Molten Globule of Human α-Lactalbumin: Hydration, Density, and Compressibility of the Interior

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ABSTRACT: Specific partial volume, partial compressibility, and sound absorption changes induced by the native-to-molten globule state (acid) transition of the human α-lactalbumin were measured by means of densitometric and ultrasonic techniques and interpreted in terms of the protein molecule phase transition and interphase water transfer. The molten globule is a highly hydrated state containing about 270 water molecules inside. Intrinsic mass density of the hydrated (swollen) interior of the protein molecule is 5% smaller and the intrinsic compressibility coefficient 2 times higher than those in the native molecule. The obtained intrinsic compressibility falls into the range of values characteristic of highly associated liquids. Water inside the molten globule interior occupies less volume and is less compressible than in solvent phase. The acoustic relaxation was found to increase indicating an appearance of pressure-dependent processes. The commonly used approach to the calculation of the volume fluctuations of protein molecules, based on the well-known relation between the volume fluctuations and compressibility, is of limited applicability to the highly hydrated molten globule state because a large, if not predominant, part of the fluctuations may be determined by the process of water exchange between the molten globule and bulk solvent.

According to the modern concept of the globular protein thermodynamics there are at least three basic thermodynamic states of protein molecules in aqueous solution (Dolgikh et al., 1981; Shakhnovich & Finkelstein, 1989; Finkelstein & Shakhnovich, 1989; Ptitsyn, 1995; Kuwajima, 1989); the native, solid-like state with a rigid tertiary structure; the unfolded, coil-like state; and a liquid-like state characterized by the absence of tight packing of side chain groups, unfrozen rotation of the groups, a high compactness, and the native-like secondary structure. The latter state, called the molten globule (Ohgushi & Wada, 1983), has been reported for dozens of globular proteins (Bychkova & Ptitsyn, 1993).

Compressibility is an important characteristic of state of condensed matter. Among particular factors determining compressibility of proteins in solution are their packing density, various relaxation processes, outer surface hydration, and penetration of water inside the globule, if this occurs. Some of these factors have been studied for *native* proteins and, to some extent, for *unfolded* polypeptide chains [for review see Sarvazyan and Hemmes (1979), Kharakoz and Sarvazyan (1993), Chalikian et al. (1994), and Kharakoz, (1996)]. For native proteins, the intrinsic compressibility of the molecule is as low as that of macroscopic organic solids; the outer surface hydration contribution to the measured partial compressibility is quite negative; while relaxation contribution is negligible. Complete *unfolding* leads to a considerable *decrease* in the partial compressibility due to the loss of chain−chain contacts and the expansion of the surface area contacting the bulk water. As to the *molten globule state*, there is no sufficient basis developed for its quantitative analysis. In particular, *transfer of water molecules into the globule*, which may contribute significantly to the parameters under consideration, has not been properly analyzed; hence, intrinsic properties of this compact state could not be accurately estimated.

Inspection of available data on the volume and compressibility changes in globular proteins upon denaturation [for review see Zamyatnin (1984), Durchschlag (1986), Sarvazyan (1991), and Chalikian and Breslauer (1996); see also recent works by Nölting and Sligar (1993), Nötling et al. (1993), and Tamura and Gekko (1995)] leads to the following conclusion of relevance to this report. If a protein is denatured under mild conditions (at moderate ionic strength and without strong chemical denaturants), then, on the one hand, an increase in partial compressibility is usually observed as if the denatured protein would remain compact but disordered and fluctuating. On the other hand, the magnitude of this increase is considerably less than would be expected by analogy to melting of macroscopic solids. The partial volume changes little. To explain these facts it has been suggested (Kharakoz, 1983) that a protein in the compact denatured state could be a *highly hydrated molten globule*, whose partial volume and compressibility are markedly reduced due to the increased hydration. Although the idea of the compensating role of water in the transition to molten globule appeared long ago and was considered theoretically (Finkelstein & Shakhnovich, 1989), until now this role was studied rather qualitatively than quantitatively.

α-Lactalbumin is a typical protein for which the molten globule state has been shown to exist at pH 2 and 0.05 M KCl. This state has been characterized by a number of physical methods (Dolgikh et al., 1981; Gast et al., 1986; Bychkova et al., 1990; Pfeil et al., 1986; Kuwajima, 1989).

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An attempt to investigate volumetric properties of the molten globule of bovine α-lactalbumin was made by Nölting et al. (1993), but in their work only sound velocity in solution was measured and thus no direct data on partial volume and compressibility could be obtained.

In our work we have (i) measured density, sound velocity, and sound absorption of dilute aqueous solutions of the human α-lactalbumin and hen eggs lysozyme (a reference protein) at 25 °C within the pH range 7−2; (ii) determined the changes in partial volume and compressibility of this typical molten globule; (iii) obtained a quantitative information on hydration by means of comparison of the partial and hydrodynamic volumes; and then (iv), taking into account the hydration, estimated the changes of the intrinsic mass density and compressibility in the interior of the protein molecule upon melting. It has been shown that the molten globule is a *liquid-like* compact structure containing a *large amount of water inside*. The results obtained contribute to an understanding of the important physical properties of the molten globule, relevant to its fluctuating structure and to protein−water interaction.

MATERIALS AND METHODS

Preparations. Human α-lactalbumin was separated, purified, and examined as described earlier (Dolgikh et al., 1981). Hen egg white lysozyme, purchased from Sigma Chemical Co. (St. Louis, MO), was used without further purification. Protein solutions were passed through a Sephadex G-25 column equilibrated against 0.05 M KCl at neutral pH. Solutions were prepared with double distilled water. Protein concentrations were determined spectrophotometrically (280.1 nm) with the 1 mg/mL extinction coefficients of 1.82 for α-lactalbumin and 2.69 for lysozyme (using molar masses 14 000 and 14 300, respectively). The error in the concentration determination was about 2%.

Measurements of Density. Density of solutions was measured using a single-channel density meter DNA-60/602 (Anton Paar, Graz, Austria) calibrated with water and air. The high quality of temperature stabilization provided for a relative reproducibility of 3 ppm during the time of one experimental run (1.5 h).

Measurements of Sound Velocity and Absorption. Sound velocity and absorption were measured at a frequency of 7.2 MHz by means of a laboratory-built differential fixed-path interferometer. The thermostabilized high-quality ultrasonic resonator cells of 0.8 cm^3 sample volume were described earlier (Sarvazyan & Kharakoz, 1981). A high degree of identity of the measuring and reference cells allowed one to perform differential titration without worry about a systematic error. Calibration procedures were described previously: for sound velocity, the accuracy of the calibration coefficient was no worse than 0.3% and the reproducibility of measurements was about 2 ppm, calibrated by NaCl solutions (Kharakoz, 1991); for sound absorption the accuracy of the calibration was about 7% and the reproducibility of measurements was about 2%, calibrated by $MnSO_4$ solutions (Kharakoz, 1993). The errors are given relative to water.

The cells were supplied with magnetic stirrers for titrating and mixing solutions directly in the cells.

Titration Procedure. Protein solutions were differentially titrated with HCl. The measuring and reference cells of the acoustic device were filled up with equal volumes (to a precision of 0.2%) of solution and solvent, respectively, and then equal (to a precision of 0.5%) small portions of a concentrated HCl solution were added step-by-step into both cells simultaneously. After each step, the specific concentration increment of relative sound velocity (hereinafter called the "specific sound velocity"), $[u]$, was determined according to the definition

$$[u] \equiv (u - u_0)/u_0 c \qquad (1)$$

where u and u_0 are the sound velocities in solution and solvent, respectively, at each new concentration of HCl; c is the protein concentration in g/cm^3. The specific concentration increment of sound absorption per wavelength ("specific sound absorption"), $[\alpha]$, was determined according to the definition

$$[\alpha] \equiv (\alpha - \alpha_0)/c \qquad (2)$$

where α and α_0 are the amplitudes of attenuation of sound per wavelength in solution and solvent at each concentration of HCl.

A slightly different procedure was applied to the densimetric titration. As the density meter was single channel, two separate runs for each titration experiment were carried out using a single measuring cell: one run with a sample of the protein solution and the other one with the same volume of solvent to obtain the base line. A possible slow drift of the device was controlled by measuring water before and after each run. To add each new portion of HCl, the sample under titration was removed from the densimetric cell by a plastic syringe and the adding and mixing of HCl were performed inside the syringe. The sample was then replaced into the cell and additionally driven in and out of the syringe to ensure a proper mixing of the whole sample with the added HCl. Evaporation during this procedure was the same in both of these runs and did not influence the final differential results. Specific relative densities, $[d]$, were determined from the results of the two runs in accordance with the definition

$$[d] \equiv (d - d_0)/d_0 c \qquad (3)$$

where d is the density and d_0 is the density in solvent.

Solutions were dispensed with Hamilton high-precision dispenser-assembled syringes calibrated with water (Bonaduz, Switzerland). The reproducibility of the added portions was about 0.2%. Dilution of the protein in the course of titration did not exceed 5% and was taken into account in calculations of specific values.

Measurements of pH. The values of pH were measured in the course of the acoustic experiment immediately in the measuring cell with a glass microelectrode (Inhold Electrodes Inc., Andover, MA). A constant KCl flow from the built-in salt bridge could cause a marked systematic drift of sound velocity. To reduce the salt-induced drift, the built-in salt bridge was tightly closed by a rubber ring and, instead of it, an external reference electrode was used whose salt bridge was made of a thin capillary (0.3 mm thick) with a piece of baked pencil graphite soldered in. The residual drift (less than 0.4 ppm of sound velocity per minute) was taken into account in calculations. Control experiments without the pH electrode were performed with the solutions of the same protein concentrations. The results were identical which indicated sufficient accuracy in the drift correction.

In the densimetric experiments, pH was not controlled experimentally (for technical restrictions) but calculated from the amount of the added HCl by using the potentiometric curve obtained during the acoustic experiment.

Errors. The total instrumental random errors for the titration-induced changes of the parameters $\Delta[d] = [d] - [d]_0$, $\Delta[u] = [u] - [u]_0$, and $\Delta[\alpha] = [\alpha] - [\alpha]_0$ (here the subscript "0" refers to the initial neutral pH) were estimated to be 0.004, 0.003, and 0.007 cm^3/g, respectively. The estimates are given for the protein concentration 1 mg/cm^3 used in this work.

RESULTS

Measured Quantities. The absolute values of $[d]$ and $[u]$ were measured in the initial protein solutions under isopotential conditions (the column equilibration conditions). The isopotential quantities may differ significantly from the isomolal ones (Durchschlag, 1986), due to non-identical salt contents in the solution and solvent. Their correct interpretation is possible only if stoichiometry of protein−ion interaction is known or if the solution is a strictly two-component protein−water system, which was not the case in our work. Therefore, here we do not consider the absolute values but only their titration-induced changes which are practically isomolal in the sense that the salt content is identically changed in both the solvent and the solution in the course of differential titration.

Solutions of about 1 mg/cm^3 protein concentration were measured at 25 °C. At this low concentration, the difference between the values measured and those at the infinite dilution limit is negligible (Durchschlag, 1986; Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986) and, therefore, the changes of partial volume ($\Delta\bar{v}$) and partial adiabatic compressibility ($\Delta\bar{k}$) in the course of titration can be calculated by a simple equation valid for highly dilute solutions (Sarvazyan, 1991):

$$\Delta\bar{v} = -\Delta[d] \tag{4}$$

and

$$\Delta\bar{k} = -2\beta_0(\Delta[u] + \Delta[d]) \tag{5}$$

where β_0 is the adiabatic compressibility coefficient of the solvent, which is practically equal to that of pure water at the low salt content used, and Δ stands for the difference between the current and initial neutral pH. Several runs of titration have been carried out for *independently prepared* protein samples. The results are plotted against pH in Figure 1. The final values (at pH 2) obtained in the course of the step-by-step titration were confirmed by a number of experiments in which pH was decreased by a jump from 7 to 2 in one step. This was done to ensure the accuracy of the results against a possible effect of slowly passing through the isoelectric point at which the reversible self-association of α-lactalbumin took place. The results of the one-step jump measurements, presented by filled symbols in the pictures, were indistinguishable from the results of the step-by-step titration within the instrumental error.

The change of the specific sound velocity upon the passage from pH 7 to 2 was found to be $\Delta[u] = -0.027 \pm 0.004$ $cm^3\ g^{-1}$, close to the value -0.020 $cm^3\ g^{-1}$ obtained by Nölting et al. (1993) for the acidic transition of the bovine α-lactalbumin.

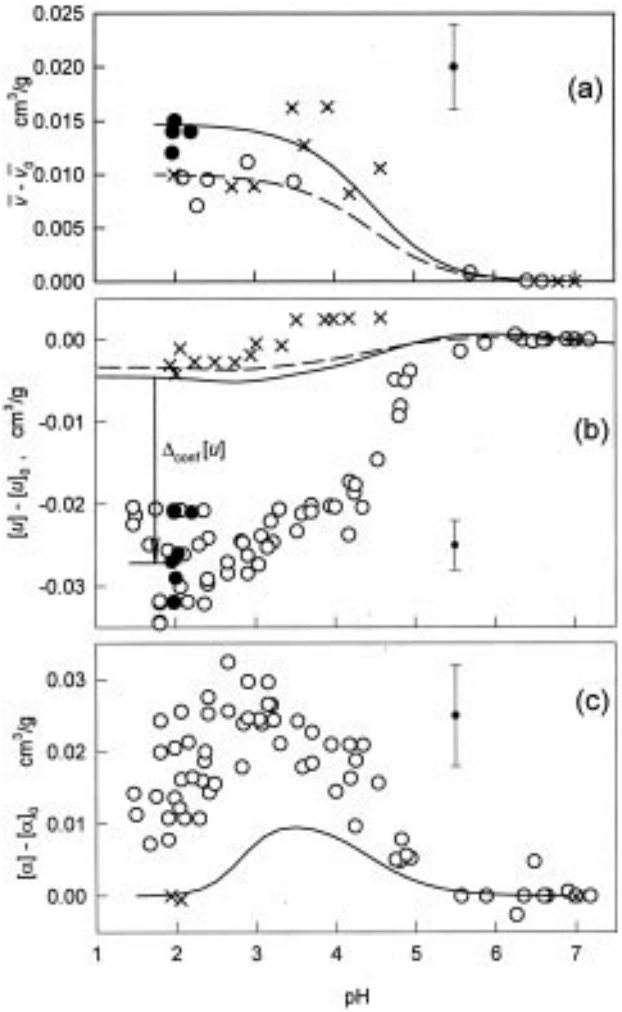

FIGURE 1: Changes in partial volume (a), specific sound velocity (b), and specific sound absorption (c) vs pH upon HCl titration of the proteins: human α-lactalbumin [step-by-step titration runs (○); one-step-jump experiments (●); and ionization contributions, calculated (—)] and hen egg lysozyme [experiment (×) and ionization contributions, calculated (- - -)]. Experimental errors are shown by the error bars on the right. The difference between the experimental and calculated data reflects the contribution of the conformational change.

Proton Binding Effects (Calculation). Not only the changes in protein conformation but also the effects of proton binding to carboxyl and imidazolyl atomic groups are reflected in the measured pH dependencies shown in Figure 1. Hence,

$$\Delta x_{\text{measured}} = \Delta_{\text{conf}}x + \Delta_{\text{H}}x \tag{6}$$

For *partial volume* ($x \equiv \bar{v}$), the term $\Delta_{\text{H}}x$ reflects the change in the ionization state of ionic groups and is proportional to the fraction of neutralized groups. For *sound velocity* ($x \equiv [u]$) and *partial compressibility* ($x \equiv \bar{k}$), this term includes not only the ionization state itself but also a relaxation contribution resulting from the pressure dependence of ionization equilibrium. For *sound absorption* ($x \equiv [\alpha]$) the ionization state itself contributes negligibly, so the term $\Delta_{\text{H}}x$ reflects only the relaxation contribution. The proton binding contributions to the measured properties were calculated from the literature data on model compounds (see Appendix for details). The calculated pH-dependencies of $\Delta_{\text{H}}x$ are shown as lines in Figure 1.

Table 1: Conformational Changes in Specific Partial Volume, $\Delta_{conf}\bar{v}$, Specific Sound Velocity, $\Delta_{conf}[u]$, Specific Partial Compressibility, $\Delta_{conf}\bar{k}$, and Specific Sound Absorption, $\Delta_{conf}[\alpha]$, of Human α-Lactalbumin upon the Native to Molten Globule Transition[a]

parameter (units)	value
$\Delta_{conf}\bar{v}$ (cm^3 g^{-1})	-0.002 ± 0.002
$\Delta_{conf}[u]$ (cm^3 g^{-1})	-0.022 ± 0.004
$\Delta_{conf}\bar{k}$ (Mbar^{-1} cm^3 g^{-1})	1.8 ± 0.4
$\Delta_{conf}[\alpha]$ (cm^3 g^{-1})	0.016 ± 0.005

[a] Comment: Standard deviations within a set of experiments are presented as estimations of errors which are close to the instrumental errors (see Materials and Methods).

Conformation Contributions. The contributions from the protein conformation change were determined as differences between the measured quantities and the calculated proton binding effects (eq 6). The values of Δx_{conf} for the whole transition (between pH 7 and 2) are presented in Table 1.

Lysozyme is a structural analog of α-lactalbumin but remains still native in the pH range studied. The conformational parts of its characteristics are unchanged, as expected (see Figure 1: the experimental curves virtually coincide with the curves reflecting proton binding contributions).

α-Lactalbumin undergoes the native-to-molten globule state transition between pH 7 and 2 (Dolgikh et al., 1981). The experimental partial volume change, however, coincides with the proton binding effect within the error (see Figure 1), showing that the conformational contribution is negligible. At the same time, the specific sound velocity decreases more than expected from the proton binding contribution (see Figure 1b). This change corresponds to an increase of partial compressibility by 1.8 Mbar^{-1} cm^3 g^{-1} (Table 1). Specific sound absorption at pH 2 is also noticeably higher than it might be expected from the proton binding relaxation (Figure 1c). It means some relaxation processes become "unfrozen" in the denatured state.

DISCUSSION

Qualitative Consideration

If the protein did unfold upon decreasing pH, its partial compressibility would decrease by 16−20 Mbar^{-1} cm^3 g^{-1} [as follows from the empirical calculations for the analogs, lysozyme and bovine α-lactalbumin performed by Kharakoz (1996)]. The observed increase in partial compressibility shows unambiguously that the native (N)[1] protein molecule is transformed not to the unfolded state (U) but to a compact structure which is the molten globule state (MG) under these experimental conditions (Dolgikh et al., 1981; Gast et al., 1986; Bychkova et al., 1990; Pfeil et al., 1986).

One of the most interesting facts is that the partial volume does not change upon the N−MG transition whereas the hydrodynamic volume is known to expand by about 35−60% (Dolgikh et al., 1981; Gast et al., 1986; Bychkova et al., 1990). This discrepancy cannot be explained by a possible change in the molecule shape (see the following section). Besides, the magnitudes of the observed changes in the partial volume and compressibility (Table 1) are considerably less than one might expect by analogy to the melting of macroscopic organic solids, where a 5−10% volume increase and a 1.5−3-fold compressibility increase are observed. These facts allowed us to assume a significantly enlarged hydration of the protein in the molten globule state. *A large amount of water must be incorporated into what is called the hydrodynamic volume of the melted protein molecule*. A semiquantitative analysis of hydration and the intrinsic density and compressibility of the hydrated molten globule is presented below.

Choice of Model for the Molten Globule

The model for the structure of the molten globule state (MG state) must fit to the following experimental facts about the N−MG transition:

(i) the conformational part of the partial volume remains constant within 0.3% during the transition;

(ii) the conformational part of the partial compressibility increases by 1.8 Mbar^{-1} cm^3 g^{-1}, i.e., by 20% of the average intrinsic compressibility k_{int}^N for native protein molecules [k_{int}^N = 9 Mbar^{-1} cm^3 g^{-1}; see Kharakoz and Sarvazyan (1993) for an analysis of the problem of the intrinsic compressibility of proteins in solution];

(iii) the hydrodynamic volume expands 1.42-fold, corresponding to the Stokes' radius increase from 1.77 to 1.99 nm as revealed from dynamic light scattering (Gast et al., 1986). Other methods give similar results: 1.35-fold from viscosity (Dolgikh et al., 1981) and 1.60-fold from diffusion coefficient (Bychkova et al., 1990);

(iv) the interatomic distances in the hydrophobic nucleus increase by 4% on average (from diffuse X-ray scattering data; Damaschun et al., 1986).

Let us now examine four simple models for the molten globule structure:

Model 1: "Dry" Molten Globule [terminology of Finkelstein and Shakhnovich (1989)]. By this model, the N−MG transition is a process of an isotropic swelling with no penetration of water inside. This model should be rejected for the reasons considered above: it cannot explain, first, why the partial volume change is not equal to the change of the hydrodynamic volume, and second, why the compressibility increase is not as large as for macroscopic organic materials upon melting.

Model 2: Mixed Model. The hydrophobic nucleus represents a "dry" melted region impenetrable to water, while the remainder of the molecule is fully unfolded and exposed to solvent. This model could explain both the significant expansion of hydrodynamic volume and the deficiency of the compressibility increase, because hydration of the exposed amino acid residues would compensate the increased compressibility of the nucleus. However, one can show that the model does not provide for a self-consistent explanation of the changes in volume and compressibility. Indeed, if (i) the volume of the nucleus is expanded by 12%, as follows from the 4% increase of interatomic distances ($\Delta v_{nucl} = 0.12 v_{int}^N = 0.086$ cm^3 g^{-1}; where v_{int}^N is the specific volume of the interior of native molecule and equal to 0.72 cm^3 g^{-1}; Pavlov & Fedorov, 1982) and (ii) the change in partial volume upon unfolding is $\Delta v_{unfold} = -0.014 \pm 0.007$ cm^3 g^{-1} (as estimated for bovine α-lactalbumin using a well-established additivity scheme; Kharakoz, 1996), then the constancy of the conformational partial volume ($\Delta_{exp} v = 0$)

[1] Abbreviations: N, native state; U, unfolded state; MG, molten globule state of protein molecules.

means that the "dry" melted nucleus represents only a small fraction of the protein molecule: $(\Delta_{exp}v - \Delta v_{unfold})/(\Delta v_{nucl} - \Delta v_{unfold}) = 8-20\%$. The value 20% is the maximum estimate from which one can obtain the minimum estimate for the compressibility coefficient of the nucleus satisfying the experimental value of the change in partial compressibility, $\Delta_{exp}k = 1.8$ Mbar^{-1} cm^3 g^{-1}. To do that, let us assume the unfolding effect to be $\Delta_{unfold}k = -16$ Mbar^{-1} cm^3 g^{-1} (the minimum change of those estimated for lysozyme and bovine α-lactalbumin; Kharakoz, 1996). It then follows that the specific compressibility of the nucleus would increase by $\Delta_{nucl}k = (\Delta_{exp}k - 0.80\Delta_{unfold}k)/0.20 =$ 73 to become 82 Mbar^{-1} cm^3 g^{-1} (Kharakoz & Sarvazyan, 1993). Therefore, even by the minimum estimate, the compressibility of the nucleus must increase 9-fold! This enormous increase seems unlikely since melting of macroscopic organic solids leads to only a 3-fold increase in compressibility, *as an upper limit*.

Thus, *the mixed model does not explain experimental observations*.

Model 3: Elongated "Dry" Molten Globule. The globule is in the "dry" melted state (no water inside) with an elongated shape of the molecule, the specific outer surface and the frictional ratio being, therefore, increased. This model is equivalent to the above considered exposure of a part of polypeptide chain (mixed model). Hence, the line of argumentation presented above remains valid, and the model cannot be accepted. Another argument against elongation comes from the fact that the protein surface tension is high (Chothia, 1975), and therefore it is unlikely that the molten globule is more elongated since it is more fluid than the native state.

Model 4: "Wet" Molten Globule (terminology of Finkelstein and Shakhnovich). This model implies that, first, the protein globule is expanded *without considerable change in the shape*, and second, the voids in the molten globule are *filled with water*. Penetration of water compensates the volume increase caused by the melting itself, so that the change of the total solution volume is very small. The penetrated water participating in the common translational movement of the molten globule increases the hydrodynamic volume.

Compressibility of the so-hydrated globule is determined not only by the enlarged interatomic distances but also by the apparent compressibility of the water penetrated inside the globule considered as a phase. This water, being partially immobilized by binding to the protein atomic groups, should be less compressible. The observed changes of partial quantities result from two major counteracting processes: melting itself and the transfer of water.

Therefore the *"wet" molten globule is the only model consistent with the experimental data*, at least on the qualitative level. A quantitative consideration is made below within the framework of this model,[2] assuming a protein molecule as a small-dimension phase separated from the bulk solvent by a more or less definite interphase boundary.

[2] In principle, one more MG state structure is possible: the "dry" molten nucleus and "wet" melted edge. However, this structure can be considered as a particular case of the "wet" molten globule model with the only difference being a specific mode of water distribution over the globule. Therefore, this possibility is not separately considered.

Hydration of the Interior

The hydrodynamic volume of a protein V_η (the volume of the hydrated molecules) is related to the partial molar volume, $\bar{V}$, and to the intrinsic molar volume, V_{int}, of the dry protein molecule by an obvious equation (Cantor & Shimmel, 1984)- where n_h is the total number of bound water molecules and

$$\bar{V} = V_\eta - n_h\bar{V}_1 = (V_{int} + n_h V_1^h) - n_h\bar{V}_1 \qquad (7)$$

$\bar{V}_1$ and V_1^h are the partial molar volumes of pure and bound water, respectively.

Spherical approximation of molecular shape gives acceptable results for the native α-lactalbumin. Indeed, in this case hydrodynamic volume of the native protein is $V_\eta^N = 14\,000$ cm^3 mol^{-1} (from the Stokes' radius 1.77 nm, see above). By comparing this value with the partial volume $\bar{V}^N = M\bar{v}^N = 10\,200$ cm^3 mol^{-1} (M is the molar mass and $\bar{v}^N = 0.73$ cm^3 g^{-1} by analogy with bovine α-lactalbumin and lysozyme; Kharakoz & Sarvazyan, 1993) it can be calculated from eq 7 that about 210 water molecules are immobilized on the surface of the native protein (the amount of internal water being neglected). This is well within the range of hydration numbers determined by various methods for native globular proteins: around 0.3 g of water per g of protein (Cantor & Shimmel, 1984).

Assuming a similar shape for the native and melted globules (see discussion of models 3 and 4 above), it can be calculated from the 1.42-fold increase of the Stoke's radius (see above) that $V_\eta^{MG} = 19\,900$ cm^3 mol^{-1}. As the partial volume remains constant upon transition, the amount of water associated with the protein is increased by 325 molecules, as follows from eq 7. A part of this increase is due to the water immobilized on the newly exposed surface. This part amounts to about 55 water molecules as estimated under the assumption of proportionality between the outer surface hydration and the Stoke's radii squared. The total increase in hydration determined above (325 water molecules) is 6 times (!) greater than the obtained 55 outer molecules and even 1.5 times higher than the total initial hydration number (210 molecules). It is unlikely that the newly exposed surface would be so much more hydrophilic than that of the native protein. Therefore *a large amount of water, about 270 molecules, penetrates into the interior of the molten globule*.

Interestingly, the number of penetrated water molecules is close to the number of internal polar atoms of the protein. For comparison, in the native lysozyme there are about 220 polar atoms buried (Lee & Richards, 1971). It might be thought, therefore, that the hydration bonding between water and internal polar groups gives an essential part of the driving force of the water penetration and the melting transition. The *protein interior "likes" water*. [See also the theoretical consideration by Finkelstein and Shakhnovich (1989).]

Density of the Interior

Within a simple approach, the volume occupied by the molten globule interior, V_{int}^{MG}, is expanded in comparison to that in the native protein, V_{int}^N, in proportion to the increased Stokes' radius cubed:

$$V_{int}^{MG} = \chi v_{int}^N M \qquad (8)$$

where $\chi = 1.42$ is the ratio of the Stokes' radii cubed. On the other hand, the melted interior contains $n_{int} = 270$ water molecules and, thus, its total molar mass is $(M + 18n_{int})$. Therefore the average mass density (ρ_{int}^{MG}) of the "wet" molten globule interior is equal to

$$\rho_{int}^{MG} = (M + 18n_{int})/(\chi v_{int}^{N} M) = 0.95\rho_{int}^{N}$$

which is 5% lower than that of the native protein ($\rho_{int}^{N} \equiv 1/v_{int}^{N} = 1.39$ g/cm^3). This change is due to both the protein melting itself and a contribution of internal water (see below).

Compressibility Coefficient of the Interior

The change in molar partial compressibility $\Delta_N^{MG}\bar{K}$, induced by the N−MG transition, is determined by three factors: (i) expansion of the hydrated outer surface of the globule $\Delta_N^{MG}K_{surf}$; (ii) loss of a portion of bulk water (n_{int}) which penetrated into the molten globule interior, $-n_{int}\bar{K}_1$ ($\bar{K}_1$ is the partial molar compressibility of pure water); and (iii) change in the interior molar compressibility, $K_{int}^{MG} - K_{int}^{N}$.[3] In principle, there is a fourth possible contribution, a relaxation one, connected with the water exchange between the molten globule and bulk water phases. Unfortunately at present there is not sufficient data to estimate this contribution correctly because the chemical potential of the internal water as a function of the internal water number is strongly required but not available. For this reason, this kind of relaxation is considered here implicitly as a part of the interior compressibility, K_{int}^{MG}. Then

$$\Delta_N^{MG}\bar{K} = K_{int}^{MG} - K_{int}^{N} + \Delta_N^{MG}K_{surf} - n_{int}\bar{K}_1 \qquad (9)$$

The following input data can be used for making an estimation:

(i) Hydration of the protein outer surface reduces compressibility. For 14 000 dalton native proteins this contribution is about $-6.5M$ Mbar^{-1} cm^3 mol^{-1}, on the average (Kharakoz & Sarvazyan, 1993). Assuming that the surface contribution is proportional to the Stokes' radius squared we obtain $\Delta_N^{MG}K_{surf} = -1.7M$ Mbar^{-1} cm^3 mol^{-1} for our protein.

(ii) The molar partial compressibility of pure water is $\bar{K}_1 = 810$ Mbar^{-1} cm^3 mol^{-1} (Kell, 1975) and hence $n_{int}\bar{K}_1 = 15.6M$ Mbar^{-1} cm^3 mol^{-1}.

(iii) The intrinsic compressibility of the native protein interior is $K_{int}^{N} = 9M$ Mbar^{-1} cm^3 mol^{-1} (Kharakoz & Sarvazyan, 1993).

(iv) The change in molar partial compressibility is $\Delta_N^{MG}\bar{K} = 1.8M$ Mbar^{-1} cm^3 mol^{-1} as obtained in this work.

Using these data from eq 9 we obtain $K_{int}^{MG} = 28M$ Mbar^{-1} cm^3 mol^{-1} and, then, the intrinsic compressibility coefficient $\beta_{int}^{MG} \equiv K_{int}^{MG}/V_{int}^{MG} = 28M/(\chi v_{int}^{N} M) = 27$ Mbar^{-1} is yielded (taking into account eq 8). The obtained value is 2-fold that for native globules ($\beta_{int}^{N} = 13$ Mbar^{-1}; Kharakoz & Sarvazyan, 1993) and falls into the range of values characteristic of highly associated liquids (Litovitz & Davies, 1965). For comparison, the compressibility coefficients for highly associated glycerol and water are 22 and 45 Mbar^{-1}, respectively, whereas for weakly associated alcohols and for the non-associated organic liquids like benzene, toluene, etc. they are around 70−100 Mbar^{-1} (Vinogradov, 1991). This is not surprising because the molten globule is a system whose elements are highly interlinked not only by covalent bonds but also by the hydrogen-bonded internal water.

Properties of the Internal Water

As discussed above, melting itself ("dry" melting) would cause an increase of partial volume of protein by *at least* 0.035 cm^3/g (5% of v_{int}^{N}). This change is completely compensated by hydration ($\Delta_{conf}v = 0$, Table 1). Neglecting the surface effect on the volume [*cf.* Finney (1975) and Pavlov and Fedorov (1982)] one can write $M\Delta_{conf}v = 0.035M + n_{int}V_{1,trans}$ and obtain $\Delta V_{1,trans} = -1.8$ cm^3/mol^{-1}. Therefore water occupies *at least* 10% less volume in the interior than in pure water phase (18 cm^3/mol; Kell, 1975).

A similar estimation can be made for compressibility. Melting itself would cause an increase in the partial compressibility of the protein by *at least* 4.5 Mbar^{-1} cm^3 g^{-1} (50% of k_{int}^{N}, by analogy to macroscopic matters melted). For the conformational compressibility one can write $M\Delta_{conf}k = 4.5M + n_{int}\Delta K_{1,trans} + \Delta_N^{MG}K_{surf}$ (here the outer surface effect cannot be neglected). Therefore $\Delta K_{1,trans} = (1.8 - 4.5 + 1.7)M/n_{int} = 52$ Mbar^{-1} cm^3 mol^{-1}, i.e., water inside the globule is *at least* 6% less compressible than in pure water (810 Mbar^{-1} cm^3 mol^{-1}; Kell, 1975).

The volume and compressibility changes obtained for water transfer into the molten globule are within the range of values characteristic of the water transfer from pure water phase to alcohols (Sakurai & Nakagawa, 1982; Nozdrev, 1958) or to organic polymers (Starkweather, 1980; Scandola & Pezzin, 1980).

Acoustic Relaxation

A part of the increased sound absorption upon the N-MG transition (Table 1) is due to the Stokes' dissipation of energy resulting from an increase in intrinsic viscosity of the protein ($\Delta[\eta] = 1.1$ cm^3 g^{-1}; Dolgikh et al., 1981). This part can be calculated using the Stokes' equation (Litovitz & Davies, 1965) rewritten here in terms of relative specific quantities for dilute solutions: $\Delta[\alpha]_{Stokes} = (8/3)\pi^2\eta_o\beta_o f \times \Delta[\eta] = 0.008$ cm^3 g^{-1} (η_o is viscosity of solvent; f is sound frequency). This value is 2 times less than that of the total experimental change. The remaining part indicates that there is a relaxation process appeared in the molten globule state. Whether this relaxation results from "unfrozen" intramolecular motions, from the water transfer, or from both is not clear. Consequently, quantitative estimation of the associated compressibility change is not possible yet.

Comparison with Other Proteins

To date, there are a few proteins for which volume and compressibility in the molten globule state have been accurately measured. The experimental data are summarized

[3] The acoustic method enables one to measure the macroscopic *adiabatic* compressibility of solution, which is, on the microscopic level, connected to the so-called *pseudoadiabatic* compressibility of a protein molecule and its hydration shell. For the purposes of this paper the difference between the pseudoadiabatic, isothermal, and adiabatic terms is not essential and is neglected. See Supplemental Material to the paper by Kharakoz (1991) for the physical meaning of the term "pseudoadiabatic compressibility", the paper by Kharakoz and Sarvazyan (1993) for an empirical estimate for proteins, and also a recent work by Nölting (1995).

Table 2: Comparison of Results from This Work with Literature Data on Other Proteins

	volume ratio		specific sound velocity change	partial compressibility change	
	hydrodynamic V_η^{MG}/V_η^N	partial (conformational) $\bar{v}^{MG}/\bar{v}^N$	$[u]^{MG} - [u]^N$ ($cm^3\ g^{-1}$)	$\bar{k}^{MG} - \bar{k}^N$ ($Mbar^{-1}\ cm^3\ g^{-1}$)	reference
α-lactalbumin, human[a]	1.42	1.019	−0.027		this work
		0.997[b]	−0.022[b]	1.8[b]	
α-lactalbumin, bovine[a]	1.5		−0.020		Nölting et al. (1993), ultrasonic data; Bychkova and Ptitsyn (1993), hydrodynamic data
			−0.013[b]		
cytochrome *c*[a]		1.00		2.2	Nölting and Sligar (1993)
	1.6	1.019		3.8	Chalikian et al. (1995)
		0.996[b]		1.5[b]	
cytochrome b_{562}[a]		1.00		0.7	Nölting and Sligar (1993); Brandts et al. (1970)
ribonuclease A (pH 1.9)[c]	2	0.996[b]		1.5[b]	
		0.946[b]		3.9[b]	Tamura and Gekko (1995)

[a] Acidic denaturation at moderate ionic strength, 25 °C. [b] The ratio of the *conformational parts* was estimated by a manner similar to that described in this work for α-lactalbumin. For ribonuclease, denatured thermally, the conformational parts are considered to be equal to the experimentally observed partial values, under the assumption that dissociation constants of ionizable groups change negligibly upon denaturation. Literature data have been reconsidered here with use of the same data on ionization effects (in order to unify the compared results). [c] Denaturation by heat at moderate ionic strength, temperature about 40 °C.

in Table 2 and compared with our results. Within an order of magnitude the volumetric parameters change similarly in all proteins. A relatively large (50%) expansion of the hydrodynamic volume is accompanied by a negligible, or even negative, change in the conformational part of partial volume. The conformational compressibilities increase by 10−40% of the average intrinsic compressibility of the native molecules. *Probably in all of these cases the protein interior is highly hydrated in the compact denatured state*, similar to what is observed in human α-lactalbumin.

Comment on the Volume Fluctuations of Proteins

Let us consider an important consequence of the fact of the high internal hydration of molten globule. After Cooper's (1976) work it has become traditional in the studies of protein solution compressibility to calculate the mean-square volume fluctuations, $\langle(\delta v)^2\rangle$, of a protein molecule by means of the well-known general thermodynamic equation:

$$\langle(\delta v)^2\rangle = \beta_T v k_B T \qquad (10)$$

(v is the volume of a system; β_T is the isothermal compressibility coefficient; k_B is the Boltzmann's constant; T is the absolute temperature). This equation was applied not only to the native state *but also to the molten globule state of proteins*. We should stress, however, that the equation is related to a system with a *constant number of particles* (Landau & Lifshitz, 1964). Therefore it is more or less valid only in the case of *native* proteins (for which Cooper did propose to use it), the case when the water exchange between the protein interior and bulk solvent is evidently negligible. At the same time the molten globule is a highly hydrated structure, and open to water exchange between the interior and bulk solvent. Therefore an essential, if not predominant, part of the volume fluctuations may be caused by the fluctuations in the number of internal water molecules, n_{int}. In this case, eq 10 may account for only a small part of $\langle(\delta v)^2\rangle$. To calculate the contribution from the fluctuations in n_{int} another equation must be used (Landau & Lifshitz, 1964):

$$\langle(\delta n_{int})^2\rangle = k_B T/(\partial\Delta\mu/\partial n_{int}) \qquad (11)$$

and then for the corresponding volume fluctuations:

$$\langle(\delta v)^2\rangle_{\text{water exchange}} = \langle(\delta n_{int})^2\rangle \times (\bar{v}_{1,int})^2 \qquad (12)$$

Here $\Delta\mu$ is the chemical potential of transfer of water from the bulk solvent to the globule interior; $\bar{v}_{1,int}$ is the partial molecular volume of water in the interior. To convince oneself that this contribution can be large, one may roughly assume that $\delta\Delta\mu/\partial n_{int} = k_B T/n_{int}$ (as if activity coefficient of internal water were independent of n_{int}) and that $\bar{v}_{int} = 30$ Å^3 (as in pure water). Then, taking $n_{int} = 270$ and molecular volume of a protein $v = 16\,700$ Å^3 (such as α-lactalbumin) one can obtain using eqs 11 and 12 $|\delta v_{\text{water exchange}}| = 490$ Å^3, which is 3 times higher than the contribution calculated from eq 10 (170 Å^3). Actually, the magnitude of these fluctuations *can be either greater or smaller, depending on the mechanism of internal hydration*. Unfortunately, there are no available data on the real value of $\delta\Delta\mu/\partial n_{int}$ for the molten globule hydration, and, hence, the presented estimate has only an illustrative meaning.

CONCLUSIONS

(i) A marked increase in hydration of the interior of human α-lactalbumin occurs during the native to molten globule state transition. *The molten globule interior is filled up with about 270 molecules of water*. This number is close to the number of internal polar atoms in the protein which indicates that a stoichiometric water−polar group interaction might be an essential part of driving force of the internal hydration and melting.

(ii) Physical properties of the molten globule state cannot be understood and accurately evaluated if the internal water is not taken into consideration. Expansion of hydrodynamic volume upon melting is mainly due to the water penetrated into the globule. A large increase in volume and compressibility of solution, which would be expected from the melting itself, is considerably compensated by the *negative contributions* of the water transfer.

(iii) Intrinsic compressibility coefficient for the hydrated molten globule state of α-lactalbumin is significantly higher and the *intrinsic mass density* is less than those in the native state. The compressibility, being two times greater than that of the solid-like native molecule, falls into the range of *compressibilities of highly associated liquids*.

(iv) Acoustic relaxation is increased in the molten globule state. Whether this relaxation results from unfrozen intramolecular motions or from water transfer between the protein interior and bulk solvent is not yet clear and is a subject of further investigations.

(v) Volume fluctuations of protein molecules, important characteristics of mechanical stability of a molecular system, cannot be calculated for the highly hydrated molten globule state at present because of the absence of data on the thermodynamics of water transfer into the globule. It is evident that the *commonly used approach to such calculations is not correct for the molten globule state* because the water transfer contribution to the volume fluctuations cannot be generally neglected.

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APPENDIX

Ionization and Acoustic Relaxation

The partial volume effect, $\Delta_H\bar{v}$, of proton binding

$$A^- + H^+ \leftrightarrow AH \text{ (or } A + H^+ \leftrightarrow AH^+) \qquad \text{(A1)}$$

is given by

$$\Delta_H\bar{v} = (1/M)[\sum_i n_i\theta_i(\Delta_H\bar{V}_i)] \qquad \text{(A2)}$$

where n is the number of ionizable groups of sort "i" in the protein; $\theta_i = 10^{(pK-pH)}/(1 - 10^{(pK-pH)})$ is the degree of association of the groups with proton ($K = [A^-][H^+]/[AH]$ being the dissociation constant); $\Delta_H\bar{V}_i$ is the molar volume change upon proton binding.

The change in specific sound velocity, $\Delta_H[u]$, upon proton binding is given by the following equation rewritten here to a more appropriate form (Kharakoz, 1992)

$$\Delta_H[u] = (1/M)(\sum_i n_i\theta_i\Delta_H[U]_i) - (1/4\pi^2 f)(\sum_i [A]_{H,i}/\tau_i) \qquad \text{(A3)}$$

where $\Delta_H[U]_i$ is the change in molar sound velocity for the ith group; f is the sound frequency; τ is the relaxation time; $[A]_{H,i}$ is relaxation contribution of the reaction A1 to the molar sound absorption. Equations for the values of τ and $[A]_H$ can be found elsewhere (Kharakoz, 1992; Stuehr & Yeager, 1965).

The quantitative parameters used (i) for carboxylic groups, the molar changes 13 and −3 cm^3 mol^{-1} for molar partial volume and molar sound velocity, respectively, were taken from the data on polyglutamic acid by Noguchi and Yang (1963, 1971) reconsidered by Kharakoz (1983); (ii) for imidazolyl groups, the molar characteristics (−2 and 11 cm^3 mol^{-1} for molar partial volume and molar sound velocity, respectively) were taken from the data on histidine (Kauzmann et al., 1962; Kharakoz, 1983) and its analogs piperidine and piperazine (Høiland, 1986a,b); (iii) the pK values 4.5 and 6.8 were used for the carboxyl and imidazolyl, respectively; (iv) the numbers of these groups in the proteins studied were taken from Findley and Brew (1972); (v) the rate constant of the proton binding to carboxylic groups, required for calculation of τ_i, was assumed to be 0.03 ps^{-1} M^{-1}, as it had been obtained for monocarboxylic acids (Kharakoz, 1992); (vi) the acoustic relaxation of the imidazolyl group was not considered because of its negligible relaxation strength (Sarvazyan et al., 1979); (vii) the influence of ionic strength (0.05 M KCl) and the difference between activity and concentration were neglected.

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