reasons. First, tripeptides with bulky side chains may tend to fold, as follows from the volumetric study performed by Nikitin (1983). Second, to date, the collection of data on volumetric properties of peptides is extremely scanty to allow evaluation of contributions of all the amino acid residues.

The original experimental data used for calculations were obtained at 25 °C in concentration ranges above 0.01 M. The data were extrapolated to zero concentration or measured in highly dilute solutions where the deviation from the limiting value is negligible. Measurements were normally performed in strictly two-component solutions (i.e., at isoionic conditions) except for the cases when ionization effects were determined (for details, see footnotes to the tables in the Supporting Information).

Contributions of Uncharged Residues, $\tilde{X}_i^\circ$. Beginning with triglycine onward, the partial volumes and compressibilities of oligoglycines are additive functions of the number of monomers (Zamyatnin, 1972; Nikitin, 1983; Chalikian et al., 1994); the increments per residue $\tilde{x}(\text{Gly}) \equiv \bar{X}_2(^+\text{H}_2\text{-Gly}_n\text{-O}^-) - \bar{X}_2(^+\text{H}_2\text{-Gly}_{n-1}\text{-O}^-)$ are virtually constant when $n \geq 3$. These increments, $\tilde{V}(\text{Gly}) = 37.5 \pm 0.1$ cm^3/mol and $\tilde{K}(\text{Gly}) = -0.56 \pm 0.32$ kbar^{-1} cm^3 mol^{-1} (averaged values from five literature sources each, see the Supporting Information), are used here as the basic values for estimation of $\tilde{X}_i^\circ$.

The contribution of the ith residue, $\tilde{X}_i^\circ$, is calculated as a sum of the glycyl residue's contribution, $\tilde{x}$(Gly), and the increment per nonionized side group in the corresponding amino acid. The side group increment is obtained as a difference between the partial quantities of a given free amino acid, $\bar{X}_{2,i}$, and free glycine, $\bar{X}_2$(Gly), given that both the amino acid and glycine are in the same standard zwitterionic state. Here, the standard zwitterionic state is the state in which both the α-amino (+) and α-carboxylic (−) groups are completely ionized while the side chain is not (o). This state is designated hereafter by the triplet (+o−). Therefore, $\tilde{X}_i^\circ = \tilde{x}(\text{Gly}) + \bar{X}_{2,i}(\text{amino acid, +o-}) - \bar{X}_2(\text{Gly, +o−})$. For nonionizable amino acids in pure water at commonly used concentrations, above 0.01 M, the fraction of nonzwitterionic forms of the amino acids is negligible, and thus, the increments per side chain can be obtained simply by using experimental partial quantities $\bar{X}_{2,i}$ and $\bar{X}_2$ (Gly). For glutamic acid, the standard zwitterion (+o−) is the dominant form, the fraction of the alternative zwitterionic form (+− o) being 11%, as it follows from the dissociation constants of glutamic acid (the fraction of other ionic states is negligible). The fraction of the (+−o) form causes only a small decrease in the partial volume (by 0.2 cm^3/mol) and in the partial adiabatic compressibility (by 0.04 kbar^{-1} cm^3 mol^{-1}) which is comparable to the scatter of the experimental data. These estimates are based on a comparison between α- and γ-aminobutyric acids (Høiland, 1986a,b) and on an inspection of the data for the α,ω-amino acid family (Chalikian et al., 1993), which show that the decrease in partial volume and compressibility is about 2 cm^3/mol and 0.4 kbar^{-1} cm^3 mol^{-1}, respectively, upon complete transition from the (+o−) state to the (+−o) state. For aspartic acid, the difference between the two ionic forms contributes negligibly, because in this case the two carboxylic groups are closer to each other and therefore their distances from the charged α-amino group differ less than in the glutamic acid. The contribution of the nonstandard zwitterions can also be neglected in histidine, tyrosine, and cysteine, as their fraction is 3% in the former and less than 1% in the latter two.

Table 1: Molar Volumes ($\tilde{V}°$) and Adiabatic Compressibilities ($\tilde{K}°$) of Amino Acid Residues, Some Inorganic Compounds, and Terminal Group Corrections ($X°_{term}$ and $\delta X_{dipeptides}$)

residue	molecular mass (g/mol)	$\tilde{V}°$ ($cm^3\ mol^{-1}$)	$\tilde{K}°$ ($kbar^{-1}\ cm^3\ mol^{-1}$)
amino acid residues[a]			
glycine	57.05	37.5	−0.56
alanine	71.07	54.7	−0.37
α-aminobutyric acid	85.10	69.82	−0.64
valine	99.13	85.05	−0.90
norvaline	99.13	85.96	−0.79
leucine	113.16	101.92	−0.98
isoleucine	113.16	99.86	−1.08
norleucine	113.16	102.01	−0.98
proline	97.12	74.8	−0.51
hydroxyproline	113.12	76.8	−0.02
phenylalanine	147.18	116.06	−1.28
tyrosine	163.18	118.0	−1.32
tryptophan	186.22	138.16	−0.90
histidine	137.14	93.06	−1.09
methionine	131.20	99.62	−0.96
cysteine	103.14	67.71	−1.18
serine	87.08	54.95	−0.87
threonine	101.11	71.12	−0.98
asparagine	114.10	71.56	−1.22
glutamine	128.13	88.16	−0.76
aspartic acid	115.09	68.56	−1.18
glutamic acid	129.11	84.0	−0.78
lysine	128.17	105.7	−1.21
arginine	156.19	121.2	0.01
terminal group corrections			
for uncharged groups ($X°_{term}$)	18.02	16.6	1.20
for interaction of the ionized terminal groups in dipeptides ($\delta x_{dipeptides}$)		1.7	0.06
inorganic compounds[b]			
water	18.02	18.1	0.81
HCl	36.46	17.8	−0.84
HBr	80.91	24.7	−0.09
NaOH	39.99	−5.2	−8.53
KOH	56.11	5.0	−7.83
$CaCl_2$	110.99	17.8	−9.47

[a] References to literature sources, input values, and details of calculations are presented in the Supporting Information. [b] Partial molar volumes and adiabatic compressibilities of pure water and inorganic compounds in dilute solution (Kell, 1975; Del Grosso & Mader, 1972; Millero, 1972; Millero et al., 1977; Mathieson & Conway, 1974). These data are required for calculation of the volumetric properties of peptide salts.

A different situation takes place for lysine and arginine whose dominant zwitterionic form in solution is (o+−). The difference between the (o+−) and (+o−) forms is considerable because in the latter the unlike charges are further apart from each other than in the former. The side chain contributions for these residues were evaluated in a more complex way. The partial volumes and compressibilities of the hypothetical standard zwitterionic forms were first calculated from experimental data, and then the residue contributions were determined as described above. A different way was also used for proline, whose backbone differs in chemical structure from that of glycine.

The set of experimental data used and calculation details are presented in the Supporting Information. Results are summarized in Table 1.

Contribution of Terminal Groups, $X°_{term}$. The contribution of uncharged terminal groups (see Table 1) is determined as the difference between triglycine in the hypothetical nonzwitterionic state (H-Gly_3-OH) and the sum of three glycyl residues: $X°_{term} = \bar{X}_2$(H-Gly_3-OH) − $3\tilde{X}$(Gly). The values of $\bar{X}_2$(H-Gly_3-OH) were obtained from experimental data on oligoglycines (Supporting Information) with consideration of the effect of zwitterion formation (calculated from the data collected in Table 2).

Contribution of Ionization. The above terms describe the peptides in the hypothetical state with all atomic groups uncharged. The ionization contribution, $\Delta_{ion}X$, results from the proton release and binding in solution which include reactions of the protein atomic groups, water, and low-molecular mass constituents of the buffer solution in which the protein is dissolved. The ionization term is evaluated as follows. First, the pH of the isoionic solution is calculated, and the fractions of protonated atomic groups and the amount of dissociated water for this pH value are determined by a standard approach applicable to dilute solutions [see, e.g., Cantor and Schimmel (1980)]. Second, the volume and compressibility changes caused by the ionization reactions are calculated. The required pK values and volumetric effects of the reactions are given in Table 2 (they are the model values valid for the atomic groups separated from other charged groups). Third, if a protein is dissolved in a solution containing a pH buffer, a strong acid or base, the corresponding contributions of the ionization of these components and the corresponding changes in protein ionization should be taken into account. Volumetric data for the phosphate buffer are presented in Table 2, and the data on some inorganic compounds are given in Table 1.

In the case of natural proteins, the total ionization contribution results in a decrease in partial volume and com-

Table 2: Molar Changes in Partial Volume ($\Delta\tilde{V}_2$) and Compressibility ($\Delta\tilde{K}_2$) upon Proton Release from the Side and Terminal Groups, Water, and Phosphate[a]

side chains and ionizable substances	reaction type	pK^b	$\Delta\bar{V}_2^c$ ($cm^3\ mol^{-1}$)	$\Delta\bar{K}_2^c$ ($kbar^{-1}\ cm^3\ mol^{-1}$)
glutamic acid	$AH \rightarrow A^- + H^+$	4.5	−13	−1.60
aspartic acid	$AH \rightarrow A^- + H^+$	4.5	−13[d]	−1.60[d]
cysteine	$AH \rightarrow A^- + H^+$	8.6	−13[d]	−1.60[d]
tyrosine	$AH \rightarrow A^- + H^+$	9.8	−13[d]	−1.60[d]
lysine	$BH^+ \rightarrow B + H^+$	10.1	4	2.30
arginine	$BH^+ \rightarrow B + H^+$	12	4[e]	2.30[e]
histidine(I)	$BH^+ \rightarrow B + H^+$	6.8	2.1	1.20
histidine(II)	$AH \rightarrow A^- + H^+$	11.5	−13[d]	−1.60[d]
α-NH_3^+	$BH^+ \rightarrow B + H^+$	7.9	4	2.30
α-COOH	$AH \rightarrow A^- + H^+$	3.4	−13[d]	−1.60[d]
H_2O	$AH \rightarrow A^- + H^+$		−22.1	−5.13
$H_2PO_4^-$	$AH^- \rightarrow A^{2-} + H^+$	7.2	−23.8	−4.09

[a] Volumetric data for other pH buffers can be found elsewhere [e.g., Høiland (1986a,b)]. [b] The dissociation constants are taken from the book by Cantor and Schimmel (1984), except for the second ionization of histidine (B. Atanasov, personal communication). [c] The volume and compressibility effects are obtained from experimental data of Noguchi and Yang (1971) and Makino and Noguchi (1971) on coiled polypeptides; Kauzmann et al. (1962), Høiland (1986a,b), and Kharakoz (1983) on amino acids and other model compounds; and Heppler and Wooley (1973), Mathieson and Conway (1974), Millero (1972), Kharakoz (1983), and Khare (1962) on water and sodium phosphate. [d] Assumed to be equal to those for the carboxyl groups of polyglutamic acid. [e] Assumed to be equal to those for the lysyl residue.

pressibility of ∼3.5 and ∼20%, respectively (relative to the final calculated values for the unfolded state). The pressure-induced relaxation of ionic equilibrium can additionally increase compressibility. This contribution may be calculated by standard approaches [see Kharakoz and Bychkova (1997) and Sarvazyan and Hemmes (1979) and references therein], but here it will not be considered for two reasons. First, in most cases, it is negligible [at least at pH values ranging from 5 to 11 and also below 2.5; see, e.g., Sarvazyan and Hemmes (1979)]. Second, only the structural part of partial compressibility is of main concern here.

Corrections for Dipeptides, $X_{dipeptides}$. The fourth term of the right-hand part of eq 1 is introduced specifically for calculation of dipeptides. It takes into account the interaction between opposite charges of the terminal groups positioned closely in dipeptides. The interaction reduces total hydration, thus resulting in a molar volume and compressibility increase (by 1.7 $cm^3\ mol^{-1}$ and 0.06 $kbar^{-1}\ cm^3\ mol^{-1}$, respectively, as determined from comparison of the increments of volume and compressibility on going from di- to triglycine with the glycyl increments obtained for longer olygoglycines; see the Supporting Information for numerical data).

In principle, the charged terminal groups interact also with the closest side chains, even nonionized ones, as concluded by Nikitin (1983) and Hedwig et al. (Hedwig & Høiland, 1991; Hedwig et al., 1991; Reading & Hedwig, 1990). The corresponding deviations are on the order of 1% for partial volumes and compressibilities of short peptides. They are not considered here for two reasons. Their contributions are not yet available for all the side groups occurring in proteins, and they are negligible for long peptides which are the focus of our attention.

Assessment of the Additivity Scheme

In Figure 1, the available experimental data on specific partial volumes and compressibilities of di- to pentapeptides are plotted versus calculated values (numerical data can be found in the Supporting Information). The root-mean-square deviations from the lines of identity in panels a and b of Figure 1 are satisfactorily small: 0.006 $cm^3\ g^{-1}$ for volume (0.9%) and 0.0023 $kbar^{-1}\ cm^3\ g^{-1}$ for compressibility (12% for short peptides and 18% for unfolded natural proteins).

Unfortunately, there are only a few coiled polypeptides studied volumetrically in aqueous solution. They are also presented in Figure 1 and show a good agreement between calculation and experiment. Good accuracy of the additivity scheme for extended polypeptides is thus demonstrated.

Comparison with Other Additivity Schemes

The most developed additivity scheme for the partial volume of peptides is that of Zamyatnin (1984). His estimates for the contributions of amino acid residues are systematically lower (by 3.4%, on average) than those presented in Table 1. This is primarily due to his underestimated value for the glycyl residue: 34.8 cm^3/mol, obtained indirectly in his work, compared to 37.5 cm^3/mol, obtained here from the oligoglycine series. On the other hand, ionization of side groups was not considered in Zamyatnin's scheme at all. Therefore, underestimation of the residue contributions was partially compensated for by neglecting the negative ionization effects.

Partial volume increments obtained by the method of Reading and Hedwig (1990), mentioned above, have been reported for serine and aliphatic residues. They are systematically higher (by ∼1.5%) then those reported here. The reasons for this discrepancy may be explained only when the data for all biological amino acids are available. (See also a comment in Basic Points.)

Iqbal and Verrall (1988), in their additivity scheme, used the same value for the glycyl volume contribution as used in the present work, 37.5 cm^3/mol. But they also did not consider ionization, and besides, they estimated increments per amino acid residue without consideration of essential differences in the zwitterionic states of ionizable amino acids in solution. The partial volumes calculated for proteins are in their work 1.5% higher than those in this work. Most striking is the difference between the two schemes in the calculated compressibilities. They obtained −0.059 $kbar^{-1}\ cm^3\ g^{-1}$ for unfolded globular proteins. This is 4.5 times less than −0.013 $kbar^{-1}cm^3g^{-1}$obtained in this work. Such a large discrepancy is difficult to rationalize because basically we used similar methods of calculation. It cannot be explained by the fact that Iqbal and Verrall used a lower

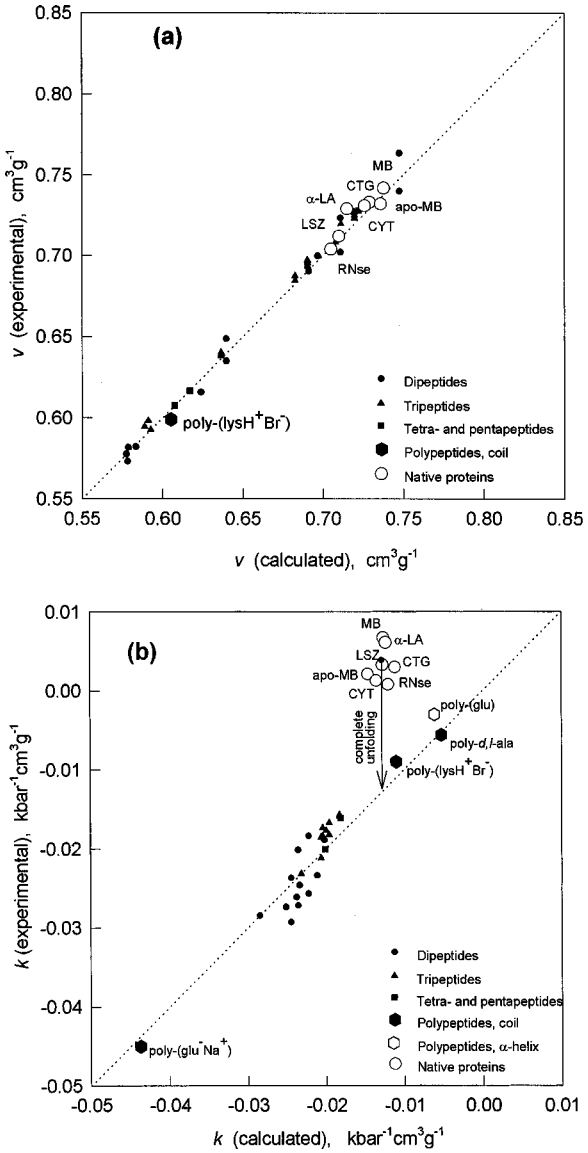

FIGURE 1: Plots of experimental versus calculated specific partial volumes (a) and compressibilities (b) of peptides, polypeptides, and native proteins. The dotted line is the line of identity. Abbreviations: LSZ, lysozyme; α-LA, α-lactalbumin; CYT, ferricytochrome *c*; MB, holometmyoglobin; apo-MB, apometmyoglobin; CTG, chymotrypsinogen; RNse, ribonuclease A. For amino acid residues in polypeptides, conventional abbreviations are used. Numerical data, literature sources, and details of calculations are presented in the Supporting Information.

value for the glycyl residue contribution (-0.85 kbar^{-1} cm^3 g^{-1} instead of -0.56).

Chalikian et al. (1995) made an estimation of partial compressibility of unfolded cytochrome *c* on the basis of the assumption of proportionality between partial compressibility and accessible surface area. The coefficient of proportionality was derived from experimental data on tripeptides. They obtained the value -0.018 ± 0.003 kbar^{-1} cm^3 g^{-1} which is lower than the estimate obtained here for the same protein, -0.0135 kbar^{-1} cm^3 g^{-1}. It is likely that this underestimation was due to the fact that the fraction of charged groups in tripeptides (0.0045 mol/g as expressed in pairs of opposite charges per unit molecular mass) is 3 times higher than that in cytochrome *c* (0.0015 mol/g). The hydration of charged groups significantly reduces the partial compressibility due to the electrostriction of water, and therefore, tripeptides cannot be considered good average representatives of real proteins. The latter conclusion can also be revealed from inspection of Figure 1b.

RESULTS AND DISCUSSION

Calculated Effects of Complete Unfolding of Native Globular Proteins

The partial volumes and compressibilities of a number of native globular proteins are plotted in Figure 1 versus the calculated values for the completely unfolded state. Surprisingly, the native and unfolded proteins have almost the same partial volumes; deviation of their points upward from the line of identity (Figure 1a) is within the accuracy of the calculation. Therefore, the volume change upon complete unfolding is very small with a tendency toward negative values. This is in accord with the conclusion of Durchschlag (1986), who found that denaturation causes only small changes in partial volume. The similarity of the volumes occupied by amino acid residues in the aqueous environment and in the native proteins was noticed long ago (Cohn & Edsall, 1943). It indicates that there is some compensation by different factors determining the partial volumes of the two quite different states of proteins [see Comments on Hydration below, and also see Kauzmann (1959)].

The partial compressibility, in contrast, is definitely larger in the native state than in the unfolded one. Its value, positive for native proteins, becomes negative for the completely unfolded state. The decrease in compressibility is due to the fact that compressible interatomic contacts in the interior of native globule are replaced by atomic group−water contacts (hydration) in the unfolded molecule. The hydration of protein surfaces (be they charged, polar, or nonpolar ones) is known to significantly decrease the compressibility of the solution at room temperature (Gekko & Noguchi, 1979; Kharakoz, 1991; Kharakoz & Sarvazyan, 1993) because of the reduced compressibility of water in the hydration shells.

Comparison with Experimental Data on Protein Denaturation

The calculated volumetric effects of complete unfolding and the available experimental data on protein denaturation are collected in Table 3 and discussed below.

Complete Unfolding (Expectations). Completely unfolded proteins differ from native ones in their partial volume only within the error (see above), with a weak tendency of the volume to decrease upon unfolding. At the same time, the partial compressibility significantly decreases upon complete unfolding, by 0.016 kbar^{-1} cm^3 g^{-1}, on average, and drops into the negative region of the scale of partial compressibilities as shown in Figure 2.

Experimental Unfolding. Tamura and Gekko (1995) studied denaturation of ribonuclease A by guanidine hydrochloride and found that $\Delta\bar{k}_2 = -0.016$ kbar^{-1} cm^3 g^{-1} (extrapolated to zero concentration of the denaturant), which is in accord with the expected value for complete unfolding, -0.013 (Table 3). The partial volume decreased in their experiment by 0.06 cm^3/g which is surprisingly greater than expected from additive calculations (the reason for this discrepancy is not clear).

There are only two proteins, metmyoglobin (Kharakoz & Karshikov, 1984) and ferricytochrome *c* (Chalikian et al.,

Table 3: Experimental Data on Specific Partial Volume ($\Delta\bar{v}_2$ in cm^3 g^{-1}) and Specific Partial Compressibility ($\Delta\bar{k}_2$ in kbar^{-1} cm^3 g^{-1}) Changes upon Protein Denaturation of Different Types in Comparison with Calculated Effects of Complete Unfolding

	calculation for complete unfolding at 25 °C (reference values)[a]		experimental data			
protein (denaturing factor)	$\Delta\bar{v}_2$	$\Delta\bar{k}_2$	$\Delta\bar{v}_2$	$\Delta\bar{k}_2$	experimental conditions	comments
Unfolding Transitions						
metmyoglobin (acid)	−0.004[b]	−0.0193[b]	−0.007[c]	−0.0073[c]	10 mM KCl, transition pHs of 4.2−2.8, 25 °C	*d*
ferricytochrome *c* (acid)	−0.0085[b]	−0.0165[b]	−0.004[c] (−0.001)	−0.0037[c] (−0.0039)	no salt, 25 °C	*e*
ferricytochrome *c* (base)	−0.005[b]	−0.0152[b]	−0.013[c] (−0.014)	−0.0058[c] (−0.038)	no salt, 25 °C	*f*
ribonuclease A (guanidine hydrochloride)	0.001	−0.0128	−0.06	−0.016	water, pH 2.0, 15 °C results are extrapolated to a zero GuHCl concentration	*g*
Native to Molten Globule Transitions						
metmyoglobin (acid)	−0.004[b]	−0.0193[b]	−0.0015[c]	−0.0022[c]	53 mM KCl and higher, transition pHs of 4.3−4.0 or higher (depending on salt concentration), 25 °C	*d*
ferricytochrome *c* (acid)	−0.005[b]	−0.0152[b]	−0.003[c]	0.0015[c]	200 mM CsCl, 25 °C	*e*
cytochrome b_{562} (acid)			(0)	(0.0005)	50 mM potassium phosphate, pH 7, 25 °C	*h*
lysozyme	−0.002	−0.0160				
α-lactalbumin, bovine	−0.014[i]	−0.0195				
α-lactalbumin, human (acid)	−0.008[j]	−0.018[j]	−0.002[c]	0.0018[c]	50 mM KCl, 25 °C	*k*
ribonuclease A (heat)	0.001	−0.0128	−0.035	0.0039	no salt, pH 1.9, 25 °C	*g*
chymotrypsinogen (heat)	−0.004	−0.0141	0.005	0.0026	pH 1.8, 41 °C	*l*
			0.006	0.0032	pH 2.6, 52 °C	
High-Pressure Denaturation						
ribonuclease A	0.001	−0.0128		0.0015	pH 2.0, 0−70 °C, 1−5000 bar	*m*
chymotrypsinogen	−0.004	−0.0141		0.0012	pH 2.07, 5−70 °C, 1−5000 bar	*n*
metmyoglobin	−0.004[b]	−0.0193[b]		>0	pH 5 and 9, pressure below 2 kbar	*o*

[a] Determined as differences between the values for the native (experimental) and completely unfolded (calculated using the additivity scheme) states. See the Supporting Information for numerical data and references. [b] Specific volume and compressibility are calculated as for the apoprotein. [c] Ionization effects are subtracted. [d] From ultrasonic data of Kharakoz and Karshikov (1984) and densitometric data of Katz et al. (1973). [e] From densitometric and ultrasonic experimental data reported by Chalikian et al. (1995) reconsidered here as follows. (i) The effects of proton binding by 13 carboxyl groups are taken from Table 2 instead of the values used by the authors; (ii) for the effects of two Cl^- ions captured by positively charged groups upon the native to molten globule transition, the Cl^- ion is approximated by COO^-, and the corresponding correction is made according to the reaction $NH_3^+ + COO^- \rightarrow NH_2 + COOH$ (numerical data are taken from Table 2). The results calculated in the original work are presented in parentheses, for comparison. [f] From densitometric and ultrasonic experimental data reported by Chalikian et al. (1996a) reconsidered here as follows. (i) Effects of proton binding by atomic groups are taken from Table 2 instead of the values used by the authors; (ii) ionization of the histidyl residue is also taken into consideration. The values calculated by Chalikian et al. are presented in parentheses, for comparison. [g] Tamura and Gekko, (1995). [h] From comparison of the native holoprotein with the molten globule state of the apoprotein (Nölting & Sligar, 1993). [i] An average of two values presented in the Supporting Information, −0.007 and −0.021 cm^3 g^{-1}. [j] Averaged value for two homological proteins, lysozyme and bovine α-lactalbumin. [k] Kharakoz and Bychkova (1997). [l] Dilatometric and ultrasonic measurements (Kharakoz & Sarvazyan, 1980; Kharakoz, 1983). [m] From a two-state transition analysis of the *P*−*T* phase diagram obtained by Brandts et al. (1970) and reconsidered by Hawley (1971). [n] From a two-state transition analysis of the *P*−*T* phase diagram (Hawley, 1971). [o] A qualitative conclusion from the shape of the *P*−*T* phase diagrams [Figure 4 in Zipp and Kauzmann (1973)].

1995, 1996a), for which the volumetric changes upon unfolding have been studied in aqueous solutions without strong denaturants. Unfolding was observed in these cases at a low ionic strength (less than 10 mM) at acidic pH. The most essential though indirect evidence showing that the proteins did unfold under these conditions was the fact that their partial compressibility strongly depended on the ionic strength, until a limiting compact state (molten globule) was reached. The decrease in the partial volume shown in Table 3 is small and comparable to that calculated for complete unfolding. The partial compressibility decreases significantly but not as much as would be expected for complete unfolding; the change is 3 times less (Figure 2). This evidently means that the protein does not completely unfold; a significant fraction of its molecular surface remains poorly accessible to water. This conclusion is qualitatively in accord with that derived by Chalikian et al. (1995, 1996) for cytochrome *c*, despite the fact that the compressibility of the completely unfolded cytochrome *c* estimated here is higher than in the cited papers. Unfortunately, a quantitative estimation of the extent of unfolding is not possible without speculation as to what the moiety that remains poorly accessible to water is. One can reasonably assume that this moiety is a molten globule-like structure (see below) and use the partial compressibility of the molten globule and the completely unfolded states as two opposite reference points for the estimation of the extent of unfolding. Then, the positions of experimental points for unfolded proteins in Figure 2 indicate that the extent of unfolding is only about 30% for metmyoglobin and ferricytochrome *c* denatured by

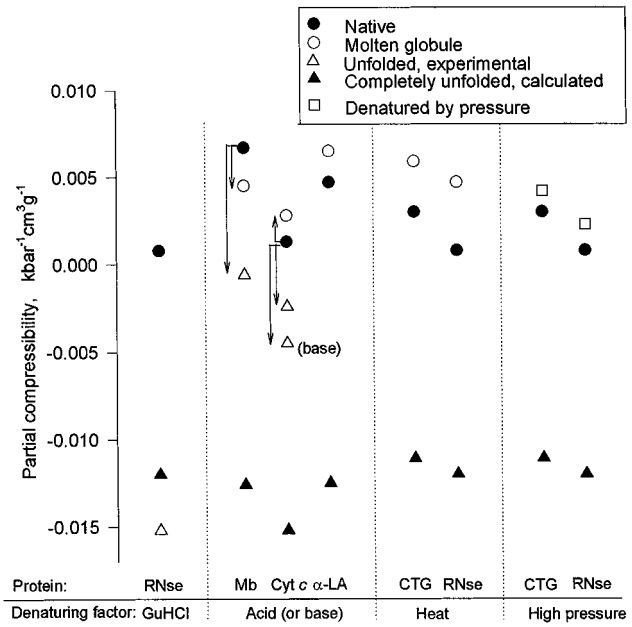

FIGURE 2: Scale of specific partial compressibilities of proteins in different states. Numerical data for the native state are presented in the Supporting Information, and the denaturation-induced changes are taken from Table 3. Abbreviations are the same as in Figure 1.

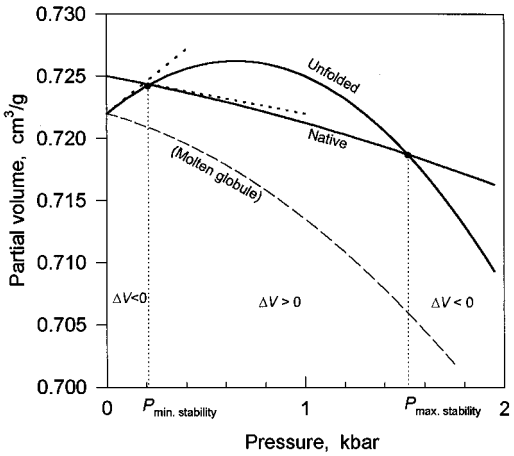

FIGURE 3: Pressure dependence curves for the partial volume of a protein in the native and completely unfolded states (solid lines) calculated by eq 2 with the following parameters: $\bar{v}_2(P_0) = 0.725$ and 0.722 cm^3 g^{-1}; $\bar{k}_2(P_0) = 0.003$ and -0.016 kbar^{-1} cm^3 g^{-1}, and $\partial\bar{k}_2/\partial P = 0.0015$ and 0.02 kbar^{-2} cm^3 g^{-1} for the native and unfolded states, respectively. The dashed line shows the curve for the molten globule state (a qualitative estimate, see the text).

acid and 40% for ferricytochrome *c* denatured by base. The major part of a protein molecule remains, thus, poorly accessible to water. These estimates significantly differ from those obtained by Chalikian et al. (1995, 1996a) because in their works the moiety remaining folded was assumed to be completely inaccessible to water and was modeled by highly compressible nonpolar organic liquids. In my opinion, it is unlikely that a disordered and rather polar protein interior is impenetrable to water [see Kharakoz and Bychkova (1996)].

The compressibility data discussed here are consistent with the expectation that hydrophobic interactions should disfavor complete unfolding and, thus, a true random coil conformation can be reached only in the presence of strong chemical denaturants (Richards, 1992; Creighton, 1992).

Molten Globule Transition. A molten globule is a compact denatured state with an increased hydrodynamic volume, having loosely packed and mobile side chains (Ptitsyn, 1995) and a highly hydrated interior (Kharakoz & Bychkova, 1997). Upon the native-to-molten globule transition, the increase in volume and compressibility caused by the loss of tight packing and the enhanced mobility of side chain groups is considerably compensated for by the interior hydration. The overall changes in the partial volume and compressibility result from an interplay between these counteracting factors. As seen from the experimental data presented in Table 3, the partial volume and compressibility changes can, in principle, be both positive and negative. However, when they are negative, they are still of significantly smaller magnitudes than expected for unfolding of the same protein. It has to be stressed also that the partial compressibility noticeably increases (metmyoglobin is an exception).

Comments on Denaturation by High Pressure: Can It Be Unfolding?

Denaturation caused by pressure is accompanied by an increase in partial compressibility (Table 3, Figure 2). At first glance, this indicates that a native-to-molten globule transition occurs rather than an unfolding, because the latter would cause the compressibility to decrease, as expected from the above consideration. However, such an expectation is valid only in the case of low pressure. The difference in compressibility between the two states can reverse at high pressure. This conclusion follows from the experimental data of Sarvazyan and co-workers (Sarvazyan & Chalikian, 1989; Chalikian, 1989; Sarvazyan et al., 1990) on the pressure dependence of partial compressibilities of native proteins and model compounds. The adiabatic pressure derivative, $(\partial\bar{k}_2/\partial P)_S$, has been determined from ultrasonic measurements under a pressure of 1−200 bar. For globular proteins of about 15 kDa, this value is, on average, 0.0015 kbar^{-2} cm^3 g^{-1}. While for low-molecular mass compounds, it is 1 order of magnitude higher and ranges from 0.006−0.008 for sugars to 0.017−0.025 kbar^{-2} cm^3 g^{-1} for amino acids and nucleotides. This large difference is due to a considerable hydration contribution to this property (see the cited papers). As revealed from the data reported therein, the average atomic group contributions to $(\partial\bar{k}_2/\partial P)_S$ are 0.030 for nonpolar, 0.007 for polar, and 0.017 kbar^{-2} cm^3 g^{-1} for charged atomic groups (the latter value is calculated for a pair of groups bearing unlike charges). Using these values, one can make an estimation for an unfolded protein, taking into account that nonpolar groups amount to about one-half of all the protein atomic groups and that a protein of 15 kDa contains about 20 charged pairs. Thus, for the completely unfolded state, one obtains $(\partial\bar{k}_2/\partial P)_S = 0.02$ kbar^{-2} cm^3 g^{-1}.

Taking into consideration the values obtained, one can estimate the pressure dependence of $\bar{v}_2$ for the native and unfolded states, by means of eq 2

$$\bar{v}_2(P) = \bar{v}_2(P_0) - \bar{k}_2(P_0)(P - P_0) - {}^1/_2(\partial\bar{k}_2/\partial P)_T(P - P_0)^2 \quad (2)$$

(P_0 is normal pressure), the difference between the isothermal and adiabatic derivatives being neglected. The $\bar{k}_2$ values for native and completely unfolded states at normal pressure are assumed to be 0.003 and -0.016 kbar^{-1} cm^3 g^{-1}, respectively. The results of the estimation, presented in Figure 3, allow the following qualitative conclusions.

(i) The partial compressibility of the completely unfolded state, being negative at low pressure [it corresponds to the positive slope of the $\bar{v}_2(P)$ curve], increases very rapidly, and its sign is changed to positive at a pressure which is only 0.7 kbar (the point of maximum volume). It becomes higher than the compressibility of the native state at pressures above 0.8 kbar. Therefore, an experimentally observed positive change of compressibility upon high-pressure denaturation (Table 3 and Figure 2) does not contradict the assumption that the denatured protein is unfolded.

(ii) If the initial volume effect of complete unfolding is slightly negative, the curves for the native and unfolded state intersect at two points, the left being the point of minimum stability and the right one the point of maximum stability of the native state. Between these points, the transition volume effect is positive, and thus, an elevated pressure can only stabilize the native state. The complete unfolding can be reached at pressures considerably above the point of maximum stability.

(iii) If the initial volume effect of transition is a large negative value, $\Delta\bar{v}_2(P_0) < -0.007$ cm^3/g, the complete unfolding is favored by any pressure because in this case the curves never intersect (such a curve is not shown in Figure 3 but is easy to imagine).

Now let us consider the native-to-molten globule transition. The molten globule state is considerably more hydrated than the native state (Kharakoz & Bychkova, 1997) yet is less hydrated than the completely unfolded state. As mentioned above, the nonlinearity coefficient, $\partial\bar{k}_2/\partial P$, is highly sensitive to hydration. Therefore, one can assume that the value $\partial\bar{k}_2/\partial P$ is intermediate for the molten globule, and hence, it can be expected that the partial volume of the molten globule behaves as shown qualitatively by the dashed line in Figure 3. It is always less than the volume of the native state, and thus, an elevated pressure favors this state over the whole scale of P (except for some range of very low pressures if the initial volume change is positive).

Therefore, the question posed in the title of this section is answered positively with some refinement. A high pressure can unfold native proteins; however, first, in some cases, considered above, this cannot happen at moderate pressures (near 1−2 kbar), and second, this can be associated with an increase in partial compressibility only at a pressure higher than 1 kbar or otherwise the positive sign of $\Delta\bar{k}_2$ means that this is a native-to-molten globule state transition.

Comments on Protein Hydration and Its Modeling

The partial volume of a solute can be described as a sum of the following three basic contributions (Kharakoz, 1992, and references therein): (i) the volume of the van der Waals core of the solute [or, in the case of tightly packed native proteins, the globular volume that is impenetrable to solvent in the static sense, i.e., a static "molecular volume" in the terminology of Richards (1977); thus, this term is a sum of the atomic van der Waals cores and the voids between them); (ii) the "thermal motion" contribution, a space required for heat motion at non-zero temperatures; and (iii) the so-called "interaction volume", the effects of solute−solvent interactions caused by electrostriction around charged groups or by the hydrogen bonding of polar groups with water. There is one more contribution, that resulting from translational thermal motions of a molecule as a whole, but it is negligible (1.1 cm^3/mol) in large molecules like proteins.

One can qualitatively explain in terms of the listed factors why the total partial volume of a native protein practically does not change upon unfolding. The van der Waals volume is a conserved property of atomic groups insensitive to intermolecular interactions. Therefore, it does not contribute to the volume change. Also, we may not consider the contributions associated with charged groups because most of them remain solvent-exposed in both native and unfolded states and thus do not contribute significantly to the volume change. The remaining factors can make both positive and negative contributions. Let us consider them.

The positive contributions result from (i) the thermal motion (its contribution is associated with and approximately proportional to the newly exposed molecular surface; Kharakoz, 1992) and (ii) broken intramolecular hydrogen bonds, which cause an increase in partial volume due to the increased atom−atom distance. The negative contributions result from (i) the lost intraglobular voids when a native globule unfolds and (ii) the polar group−water hydrogen bonding for newly exposed polar groups. The listed opposite contributions may compensate for each other. This qualitative picture needs to be made more quantitative which will be done elsewhere. Here, I only stress that the partial volume is weakly dependent on intermolecular interactions for two reasons. First, its major part is determined by a conserved parameter, the van der Waals volume. Second, the volume effect of hydrogen bonding is also a conserved parameter (its value is the same even in such different cases as the polar group−water and the water−water bonds; Kharakoz, 1992).

The compressibility is determined mainly by the rigidity of intermolecular interaction potentials and by the flexibility of the liquid structure. This is why the compressibility is essentially more sensitive than the volume to the changes in the molecular structure and interactions. At room temperature and below, the hydration of most protein atomic groups results in a compressibility decrease (Kharakoz, 1991). This is the reason for the large negative values of partial compressibilities of fully unfolded polypeptides at normal conditions (Figure 1b).

The fact shown in this paper that simple additivity rules are fulfilled for the partial compressibility of fully unfolded polypeptides means that there are no specific features in the hydration of polypeptides which could cause a large difference between polypeptides and small model molecules. This conclusion is valid at least at 25 °C where the additivity scheme has been developed. The physical reasons for the good additivity are, first, the local character of hydration changes in water around separate atomic groups (Buckin et al., 1989) (they overlap no more than two coordination spheres) and, second, the accessible surface of unfolded polypeptides is a randomly fluctuating structure and, thus, cannot serve as a rigid regular matrix to exert a cooperative immobilizing effect on the surrounding water. Therefore, it follows from the results obtained that aqueous solutions of low-molecular mass compounds can model the hydration and compressibility of unfolded proteins.

A different situation could, in principle, be the case for native protein molecules. The surface of a native protein might serve as a rigid matrix immobilizing more than one molecular layer of water, due to a cooperative effect of the large surface. However, even in this case, simple additivity rules are fulfilled for the hydration part of the partial

compressibility to a satisfactory precision (Kharakoz & Sarvazyan, 1993). It means there is no significant cooperative effect in hydration. Therefore, the hydration compressibility of native proteins can be modeled by small molecules. [An opposite point of view has been reported recently by Chalikian et al. (1996b), who concluded that the hydration of polar groups is different in small molecules and in the native globular proteins. This point of view will be discussed elsewhere.]

The molten globule state represents an interesting case of internal hydration, where a large amount of water penetrates inside the globule (Kharakoz & Bychkova, 1997). The internal water may differ essentially from that bound to the outer protein surface. Therefore, small molecules in aqueous solution are poor models for internal hydration. Solutions of water in organic liquids seem to be more pertinent in this case [see Discussion in Kharakoz and Bychkova (1997)].

CONCLUDING REMARKS

(1) The empirical additivity scheme developed here for calculation of partial volumes and compressibilities of completely unfolded polypeptides gives results significantly more accurate than those of the approaches developed earlier. This is primarily due to the explicit counting of the ionization effects neglected earlier.

(2) An estimation of the extent of unfolding of globular proteins is possible, on the basis of the experimental and calculated values of partial compressibility. According to this estimation, only 30−40% of the buried surface of a protein molecule becomes fully exposed to solvent when the protein unfolds in aqueous solution free of strong denaturants.

(3) Interesting conclusions have been reached from the recently reported data on the volume−pressure nonlinearity of protein solutions. One of the most important issues of the nonlinearity is that unfolding at high pressure would cause an increase in partial compressibility, contrary to what is observed at normal pressure. Therefore, the increase in compressibility observed earlier for high-pressure denaturation does not contradict the assumption that denaturation is unfolding. On the other hand, if an increase in compressibility is observed at low pressure (below 1 kbar), the process is, most probably, a native-to-molten globule state transition.

With respect to these observations, the old $P-T$ phase diagrams of proteins, obtained in the pioneering works by Brandts et al. (1970), Hawley (1971), and Zipp and Kauzmann (1973), might be reinterpreted. It is probable that they were not two-state ("native-denatured") but rather three-state diagrams, the area of the denatured state being subdivided into two regions: one for the molten globule state at low pressure and the other for the unfolded state at high pressure. An indication favoring this assumption can be found by inspecting the shape of the most detailed phase diagram, namely that obtained for metmyoglobin (Zipp & Kauzmann, 1973). A family of isopotential lines in the $P-T$ space has been determined for the native-to-denatured state transition at various pHs. The lines obtained at pH 5 and 9 are sharply bent at a certain pressure (about 4 kbar), and the shape of the lines shows that the compressibility increases at low pressure as would be expected for a transition to the molten globule state. These properties may indicate the existence of a triple point at which the three states (native, unfolded, and molten globule) coexist. [For the mentioned experimental diagram, readers are referred to the original work or to a recent review by Royer (1995) where it has been reproduced.]

(4) The directions of further studies which could contribute significantly to the empirical basis for volumetric investigations of proteins are clear.

(i) The accuracy of additive calculations can be improved, if experimental data on ionization effects for amino acid residues are used instead of model values. To date, they are available only for the carboxylic group of glutamic acid and for histidine (see footnotes to Table 2).

(ii) The experimentally based corrections for the interactions between the charged terminal groups and the closest side chains must be taken into account, especially when short peptides are considered. Only a few papers, cited in this work, were devoted to volumetric studies of these interactions. They indicated that the corrections might be noticeable, but to date, there are no systematic data available for all types of side groups which could allow one to formulate the rules for practical calculations.

(iii) Measurements of synthetic unfolded polypeptides are required to determine more strictly the completely unfolded state reference point for volumetry. Unfortunately, the last measurements of this kind were made more than 10 years ago, when the measuring techniques were far less developed.

(iv) Further studies of the volume−pressure nonlinearity are necessary, especially concerning the molten globule state of proteins. Otherwise, one cannot extrapolate the rich collection of the normal-pressure volumetric data to the high-pressure regime in which proteins denature.

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SUPPORTING INFORMATION AVAILABLE

Numerical literature data on partial volumes and compressibilities used for evaluation of parameters and for assessment of the additivity scheme, details of calculations, data for native proteins, and the corresponding references in five tables: (1) oligoglycine series and volumetric increments per residue, (2) experimental solution data and the values corrected for contribution of the nonstandard zwitterions for amino acids, (3) calculated and experimental values for short peptides (32 items, from di- to pentapeptides), (4) calculated and experimental values for polypeptides; and (5) calculated (for the unfolded state) and experimental (for the native state) values for proteins (11 pages). Ordering information is given on any current masthead page.

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