VOLUME 115, NUMBER 22
NOVEMBER 3, 1993

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

The Acidic Molten Globule State of α-Lactalbumin Probed by Sound Velocity

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Received April 12, 1993®

Abstract: We have investigated the structure and the chemical and conformational relaxation in the acidic molten globule state of α-lactalbumin (A-state) by using ultrasonic velocimetry. The A-state of the α-lactalbumin at pH 2 exhibits an 11% lower sound-velocity increment than that of the native state at neutral pH and 2-MHz sound frequency. An excess of calcium decreases this effect. For lysozyme, the difference in sound velocity between pH 7 and 2 is significantly smaller. Lysozyme has a large sequence homology and structure similarity to the native α-lactalbumin, but no molten globule state in aqueous solution has been described. The destabilized apo-state of α-lactalbumin at neutral pH exhibits a small decrease of roughly 4% in its sound-velocity increment relative to the native state. The large sound-velocity decrease of the A-state relative to that of the native state of α-lactalbumin indicates significant conformational relaxation at a time scale faster than 500 ns in the acidic molten globule at pH 2.

Introduction

The "molten globule" state of proteins is characterized by a moving tertiary structure but a higher degree of compactness and a larger content of secondary structure than the unfolded state,[1-3] and it is assumed to be a general intermediate in protein folding.[4] Since the folding of most small globular proteins is a highly cooperative process,[5,6] the understanding of molten-globule-equilibrium intermediates is considered a key to the understanding of protein folding. Molten globules have been described for more than 40 natural and modified proteins. One of the most extensively investigated is the calcium-binding protein α-lactalbumin.[7-11] There exist at least two molten globule-like states for this protein, the calcium-free (apo) protein and the acidic protein at about pH 2. The acidic molten globule (A-state) does not show cooperative thermal unfolding.[11] Apo α-lactalbumin (apo-state) exhibits cooperative thermal unfolding but a reduced thermal stability at neutral pH in the absence of denaturants.[8-11] It represents a more native-like molten globule.

The differences between molten globules, the structural

* Abstract published in *Advance ACS Abstracts*, October 15, 1993.

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0002-7863/93/1515-9879$04.00/0

dynamics, and the chemical relaxation processes in molten globules are still poorly understood. Ultrasonic velocimetry is a sensitive method for the studies of tertiary and quaternary structure differences,[12,13] adiabatic compressibility,[14,15] protein hydration,[16] and chemical and conformational relaxations in proteins.[12,17]

The relative sound velocity of an aqueous protein solution, compared to that of the solvent alone, includes contributions from two sources.[12,17] First, the interior of the protein and the hydration shell have a higher sound velocity than bulk water. Second, conformational and chemical relaxations in the protein and its hydration shell decrease the sound velocity.[17] Propagation of a sound wave through a protein solution leads to a very small change of pressure, density, and temperature, which results in a slight perturbation of the thermodynamic equilibrium chemically and conformationally. Relaxation leads to a new equilibrium of the system under the perturbation and results in a retardation of the sound wave. Only if the relaxation is not much slower than the period of the sound wave can it significantly decrease the sound velocity.[17]

Probably due to experimental difficulties, only a few proteins have been investigated by sound velocity.[15,16] The development of precise sound-velocity chambers with small volumes[14,18] has made it possible to perform ultrasonic velocimetry on scarce biochemical samples. We present the first investigation of the acidic molten globule state and the apo-state of α-lactalbumin by sound velocity and compare the results with similar measurements on lysozyme, which has a high degree of structural similarity in the native state and about 40% sequence homology[19] to α-lactalbumin but does not exhibit a molten globule state under the same conditions. We found a sound-velocity increment about 11% lower for the acidic molten globule at pH 2 than for the native protein at neutral pH. This difference is significantly larger than the sound velocity difference in lysozyme between pH 7 and 2, and it decreases in the presence of excess calcium. The destabilized state of apo α-lactalbumin exhibits a significantly smaller sound-velocity difference relative to the native state than does the acidic molten globule. The difference between the native protein and the acidic molten globule is larger than the changes we would expect due to the titration of carboxyl groups in the protein. Significant contributions of conformational relaxation to the sound velocity of the acidic molten globule are indicated.

Materials and Methods

The sound-velocity apparatus is designed essentially as described by Gavish *et al.*[20,21] A new sample chamber of 0.6-mL volume[14] was used as an acoustic resonator for the sound-velocity measurements. PXE5 piezoelectric transducers were from Philips. The resonance frequency of the sample chamber, which can be measured with high precision, is proportional to the sound velocity of the sample. The sound frequency used was about 2 MHz. Calibration of the system utilized standard sodium chloride solutions.[22] Bovine apo α-lactalbumin (Type III) and chicken egg-white lysozyme (Lot 6876) were from Sigma. Holo α-lactalbumin was reconstituted by dissolving in aqueous calcium chloride solutions. All measurements were conducted at 25.0 °C with a protein concentration of 9.6 g L^{-1} (0.7 mM). Concentrations were determined by using the extinction coefficients of 2.09 L g^{-1} cm^{-1} for holo α-lactalbumin[1] and 2.69 L g^{-1} cm^{-1} for lysozyme,[23] both at 280 nm and pH 6. The experimental error of the sound-velocity increment $\Delta u/c$ is estimated to be ±0.02 m s^{-1} L g^{-1}, where Δu is the sound-velocity difference between the protein solution and the solvent and c is the protein concentration. The accuracy of the measurement of the sound-velocity increment was limited by systematical errors in the determination of the concentration of protein and its impurities. Thus, differences of the sound-velocity increment are more accurate than absolute values. We estimate the error for the difference in the sound-velocity increment between apo and holo α-lactalbumin at pH 7 at ±0.01 m s^{-1} L g^{-1}. Protein solutions with differing pH's were prepared by titration of the proteins with 1 M HCl, and the concentration was corrected for the small dilution of the protein solution. The error of differences in the sound-velocity increment for the same solvent at different pH's was ±0.005 m s^{-1} L g^{-1}, corresponding to an error of ± 0.02 in the relative sound-velocity changes $\delta u_{rel} = (\Delta u - \Delta u_0)/\Delta u_0$, where Δu_0 is measured at pH 7 and Δu at the actual pH. Base- lines obtained with the resonance chamber filled with protein-free solvent were substracted from each experimental run.

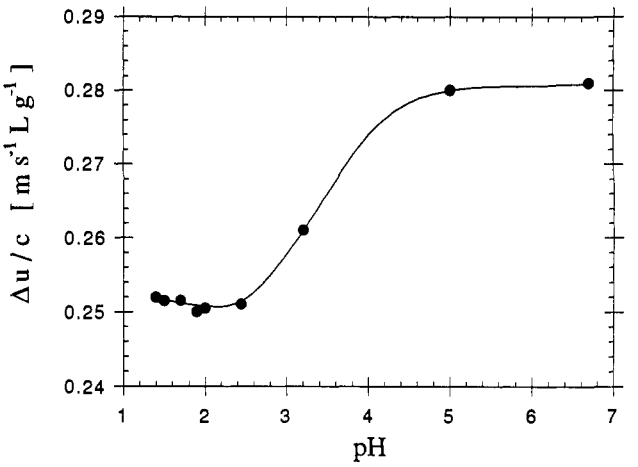

Figure 1. Sound-velocity increment $\Delta u/c$, where Δu is the sound-velocity difference between the protein solution and the solvent and c is the protein concentration, of 0.7 mM α-lactalbumin in 0.7 mM $CaCl_2$ and 23 mM KCl.

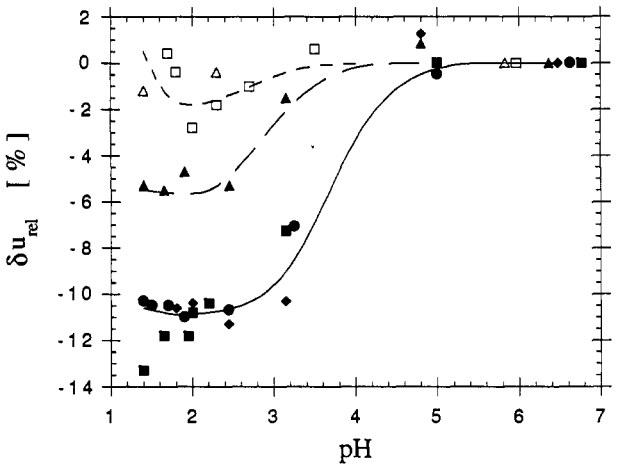

Figure 2. Relative sound-velocity changes $\delta u_{rel} = (\Delta u - \Delta u_0)/\Delta u_0$, where Δu_0 is measured at pH 7 and Δu at the measured pH: open symbols, 0.7 mM lysozyme; closed symbols, 0.7 mM α-lactalbumin; circles, solvent A, 0.7 mM $CaCl_2$ and 23 mM KCl; squares solvent B, 5 mM $CaCl_2$ and 10 mM KCl; triangles, solvent C, 70 mM $CaCl_2$; and diamonds, solvent D, 0.7 mM $CaCl_2$ and 200 mM KCl.

Results

Figure 1 shows the sound-velocity increment $\Delta u/c$ as a function of pH for α-lactalbumin in 0.7 mM $CaCl_2$ and 23 mM KCl (solvent A). The sound-velocity increment of the native holo protein at pH 6.8 is about 0.28 m s^{-1} L g^{-1}. The acidic molten globule state at pH 2 shows a significant decrease in $\Delta u/c$ by about 0.03 m s^{-1} L g^{-1}.

Figure 2 reveals the relative sound-velocity changes δu_{rel} of holo α-lactalbumin in solvents of different compositions. In solvent A, δu_{rel} is about −11% at pH 2. First, we changed the concentration of calcium from the stoichiometric amount (0.7 mM $CaCl_2$, solvent

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A) to a 7-fold excess (5 mM $CaCl_2$, defined as solvent B). To maintain the same ionic strength as solvent A, the concentration of KCl in solvent B was decreased to 10 mM. Within experimental error, no significant difference was observed in δu_{rel} for holo α-lactalbumin in solvent A and B from pH 6.8 to 1.4. In contast, at a 100-fold excess of calcium (70 mM $CaCl_2$, defined as solvent C), the absolute value of δu_{rel} at pH 2 is significantly decreased and the transition of δu_{rel} is shifted toward lower pH. In order to investigate whether that decrease is due to the higher ionic strength, we conducted the same experiments in solvent D (0.7 mM $CaCl_2$ and 200 mM KCl), which has the stoichiometric amount of calcium as solvent A but the same ionic strength as solvent C. For solvent D, we found that from pH 6.8 to 1.4, within experimental error, δu_{rel} was the same as for solvents A and B which have an 8-fold lower ionic strength. Figure 2 also presents the measurement of δu_{rel} for lysozyme in solvents B and C. No significant difference between these two buffer conditions was noted. Lysozyme shows a much smaller absolute δu_{rel} at pH 2. Finally, we measured the sound velocity of apo α-lactalbumin in 23 mM KCl, pH 7. We found a decrease in the sound-velocity increment of about 0.01 m s^{-1} L g^{-1} relative to that of the native holo protein in solvent A.

Discussion

Unfolded protein exhibits a significantly increased sound velocity relative to that of native protein.[24] The observation of a decrease in the sound velocity of α-lactalbumin solutions during transition from the native state at pH 6.8 to the molten globule state at pH 2 in solution A is consistent with the maintenance of a high degree of secondary structure and an overall compactness of the molten globule. The possible reasons for this decrease are changes in the hydration shell, changes in the intrinsic sound velocity of the molecule, conformational or chemical relaxation, and a combination of these factors.[12,17]

The differences in the hydration shell contribution between the native protein and the molten globule can be estimated from the changes in the size of the molecule. From quasi-elastic light scattering, Gast *et al.*[25] found that the linear dimensions of the acidic molten globule state of α-lactalbumin are about 10% larger than those of the native state. This corresponds to roughly a 20% increase in the hydration shell contribution to the sound velocity.[16] Using the adiabatic compressibility $\beta = 6 \times 10^{-11}$ Pa^{-1} and the partial specific volume $v = 0.736$ mL g^{-1} for native α-lactalbumin[15] and the average intrinsic adiabatic compressibility $\beta_I = 13 \times 10^{-11}$ Pa^{-1} of small native globular proteins,[16] we find that the hydration shell contribution to the sound-velocity increment is roughly 0.1 m s^{-1} L g^{-1} (see Appendix). Thus, the increase in the sound-velocity increment $\Delta u/c$ due to the enlarged hydration shell contribution is roughly 0.02 m s^{-1} L g^{-1}. Since the overall sound-velocity increment decreases by about 0.03 m s^{-1} L g^{-1}, a decrease of $\Delta u/c$ by about 0.05 m s^{-1} L g^{-1} is to be attributed to decreased intrinsic sound velocity and enhanced relaxation. Assuming the main contribution to the sound-velocity changes is connected with the titration of acidic side chains, we would expect that these changes would depend more on pH and less on ionic strength. In contrast, assuming the main contribution to changes in sound velocity is connected with alterations of nonpolar and weak hydrogen-bond interactions, we would expect a larger dependency on the ionic strength. This similarity of δu_{rel} in solvents A and D suggests that the sound-velocity increments in the acidic molten globule have little connection with changes in the nonpolar interactions and weak hydrogen bonds. It also indicates that, besides the changes which are directly connected with the titration of the carboxyl side chains, there are no large changes in the intrinsic sound velocity.

At acidic pH, a significant part of the relaxation contribution to the sound velocity in the 0.1–10-MHz frequency range is a proton-exchange reaction of the water-accessible carboxyl side chains:[17,26]

$$-COO^- + H_3O^+ \leftrightarrow -COOH + H_2O \qquad (1)$$

For amino acids in aqueous solution, the decrease in sound velocity at pH 1.5–2 relative to that at neutral pH that arises from changes in the hydration and the proton-exchange reaction is roughly 10 m s^{-1} L mol^{-1}.[27] Bovine α-lactalbumin contains, besides the α-carboxyl group, 17 β- and γ-carboxyl groups per molecule.[28] Consequently, the upper limit to the contribution of carboxyl group titration to the decrease in sound velocity observed at pH 2 is roughly 0.01 m s^{-1} L g^{-1} (180 m s^{-1} L mol^{-1}). The larger expected decrease of $\Delta u/c$ of 0.05 m s^{-1} L g^{-1} at pH 1.5–2 in solvent A indicates the presence of other important contributions. It should be noted that chicken egg-white lysozyme contains only eight carboxyl groups per molecule,[26] corresponding to an upper limit for the sound-velocity decrease due to titration of these side chains of roughly 0.006 m s^{-1} L g^{-1}. Within experimental error, the measured sound-velocity changes are not outside this limit, as expected from the known structural stability of lysozyme at acidic pH.

Another important relaxation term for consideration is the calcium-binding equilibrium:

$$\text{apo } \alpha\text{-lactalbumin} + Ca^{2+} \leftrightarrow \text{holo } \alpha\text{-lactalbumin} \qquad (2)$$

The binding constant for this reaction is 10^6–10^{10} L mol^{-1} at neutral pH and room temperature.[29] A second binding site with lower affinity (3×10^5 L mol^{-1} at pH 7.4) has been found.[30] It has been suggested that the formation of the acidic molten globule is due to replacement of the bound calcium by three hydrogen ions.[31] However, the binding constant for calcium for this state is about 10^5 L mol^{-1}.[31] Considering the lower molar ratio of bound calcium in comparison to that of the carboxyl groups, it is unlikely that the calcium-binding equilibrium makes a significant contribution to the sound velocity at pH 2.

Ikeguchi *et al.*[29] have described a large suppression of the guanidine-hydrochloride-induced intermediate state at a calcium concentration of 1 mM and a concentration of α-lactalbumin of 32–37 μM, pH 7. Our measured decrease in the absolute value of δu_{rel} with increasing calcium concentration (Figure 2, solvents A, B, and C) also indicates the suppression of the acidic molten globule state by excess calcium, however at significantly higher calcium concentrations. This suppression is a further indication that the formation of the acidic molten globule depends on the competitive replacement of bound calcium.[31]

Characteristics of the molten globule state are its nonfixed tertiary structure and enhanced side-chain mobility. However, little is known about the frequency scale of structural fluctuations. From polarization luminescence measurements, a time scale of >100 ns for the tertiary structure fluctuations has been suggested.[1] Our results indicate a significant contribution of conformational relaxation to the sound velocity at 2 MHz, corresponding to a time scale of faster than about 500 ns. Probably, a component of roughly –0.04 m s^{-1} L g^{-1} in $\Delta u/c$ is due to this conformational relaxation. The destabilized apo-state of α-lactalbumin differs

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by about 0.01 m s^{-1} L g^{-1} in $\Delta u/c$ from that of the native protein at neutral pH. Assuming an increase of 10% in the linear dimensions[25] for this molecule, we can account roughly 0.03 m s^{-1} L g^{-1} in $\Delta u/c$ for changes in the intrinsic sound velocity and enhanced relaxation. Thus, as quantitated by sound velocity, the apo-state of α-lactalbumin is more native-like than the acidic molten globule.

In summary, we found that the difference in the sound-velocity increment of 0.03 m s^{-1} L g^{-1} between the acidic molten globule at pH 2 and the native state at neutral pH cannot be explained by changes in the hydration of the molecule and proton-exchange relaxation. It is likely that conformational relaxation accounts for a decrease in $\Delta u/c$ of roughly 0.04 m s^{-1} L g^{-1}. Our data indicate a significant amount of structural relaxation in the A-state of α-lactalbumin at a time scale of faster than 500 ns.

Acknowledgment. We acknowledge gratefully many helpful discussions with Dr. G. Weber and Dr. A. J. Wand. We are indebted to Dr. E. Gratton, who supplied his sound-velocity measurement device as a model for our improved instrument. This work was supported by NIH GM31756.

Appendix

The adiabatic compressibility β of a protein in solution can be decomposed into the intrinsic compressibility β_I and a contribution of the hydration shell $\Delta\beta_H$.[16] β_I and $\Delta\beta_H$ contain also relaxation terms in the interior of the molecule and in the hydration shell, respectively.

$$\beta = \beta_I + \Delta\beta_H \tag{3}$$

$\Delta\beta_H$ depends on the difference of the compressibility between water in the hydration shell and bulk water. It is also proportional to the relative volume of the hydration shell to the protein. From $\beta = 6 \times 10^{-11}$ Pa^{-1} for native α-lactalbumin[15] and the average intrinsic adiabatic compressibility of small native globular proteins $\beta_I = 13 \times 10^{-11}$ Pa^{-1},[16] it follows that $\Delta\beta_H = -7 \times 10^{-11}$ Pa^{-1}. The concentration increment $\Delta u_H/c$ of the hydration contribution to the sound velocity can be approximately estimated with known $\Delta\beta_H$ values. All equations are correct for proteins at low concentration c. The relation between the concentration increments of adiabatic compressibility β, sound velocity u, and density ρ is approximately given by eq 4.[12]

$$\Delta\beta/(\beta_{so}c) = -2\Delta u/(u_{so}c) - \Delta\rho/(\rho_{so}c) \tag{4}$$

The subscript "so" refers to the solvent. The differences, indicated by Δ, are taken between the protein solution and the solvent. The relation between the compressibilities of protein solution β_s, solvent β_{so}, and protein β is approximately given by eq 5[32]

$$\beta_s = vc\beta + (1 - vc)\beta_{so} \tag{5}$$

where v is the partial specific volume of protein. It follows that the difference $\Delta\beta$ in compressibility between protein solution β_s and solvent β_{so} is given by

$$\Delta\beta = (\beta_s - \beta_{so}) = vc(\beta - \beta_{so}) \tag{6}$$

Inserting eqs 3 and 6 into eq 4, we obtain

$$v(\beta_I + \Delta\beta_H - \beta_{so})/\beta_{so} = -2\Delta u/(u_{so}c) - \Delta\rho/(\rho_{so}c) \tag{7}$$

Assuming that the water density of the hydration shell is approximately the density of bulk water, then from eq 7, for the concentration increment $\Delta u_H/c$ of the hydration contribution to the sound velocity follows eq 8

$$\Delta u_H/c = -0.5vu_{so}\Delta\beta_H/\beta_{so} \tag{8}$$

The partial specific volume v is 0.736 mL g^{-1} for native α-lactalbumin.[15] The sound velocity u_{so} and the adiabatic compressibility β_{so} of water at 25 °C are $u_{so} = 1496.69$ m s^{-1}[22] and $\beta_{so} = 44.77 \times 10^{-11}$ Pa^{-1}. For $\Delta\beta_H = -7 \times 10^{-11}$ Pa^{-1}, we find $\Delta u_H/c = 0.09$ m s^{-1} L g^{-1}.

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