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Compressibility Changes Accompanying Conformational Transitions of Apomyoglobin

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Abstract: *We used high-precision density and ultrasonic velocity measurements to characterize the native (N), molten globule (MG), and unfolded (U) conformations of apomyoglobin. The molten globule states that were studied in this work include the MG_{pH4}(NaCl) state observed at pH 4 and 20 m*M *NaCl, the MG_{pH4}(NaTCA) state observed at pH 4 and 20 m*M *sodium trichloracetate (NaTCA), the MG_{pH2}(NaCl) state observed at pH 2 and 200 mM NaCl, and the MG_{pH2}(NaTCA) state observed at pH 2 and 20 m*M *NaTCA. We used our densimetric and acoustic data to evaluate changes in adiabatic compressibility associated with the acid- or salt-induced N-to-MG, MG-to-U, MG-to-MG, and U-to-MG transitions of the protein. The N-to-MG_{pH4}(NaCl) and N-to-MG_{pH4}(NaTCA) transitions are accompanied by decreases in compressibility of $-(3.0 \pm 0.6) \times 10^{-6}$ and $-(2.0 \pm 0.6) \times 10^{-6}$ $cm^3\ g^{-1} bar^{-1}$, respectively. The N-to-MG_{pH2}(NaCl) and N-to-MG_{pH2}(NaTCA) transitions are associated with compressibility changes of $-(4.9 \pm 1.1) \times 10^{-6}$ and $(0.7 \pm 0.9) \times 10^{-6}$ $cm^3\ g^{-1}\ bar^{-1}$, respectively. We interpret these data in terms of the degree of unfolding of the various molten globule forms of apomyoglobin. In general, our compressibility data reveal significant disparities between the various equilibrium molten globule states of apomyoglobin while also quantitatively characterizing each of these states. Volumetric insights provided by our data facilitate gaining a better understanding of the folding pathways, intermediates, and kinetics of apomyoglobin folding.* © 2005 Wiley Periodicals, Inc. Biopolymers 79: 218–229, 2005

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INTRODUCTION

Apomyoglobin has long been used as a paradigm in kinetic and thermodynamic studies of protein folding.[1–12] Depending on solution conditions, it may exist in a native (N), molten globule (MG), or unfolded (U) conformation. At neutral pH, apomyoglobin is native. It exhibits a great deal of similarity with holomyoglobin with respect to secondary structure and overall conformation of the polypeptide

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chain and retains an extensive hydrophobic core formed by eight α-helices.[1,12] However, compared to the holoprotein, apomyoglobin is slightly less compact and exhibits a significantly reduced thermodynamic stability.[11,13,14]

At pH 4, the protein adopts a molten globule conformation.[13–16] The pH 4 molten globule intermediate of apomyoglobin contains a compact subdomain stabilized by nonspecific hydrophobic interactions within the intact A, G, and H helices of the native state.[2,15] The rest of the helices appear to be unfolded. Significantly, the equilibrium pH 4 molten globule is structurally similar to a kinetic intermediate on the folding pathway of apomyoglobin.[17–18] However, more detailed investigations from the Baldwin group have revealed that several molten globule-like intermediates of apomyoglobin may exist at pH 4.[18,19] For example, the pH 4 molten globule observed in the presence of NaTCA (sodium trichloracetic acid) is more highly structured than that observed in the presence of NaCl.[19]

At pH 2 and high ionic strength, the protein collapses into yet another molten globule conformation.[6,7,16] It is not clear if the high and low salt molten globule forms of apomyoglobin are structurally and thermodynamically similar. As pointed out by Jamin and Baldwin, ''the relation between the pH 4 low-salt and pH 2 high-salt intermediates remains to be worked out.''[18]

At pH 2 and low salt, apomyoglobin adopts an extended acid-induced unfolded conformation.[6,7,16] In this conformation, apomyoglobin is not fully extended but maintains 17–22% residual helicity within the A-, D-, E-, and H-helix regions and transiently samples compact states with native-like contacts between the N- and C-terminal regions.[8,10,20]

The energetics of conformational transitions of apomyoglobin has been extensively studied using calorimetric and spectroscopic approaches.[3,13,14,21] In contrast, volumetric characterizations have been much sparser and limited to determination of volume changes accompanying pressure-induced conformational transitions of apomyoglobin using the two-state approximation.[22–24] These investigations have produced very similar changes in volume, ΔV, accompanying pressure-induced denaturation of apomyoglobin. For example, Vidugiris and Royer have measured a volume change, ΔV, of -70 cm^3 mol^{-1} accompanying the transition from the native state to the pressure-induced molten globule-like intermediate state observed at 2000 bar.[23] The same authors have determined that the pressure-induced unfolding of the MG_{pH4} state is accompanied by a change in volume of -61 cm^3 mol^{-1}.[23]

These and other high-pressure volume determinations have been conducted at transition pressures, P_M, typically on the order of 1500 bar.[22–24] The atmospheric-pressure value of ΔV may be significantly different in magnitude and even in the sign if protein denaturation is accompanied by nonzero changes in isothermal compressibility, $\Delta K_T = -(\partial V/\partial P)_T$.[25] In fact, numerous results from our laboratory and other research groups have revealed that conformational transitions of proteins result in significant changes in compressibility that can be both positive and negative (for reviews, see Refs. 25 and 26). Consequently, the high-pressure studies of apomyoglobin provide little insight into the volumetric properties of the various conformational states of apomyoglobin at atmospheric pressure unless augmented by independent volume and compressibility measurements at ambient pressure. This deficiency is unfortunate since volume and compressibility are useful thermodynamic parameters that shed light into the hydration properties and intrinsic packing of various conformational states proteins.[25–34]

In this work, we report pH- and salt-dependent data on sound velocity and density of apomyoglobin solutions in the presence of NaCl and NaTCA. We use these data to evaluate changes in sound velocity, volume, and adiabatic compressibility accompanying the native-to-molten globule, molten globule-to-unfolded, molten globule-to-molten globule, and unfolded-to-molten globule transitions of apomyoglobin. We further use this volumetric information to characterize each of the conformational states of apomyoglobin with respect to the intrinsic packing and hydration properties, in particular, comparing the various molten globule states of the protein. In general, our data provide additional macroscopic insights that are useful in developing a general understanding of protein folding pathways and forces that stabilize/destabilize the native, unfolded, and intermediate conformations of globular proteins.

MATERIALS AND METHODS

Materials

Horse heart myoglobin was purchased from Sigma Aldrich Canada (Oakville, Ontario, Canada). The heme was removed by 2-butanone extraction following the previously described procedure.[35,36] The myoglobin solution was first adjusted to pH 1.5 by adding HCl. Then an equal volume of 2-butanone was added and thoroughly mixed on ice. The upper 2-butanone-rich layer of the mixture containing the heme was carefully removed. The procedure was repeated twice. The resulting apoprotein was extensively dialyzed

against water at 4°C and used without lyophylization. The content of holomyoglobin was assessed spectrophotometrically by measuring light absorption in the Soret region at 25°C and using an extinction coefficient, ε_{409}, of 160,000 M^{-1} cm^{-1}. The concentration of apomyoglobin was determined spectrophotometrically at 25°C using the extinction coefficient ε_{280} = 13,700 ± 200 M^{-1} cm^{-1}, which we determined by dry weight analysis. This result is in good agreement with the previously reported ε_{280} values of 13,940[37] and 14,300 M^{-1} cm^{-1}.[38] The contamination of the apomyoglobin solution by the holoprotein was less than 0.5%.

The protein was dissolved in doubly distilled water rather than buffers to avoid the need to correct for volume and compressibility changes due to the ionization-neutralization equilibria of the buffer. The ionic strengths of the protein solutions were adjusted to the desired levels by addition of known amounts of NaCl or sodium trichloracetate (NaTCA).

Aggregation State of Apomyoglobin

Since apomyoglobin is known to be prone to concentration- and time-dependent aggregation, we performed native polyacrylamide gel electrophoresis (PAGE) and dynamic light scattering (DLS) experiments to assess the aggregation state of the protein at the experimental conditions used in our investigation. PAGE electrophoretic experiments were performed in the following buffers: 10 m*M* cacodylate buffer with 20 m*M* NaCl or NaTCA adjusted to pH 5.5; 10 m*M* acetate buffer with 20 m*M* NaCl or NaTCA adjusted to pH 4.0; and 10 m*M* glycine buffer with 20 or 200 m*M* NaCl or 20 m*M* NaTCA adjusted to pH 2.0. For our low pH electrophoretic experiments, we used 5–20% polyacrylamide gel (PAAG) gradient gel polymerized by riboflavin-5′-phosphate/*N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) photopolymerization system. Instead of conventionally used bromophenol blue, we employed in the sample buffer methylene green, which exhibits a positive charge at acidic pH.

The light scattering experiments were performed using a Protein Solutions model Dynapro DLS dynamic light scattering spectrometer (Protein Solutions, Inc., Lakewood, NJ) at a wavelength of 823.7 nm. The scattering angle was 90°. The temperature of the sample holder was maintained at 25°C. All protein samples were freshly prepared. The protein concentrations were ~1 mg/mL. The aggregation state of apomyoglobin was assessed based on the hydrodynamic radii of protein particles calculated from the measured diffusion coefficients using the Stokes–Einstein equation.

Our combined native PAGE (data not shown) and dynamic light scattering measurements revealed complete lack of aggregation at pH 5.5 and 20 m*M* NaCl or NaTCA, pH 4 and 20 m*M* NaCl, and pH 2 and 20 m*M* NaCl. At these solution conditions, the protein was 100% monomeric with hydrodynamic radii, R_g, of 20 Å (at pH 5.5 and 20 m*M* NaCl), 19 Å (at pH 5.5 and 20 m*M* NaTCA), 24 Å (at pH 4 and 20 m*M* NaCl), and 30 Å (at pH 2 and 20 m*M* NaCl). These R_g values are in excellent agreement with the similar data reported by Kataoka et al. based on their solution small angle X-ray scattering (SAXS) measurements despite the fact that the SAXS measurements have been performed at much higher protein concentrations.[11]

At pH 4 and 2 and 20 m*M* NaTCA, we found rather insignificant but still detectable populations of oligomeric apomyoglobin. Specifically, at pH 4 and 20 m*M* NaTCA, apomyoglobin is 88.4% monomeric with a R_g of 28 Å and 11.6% oligomeric (probably, tetrameric) with a R_g of 115 Å; while at pH 2 and 20 m*M* NaTCA, the protein is 97.4% monomeric with a R_g of 22 Å and 2.6% oligomeric (probably, tetrameric) with a R_g of 84 Å. At pH 2 and 200 m*M* NaCl, one third of apomyoglobin molecules form extremely large water-soluble aggregates. Specifically, at these conditions, the apomyoglobin solution contains 66% monomers with a R_g of 27 Å and 34% large aggregates with a R_g on the order of 260 nm (2600 Å). It should be noted that, in agreement with this finding, Kataoka et al. also have observed large water-soluble aggregates at pH 2 and high NaCl concentrations.[11]

In the aggregate, when analyzing our volumetric data below, it is safe to assume that the protein is predominantly monomeric at all experimental conditions employed in our study except at pH 2 and 200 m*M* NaCl. At the latter conditions, the protein solution contains a significant population of large water-soluble aggregates that may influence the measured volumetric properties.

Volumetric Measurements

Solution sound velocity measurements were performed at 7.5 MHz using the resonator method as previously described.[39–42] We used an ultrasonic resonator cell with lithium niobate piezotransducers and a minimum sample volume of 0.8 cm^3.[43] For this type of acoustic resonator, the relative precision of the sound velocity measurements at frequencies near 7.5 MHz is at least $<1 \times 10^{-4}$%.[44]

The key characteristics of a solute directly derived from ultrasonic measurements is the relative specific sound velocity increment, $[u]$, which is equal to $(U - U_0)/(U_0 c)$, where c is the specific concentration of a solute (which is the solute molar concentration, C, divided by the solute molecular weight, M); U and U_0 are the sound velocities in the solution and the solvent, respectively.

All densities were measured with a precision of $\pm 1.5 \times 10^{-4}$% using an Anton Paar model DMA-60/602 vibrating tube densimeter (Anton Paar, Austria). We calculated the partial specific volume, v°, of apomyoglobin using the well-known relationship:

$$v^\circ = 1 / \rho_0 - (\rho - \rho_0) / (\rho_0 c) \qquad (1)$$

where ρ and ρ_0 are the densities of the solution and the solvent, respectively.

The relative specific sound velocity increments, $[u]$, determined as described above, were used in conjunction with the measured partial specific volume data, v°, to calcu-

late the partial specific adiabatic compressibility, k°_S, of apomyoglobin using the following relationship:

$$k^\circ_S = \beta_{S0}(2v^\circ - 2[u] - 1/\rho_0) \quad (2)$$

where β_{S0} is the coefficient of adiabatic compressibility of the solvent.[33,45]

All acoustic and densimetric titration experiments were performed at 25°C following the previously described experimental protocols (ref. 25 and citations therein). The protein concentration was within the range of 0.7–1 mg/mL. The pH values of all protein solutions were measured separately for the ultrasonic, densimetric, and optical experiments. The absolute error in these pH measurements was ±0.01 pH units. Each densimetric or ultrasonic velocimetric titration experiment was repeated three to five times, with the average values of $[u]$ and v° being used to calculate k°_S values from Eq. (2).

Optical Spectroscopy

The spectrophotometric measurements were conducted using an AVIV model 14 DS UV/Vis spectrophotometer, while fluorescence intensity measurements were performed using an AVIV model ATF 105 spectrofluorometer (Aviv Associates, Lakewood, NJ) with slits set for a 2-nm bandpass width. For the fluorescence measurements, the excitation, λ_{ex}, and emission, λ_{em}, wavelengths were set at 295 and 340 nm, respectively. Far- and near-ultraviolet (UV) circular dichroism (CD) spectra were recorded in 1 and 10 mm pathlength cuvettes, respectively, using an AVIV model 62 DS spectropolarimeter (Aviv Associates, Lakewood, NJ). Fluorescence intensity and CD titration profiles were measured by incrementally adding aliquots of HCl or NaCl to an optical cell containing a known amount of apomyoglobin. All optical spectroscopic experiments were carried out at 25°C. For the far-UV CD spectral measurements, apomyoglobin concentration was about 0.2 mg/mL, while for the near-UV CD measurements the protein concentration was about 2 mg/mL. For the fluorescence intensity measurements, the protein concentration was about 0.3 mg/mL.

RESULTS

To confirm that, at our experimental conditions, apomyoglobin in fact undergoes the requisite conformational transitions, we have conducted pH- and salt-dependent far- and near-UV CD and tryptophan fluorescence intensity spectroscopic measurements. Our spectroscopic results (not shown) are in perfect agreement with the literature.[6,7,14,16,19,46] These results suggest that the protein is native (N) above pH 5.5; adopts molten globule conformations at pH 4 and 20 m*M* NaCl [MG_{pH4}(NaCl)], pH 4 and 20 m*M* NaTCA [MG_{pH4}(NaTCA)], pH 2 and ~200 m*M* NaCl [MG_{pH2}(NaCl)], and pH 2 and ~20 m*M* NaTCA [MG_{pH2}(NaTCA)]; and exhibits an unfolded conformation (U) at pH 2 and low salt.

The initial values of the relative specific sound velocity increment, $[u]$, partial specific volume, v°, and partial specific adiabatic compressibility, k°_S, corresponding to the native state of apomyoglobin (determined at pH 5.8 and 20 m*M* NaCl) are 0.174 ± 0.005 $cm^3\ g^{-1}$, 0.747 ± 0.008 $cm^3\ g^{-1}$, and (6.4 ± 1.2) × 10^{-6} $cm^3\ g^{-1}\ bar^{-1}$, respectively. These volumetric properties are within the range typical of globular proteins and practically coincide with those corresponding to holomyoglobin.[25,26,34,47–49] At 25°C, the values of $[u]$, v°, and k°_S of myoglobin are 0.164 ± 0.003 $cm^3\ g^{-1}$, 0.745 ± 0.003 $cm^3\ g^{-1}$, and (7.1 ± 0.5) × 10^{-6} $cm^3\ g^{-1}\ bar^{-1}$, respectively.[48] The observed coincidence between the volumetric properties of apo- and holomyoglobin is consistent with the macroscopic similarity of their hydration and intrinsic packing. This notion stems from the standard interpretation of the volumetric properties of proteins in terms of the hydration and intrinsic contributions.[25,28,34,47–49] However, recall that NMR, calorimetric, and small angle X-ray scattering evidence suggests that apomyoglobin is more dynamic, less stable, and more extended than holomyoglobin.[1,11,13] Apparently, these differential properties of the apo- and holo-forms of myoglobin are not reflected (or somehow canceled out) in their partial specific volume and compressibility.

Figure 1a presents the pH dependence of the relative specific sound velocity increment, $\Delta[u]$, of apomyoglobin determined in 20 m*M* NaCl (●) and 20 m*M* NaTCA (○). In both salts, the relative specific sound velocity increment of apomyoglobin changes in two well-distinguishable steps. In NaCl, the two steps are both increasing and correspond to the N-to-MG_{pH4}(NaCl) (between pH 5.5 and ~4) and MG_{pH4}(NaCl)-to-U (between pH 4 and ~2) transitions. In NaTCA, the first, increasing step (between pH 5.5 and ~4) corresponds to the N-to-MG_{pH4}(NaTCA) transition. The second, decreasing step (between pH 4 and ~2) corresponds to the MG_{pH4}(NaTCA)-to-MG_{pH2}(NaTCA) transition. Figure 1b presents the NaCl dependence of $\Delta[u]$ at pH 2. In agreement with our and published CD data, our acoustic data reveal that apomyoglobin undergoes a salt-induced conformational transition from the unfolded conformation at low salt to the MG_{pH2}(NaCl) state (at 200 m*M* NaCl).[6,7,16]

Figure 2 shows how the partial specific volume of apomyoglobin, Δv, changes with pH at 20 m*M* NaCl

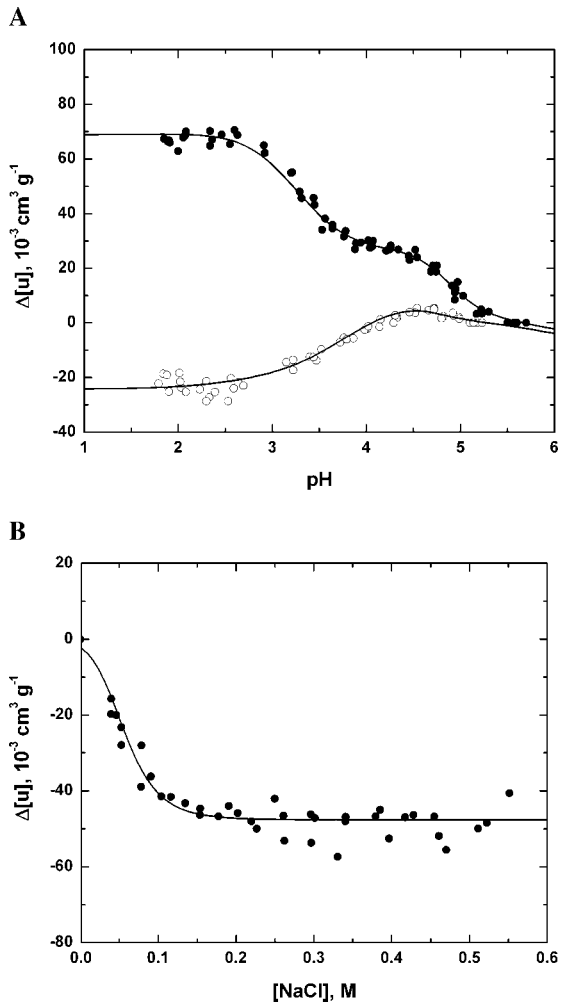

FIGURE 1 (a) The pH dependences of the relative molar sound velocity increment of apomyoglobin in 20 m*M* NaCl (●) and NaTCA (○). Experimental points were fit with Eq. (4) (solid lines). When approximating the NaCl-related data, the fit yielded the following parameters of Eq. (4): pK_1 and pK_2 are 6.5 and 4.3, respectively; $\Delta\nu_1$ and $\Delta\nu_2$ are 2.3 and 1.5, respectively; K_{10} and K_{20} are 1.7×10^{-4} and 0.031, respectively; and $\Delta[u]_1$ and $\Delta[u]_2$ are 0.023 and 0.055 $cm^3\ g^{-1}$, respectively. When approximating the NaTCA-related data, the fit yielded the following parameters of Eq. (4): pK_1 and pK_2 are 6.0 and 4.9, respectively; $\Delta\nu_1$ and $\Delta\nu_2$ are 2.5 and 1.3, respectively; K_{10} and K_{20} are 4.5×10^{-4} and 0.023, respectively; and $\Delta[u]_1$ and $\Delta[u]_2$ are 0.009 and $-0.022\ cm^3\ g^{-1}$, respectively. (b) NaCl dependence of the relative molar sound velocity increment of apomyoglobin at pH 2. Experimental points were fit with Eq. (5) (solid line). When fitting the data, the following parameters of Eq. (5) were obtained: K_0 and K_b are 0.047 and 9.4, respectively; Δn is 7.2; and $\Delta[u]$ is $-0.048\ cm^3\ g^{-1}$.

(●) and 20 m*M* NaTCA (○) (panel A) and with NaCl at pH 2 (panel B). Comparison of Figures 1 and 2 reveals that, compared to $\Delta[u]$, relative changes in volume are somewhat less pronounced and smaller (relative to experimental error). This observation is in agreement with a sizeable body of experimental evidence suggesting that protein transitions do not result

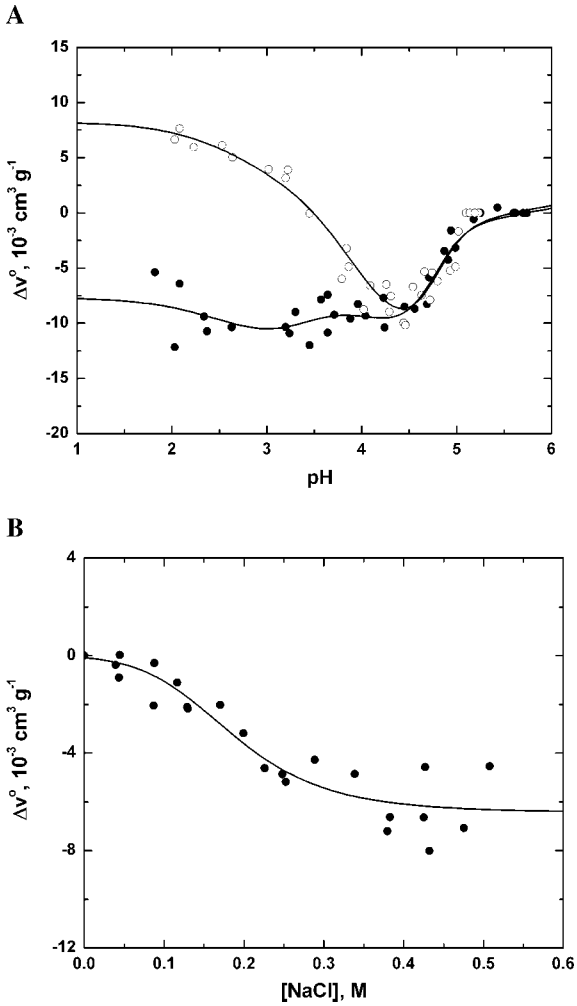

FIGURE 2 (a) The pH dependences of the partial molar volume of apomyoglobin in 20 m*M* NaCl (●) and NaTCA (○). Experimental points were fit with Eq. (4) (solid lines). When approximating the NaCl-related data, the fit yielded the following parameters of Eq. (4): pK_1 and pK_2 are 6.3 and 4.5, respectively; $\Delta\nu_1$ and $\Delta\nu_2$ are 2.5 and 1.5, respectively; K_{10} and K_{20} are 1.1×10^{-4} and 0.030, respectively; and Δv_1 and Δv_2 are -0.011 and $-0.009\ cm^3\ g^{-1}$, respectively. When approximating the NaTCA-related data, the fit yielded the following parameters of Eq. (4): pK_1 and pK_2 are 6.1 and 4.8, respectively; $\Delta\nu_1$ and $\Delta\nu_2$ are 2.4 and 1.4, respectively; K_{10} and K_{20} are 1.9×10^{-4} and 0.015, respectively; and Δv_1 and Δv_2 are -0.013 and $0.008\ cm^3\ g^{-1}$, respectively. (b) NaCl dependence of the partial molar volume of apomyoglobin at pH 2. Experimental points were fit with Eq. (5) (solid line). When fitting the data, the following parameters of Eq. (5) were obtained: K_0 and K_b are 0.013 and 5.6, respectively; Δn is 6.1; and Δv is $-0.006\ cm^3\ g^{-1}$.

in large changes in volume, the so-called protein volume paradox.[27,31,32,50]

DISCUSSION

Volumetric Contributions of Acid-Induced Neutralization of Ionizable Groups of Apomyoglobin

Apomyoglobin contains 8 aspartic acid residues, 13 glutamic acid residues, 11 histidine residues, and the C-terminal carboxylate group that become protonated upon a decrease in pH. Volumetric effects of these protonation events contribute to the measured changes in volume, Δv, and relative specific sound velocity increment, $\Delta[u]$. The protonation contributions to these volumetric characteristics can be expressed by the relationship:

$$\Delta x_{\text{prot}} = \text{M}^{-1} \sum_i \Delta X_i (1 + 10^{\text{pH}-\text{pK}_{ai}})^{-1} \qquad (3)$$

where pK_{ai} is the dissociation constant of the ith titrable group; ΔX_i is the molar change in the respective volumetric characteristics upon the complete protonation of the ith group; and M is the molecular weight of the protein (for apomyoglobin, M is 17 kDa).

We used Eq. (1) and previously described protocols to calculate the pH-dependent protonation contributions to changes in relative molar sound velocity increment, $\Delta[u]$, and volume, Δv.[51,52] In these calculations, we used the values of $\Delta[U]_i$ and ΔV_i that have been determined from investigating low molecular weight model compounds.[51,52]

In addition, changes in relative specific sound velocity increment, $\Delta[u]$, generally include the relaxation contribution. This contribution results from proton transfer reactions accompanying protonation/deprotonation of titrable amino acids, which also cause a relaxation increase in ultrasonic absorption.[26,53–56] The relaxation contribution to relative molar sound velocity increment, $\Delta[u]_{\text{rel}} = -\Delta(\alpha\lambda)_{\text{rel}}/(2\pi\omega\tau c)$, can be calculated from the relaxation increase in ultrasonic absorption expressed per wavelength, $\Delta(\alpha\lambda)_{\text{rel}}$ (α is the coefficient of ultrasonic absorption; λ is the ultrasonic wavelength; ω is the angular frequency of ultrasound; and τ is the relaxation time of the proton-transfer reaction).[53,55] For the pH-dependent investigations of apomyoglobin reported here, we measured only a very small increase in ultrasonic absorption that was within uncertainty of our measurements ($\pm 5\%$). Consequently, the relaxation contribution $\Delta[u]_{\text{rel}}$ can be ignored as negligibly small.

Volume and Compressibility Changes Accompanying Conformational Transitions of Apomyoglobin

To determine changes in relative specific sound velocity increment and volume accompanying each acid-induced transition of apomyoglobin, we fit the pH dependences of $\Delta[u]$ and Δv presented in Figures 1a and 2a with the following expression derived based on the three-state transition model:

$$\Delta x(\text{pH}) = \Delta x_{\text{prot}} + K_1([\Delta x_1(1 + K_2) + \Delta x_2 K_2)] / (1 + K_1 + K_1 K_2) \qquad (4)$$

where Δx_1 is the differential value of the volumetric observable X between the MG_{pH4}(NaCl) or MG_{pH4}(NaTCA) state and the N state; Δx_2 is the differential value of X between the U and MG_{pH4}(NaCl) states or between the MG_{pH2}(NaTCA) and MG_{pH4}(NaTCA) states; K_1 is the equilibrium constant for the acid-induced N-to-MG_{pH4}(NaCl) or N-to-MG_{pH4}(NaTCA) transition; and K_2 is the equilibrium constant for the acid-induced MG_{pH4}(NaCl)-to-U or MG_{pH4}(NaTCA)-to-MG_{pH2}(NaTCA) transition.

For the acid-induced conformational transitions of apomyoglobin, the effect of pH on the equilibrium constants K_1 and K_2 can be described by the equation $\partial \ln K/\partial \text{pH} = -2.303\ \Delta\nu$, where $\Delta\nu$ is the difference in the number of protons bound to the protein in its final (posttransitional) and initial (pretransitional) states.[57] The rigorous expression for K as derived by Tanford[57] is cumbersome and requires knowledge of the pK_a's of all titrated groups in both the initial and final states. To reduce the complexity, we use a previously described approach that, in particular, has been used for treating pH-dependent data on sperm whale apomyoglobin.[56,58] Recall that a pH-induced transition of a protein occurs as a result of a small number of ionizable amino acid residues which exhibit ''abnormal'' dissociation constants.[57] Consequently, the effect of acidic pH on protein stability can be considered in terms of the binding of protons to a relatively small number of ionizable residues. The pK_a's of these residues in the initial state may be shifted by as much as 3 or 4 pH units while changing to normal in the final state.[57] Hence, one can assume that each acid-induced conformational transition of apomyoglobin primarily occurs due to the binding of $\Delta\nu$ protons to the $\Delta\nu$ independently titratable groups. One also can reasonably assume that the dissociation constants for these $\Delta\nu$ titrated groups are abnormally low in the initial state, while being all equivalent and equal to pK_a in the final state. Under such circumstan-

Table I Volumetric Properties of Apomyoglobin Transitions

Transition	$\Delta[u]$ cm^3 g^{-1}	Δv cm^3 g^{-1}	Δk_s 10^{-6} cm^3 mol^{-1} g^{-1}
N-to-MG$_{pH4}$(NaCl)	0.023 ± 0.004	−0.011 ± 0.004	−3.0 ± 0.6
MG$_{pH4}$(NaCl)-to-U	0.055 ± 0.005	−0.009 ± 0.005	−5.7 ± 0.7
U-to-MG$_{pH2}$(NaCl)	−0.048 ± 0.003	−0.006 ± 0.004	3.8 ± 0.5
N-to-MG$_{pH4}$(NaTCA)	0.009 ± 0.004	−0.013 ± 0.004	−2.0 ± 0.6
MG$_{pH4}$(NaTCA)-to-MG$_{pH2}$(NaTCA)	−0.022 ± 0.008	0.008 ± 0.004	2.7 ± 0.6

ces, the equation derived by Tanford[57] reduces to the forms

$$K_1 = K_{10}(1 + 10^{pK_{a1} - pH})^{\Delta\nu_1} \quad (4a)$$

and

$$K_2 = K_{20}(1 + 10^{pK_{a2} - pH})^{\Delta\nu_2} \quad (4b)$$

where K_{10} and K_{20} are the respective equilibrium constants at neutral pH; $\Delta\nu_1$ is the number of abnormally titrated ionizable groups of apomyoglobin characterized by a very low dissociation constant in the native state while all exhibiting an effective dissociation constant of pK_{a1} in the MG$_{pH4}$(NaCl) or MG$_{pH4}$(NaTCA) state; $\Delta\nu_2$ is the number of abnormally titrated ionizable groups characterized by a very low dissociation constant in the MG$_{pH4}$(NaCl) or MG$_{pH4}$(NaTCA) state and with an effective dissociation constant of pK_{a2} in the unfolded or MG$_{pH2}$(NaTCA) state.

The salt dependencies of $\Delta[u]$ and Δv presented in Figures 1b and 2b were fit with the following relationships:

$$\Delta x(\text{salt}) = \Delta x K_0(1 + K_b[Cl^-])^{\Delta n} / (1 + K_0(1 + K_b[Cl^-])^{\Delta n}) \quad (5)$$

where Δx represents the differential volume or relative specific sound velocity increment between the U state and the MG$_{pH2}$(NaCl) state; K_0 is the equilibrium constant for the U-to-MG$_{pH2}$(NaCl) transition in the absence of salt; K_b is the association constant for Cl^- anion binding to apomyoglobin; and Δn is the differential number of Cl^- anions bound to the U and MG$_{pH2}$(NaCl) states.

Table I shows changes in relative specific sound velocity increment, $\Delta[u]$, volume, Δv, and adiabatic compressibility, $\Delta k_S = 2\beta_{S0}(\Delta v - \Delta[u])$, associated with the N-to-MG$_{pH4}$(NaCl), N-to-MG$_{pH4}$(NaTCA), MG$_{pH4}$(NaCl)-to-U, MG$_{pH4}$(NaTCA)-to-MG$_{pH2}$(NaTCA), and U-to-MG$_{pH2}$(NaCl) transitions determined from fitting the data in Figures 1 and 2. These values represent the volumetric signatures of each transition thereby describing the pressure-related stability characteristics of the native and molten globule conformations of apomyoglobin.

It is instructive to compare our Δv data with similar results obtained from high-pressure studies. As mentioned above, Vidugiris and Royer have measured volume changes, ΔV, of −70 and −61 cm^3 mol^{-1} to accompany the native-to-pressure-induced molten globule state transition and the MG$_{pH4}$(NaCl)-to-pressure-induced unfolded state transition of apomyoglobin, respectively.[23] Hence, the native-to-pressure induced unfolded transition is accompanied by a net value of ΔV of −131 cm^3 mol^{-1} (−70–61) determined at a midtransition of about ∼1500 bar. This value, if expressed per gram rather than per mole of protein, equals −0.0077 cm^3 g^{-1} (−131/17000), significantly larger (less negative) than our atmospheric-pressure Δv for the N-to-U transition −0.020 ± 0.007 cm^3 g^{-1}. The latter can be calculated by adding the Δv of the N-to-MG$_{pH4}$(NaCl) transition to that of the MG$_{pH4}$(NaCl)-to-U transition; −0.011–0.009 = −0.020 ± 0.007 cm^3 g^{-1} (see Table I). Understandably, such a comparison is physically meaningful only under the assumption that the acid-induced and pressure-induced unfolded states of apomyoglobin are volumetrically equivalent. With this assumption (which yet needs to be verified), the differential value of $\Delta\Delta v$ of 0.012 cm^3 g^{-1} (0.020–0.0077) between the atmospheric and elevated pressures reflects a change in isothermal compressibility accompanying the N-to-U transition of apomyoglobin, $\Delta k_T \approx \sim\Delta\Delta v/\Delta P = -8 \times 10^{-6}$ cm^3 g^{-1} bar^{-1} (−0.012/1500). This estimate is in good agreement with $-(8.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, our measured atmospheric-pressure value of Δk_S for the N-to-U transition that can be obtained by adding the Δk_S of the N-to-MG$_{pH4}$(NaCl) transition [$-(3.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}] to that of the MG$_{pH4}$(NaCl)-to-U transition [$-(5.7 \pm 0.7) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}] (see Table I). We have made a similar observation for staphylococcal nuclease.[51] This analysis underscores the notion that a change in compressibility should be carefully taken into account when a

high-pressure transition volume is extrapolated to atmospheric pressure.

Analysis of Compressibility Data

Inspection of data in Table I reveals that the N-to-MG_{pH4}(NaCl) and N-to-MG_{pH4}(NaTCA) transitions are accompanied by slightly different changes in compressibility that equal $-(3.0 \pm 0.6) \times 10^{-6}$ and $-(2.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively. The value of Δk_S for the N-to-MG_{pH2}(NaCl) transition can be obtained by adding the values of Δk_S measured for the N-to-MG_{pH4}(NaCl), MG_{pH4}(NaCl) to-U, and U-to-MG_{pH2}(NaCl) transitions. The value of Δk_S for the N-to-MG_{pH2}(NaTCA) transition is the sum of the values of Δk_S corresponding to the N-to-MG_{pH4}(NaTCA) and MG_{pH4}(NaTCA)to-MG_{pH2} (NaTCA) transitions. Such additive calculations are valid for compressibility, which is a variable of state. The N-to-MG_{pH2}(NaCl) and N-to-MG_{pH2}(NaTCA) transitions are associated with profoundly distinct changes in compressibility of $-(4.9 \pm 1.1) \times 10^{-6}$ ($-3.0 \times 10^{-6} - 5.7 \times 10^{-6} + 3.8 \times 10^{-6}$) and $(0.7 \pm 0.9) \times 10^{-6}$ ($-2.0 \times 10^{-6} + 2.7 \times 10^{-6}$) cm^3 g^{-1} bar^{-1}, respectively (see Table I). A change in compressibility upon the N-to-U transition equals the sum of the Δk_S values corresponding to the N-to-MG_{pH4}(NaCl) and MG_{pH4}(NaCl)-to-U transitions, $-3.0 \times 10^{-6} - 5.7 \times 10^{-6} = -(8.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} (see Table I).

It should be noted, however, that, at pH 2 and 200 m*M* NaCl, 34% of apomyoglobin molecules form large water-soluble aggregates (see above in Materials and Methods). It is difficult to assess the extent of the influence of protein aggregation on our determined values of Δk_S for the U -to-MG_{pH2}(NaCl) and, consequently, N-to-MG_{pH2}(NaCl) transitions. Thus, caution should be exercised in quantitative analysis of the above-determined value of Δk_S for the N-to-MG_{pH2}(NaCl) transition.

A change in compressibility associated with a protein transition, k°_S, can be conceptually rationalized in terms of the contributions from hydration, Δk_h, and intrinsic packing, k_M, since $k^\circ_S = k_M + \Delta k_h$.[25,26,28,49,59] The hydration change in compressibility, Δk_h, results from exposure to the solvent of previously buried atomic groups, as well as from the differential intensity of solute–solvent interactions in the native and denatured conformational states.[25,26] The intrinsic change in compressibility, k_M, results from the change in the size of the solvent-inaccessible protein interior as well as in the packing of atomic groups within that interior.[25,26] Based on this conceptual foundation, we have developed a simple model that analytically relates a change in compressibility accompanying a conformational transition of a protein, Δk_S, to the degree of its unfolding, σ.[26] The latter is defined as the ratio of the number of amino acid residues in the unfolded (fully solvent-accessible) domains of the protein to the total number of amino acids, thereby representing an effective measure of the interior packing and solvent exposure of protein groups. The value of σ is zero for the native state and unity for the fully unfolded state. The degree of unfolding is an operational rather than practical definition that would enable one to discriminate between the individual amino acids in the compact and unfolded protein domains. Nevertheless, the value of σ that can be determined from compressibility measurements represents a useful estimate of the effective number of amino acid residues in the random coil-like, fully solvent-exposed subdomains of the protein relative to the residues confined within the retained water-inaccessible core(s).

For our analysis below, we use a slightly modified relationship for the dependence of Δk_S on the degree of unfolding, σ that is given by the following:

$$\Delta k_S = \Delta k_M + \Delta\Delta k_h \qquad (6)$$

where Δk_M is the change in the intrinsic compressibility of the protein, $\Delta k_M = (N_A/M)$ [$1.10M(1 - \sigma)$ $(25 \times 10^{-6} + 75 \times 10^{-6}\,\sigma] - 1.10M \times 25 \times 10^{-6}$); and $\Delta\Delta k_h$ is the change in the hydration contribution to compressibility, $\Delta\Delta k_h = 4.7M^{-0.24}\,(1 - \sigma)^{0.76}$ [$\gamma_N + (\gamma_U - \gamma_N)\sigma$] + $1.45\,\sigma\gamma_U - 4.7M^{-0.24}\,\gamma_N$, where γ_N and γ_U are the average unit contributions to Δk_h (per 1 $Å^2$) of solvent-exposed atomic groups of the native and unfolded protein states, respectively.[26] At 25°C, γ_N and γ_U are equal to $(-23 \pm 3) \times 10^{-6}$ and $(-12 \pm 2) \times 10^{-6}$ cm^3 mol^{-1} bar^{-1} $Å^{-2}$, respectively.[26] Note that, in Eq. (6), the dependence of the intrinsic volume, V_M, on the molecular volume, M, of a protein is given by V_M ($Å^3$) = $1.10M$ (Da). In the original version of Eq. (6), the dependence of V_M on M was given by V_M ($Å^3$) = $1200 + 1.04M$ (Da), a relationship determined as the best fit of the experimental data on the intrinsic volumes of various globular proteins plotted against their molecular weights.[26] Although the relationship $V_M = 1.10M$ provides a slightly poorer fit of the experimental dependence of V_M on M (compared to $V_M = 1200 + 1.04M$), it yields more realistic estimates over a broad range of molecular weights; in particular, note that V_M approaches 0 as M decreases to 0.

Our developed model has enabled us to rationalize a previously made observation that the sign and mag-

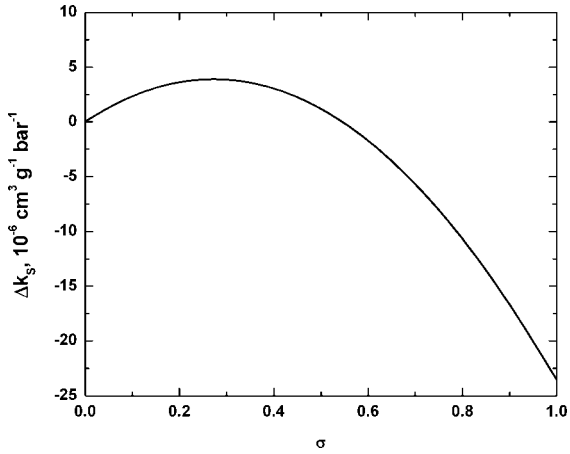

FIGURE 3 The dependence of the partial molar adiabatic compressibility of an ''average'' globular protein with a molecular weight of 17 kDa on the degree of unfolding, σ. The dependence was calculated using Eq. (6) as described in Ref. 26.

nitude of a change in protein compressibility represents a sensitive probe of the nature of the transition.[26,59] Specifically, all native-to-molten globule (N-to-MG) transitions studied to date are accompanied by increases in k°_S ranging from 1×10^{-6} to 4×10^{-6} cm^3 g^{-1} bar^{-1}. Native-to-partially unfolded (N-to-PU) transitions are accompanied by small decreases in k°_S ranging from -3×10^{-6} to -7×10^{-6} cm^3 g^{-1} bar^{-1}. Finally, native-to-fully (N-to-FU) unfolded transitions are accompanied by large decreases in k°_S that fall within the range of -18×10^{-6} to -20×10^{-6} cm^3 g^{-1} bar^{-1}.

Thus, the N-to-MG transitions of globular proteins studied to date are characterized by positive changes in compressibility.[25,26,59] In contrast to this notion, the N-to-MG$_{pH4}$(NaCl), N-to-MG$_{pH2}$(NaCl), and N-to-MG$_{pH4}$(NaTCA) transitions of apomyoglobin bring about negative changes in compressibility. This behavior is suggestive of the MG$_{pH4}$(NaCl), MG$_{pH2}$(NaCl), and MG$_{pH4}$(NaTCA) molten globule states of apomyoglobin being more extensively hydrated and less structured relative to other proteins capable of adopting a molten globule conformation (e.g., cytochrome c, α-lactalbumin, α-chymotrypsinogen A, etc.).[25,26,59]

Figure 3 shows the Δk_S-vs.-σ dependence for an ''average'' globular protein with a molecular weight of 17 kDa calculated with Eq. (6). The characteristic feature of the Δk_S-vs.-σ plot is that, due to interplay between the intrinsic and hydration effects, the change in compressibility accompanying a protein transition, Δk_S, parabolically increases upon an increase in σ from 0 to 0.27 and then decreases passing zero at σ of 0.55. Thus, around σ of 0.55, a subtle change in the degree of unfolding of the denatured protein may result in an alteration of the sign of Δk_S.

Inspection of Figure 3 reveals that, for an ''average'' 17 kDa protein, a compressibility changes of $-(3.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} [observed for the N-to-MG$_{pH4}$(NaCl) transition of apomyoglobin] and $-(2.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} [observed for the N-to-MG$_{pH4}$(NaTCA) transition of apomyoglobin] correspond to degrees of unfolding, σ, of 0.64 ± 0.02 and 0.61 ± 0.02, respectively. Based on the structural criterion, the degree of unfolding, σ, of the MG$_{pH4}$ state of apomyoglobin is 0.56 [(153 − 67)/153] since there are 67 amino acid residues in the intact helices A, G, and H compared to the total of 153 amino acid residues.[15] Thus, our determined values of σ for MG$_{pH4}$(NaCl) and MG$_{pH4}$(NaTCA) are slightly larger than the value that can be calculated based on the number of amino acid residues in the preserved A, G, and H helices. The observed disparity may suggest that some of the helical domains of A, G, and H are effectively disrupted in terms of their solvent exposure and interior packing characteristics. The compressibility changes of $-(4.9 \pm 1.1) \times 10^{-6}$ and $(0.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} accompanying the N-to-MG$_{pH2}$(NaCl) and N-to-MG$_{pH2}$(NaTCA) transitions, respectively, correspond to degrees of unfolding, σ, of 0.68 ± 0.03 and 0.51 ± 0.02, respectively. Note, however, that the value of Δk_S, and hence that of σ for the N-to-MG$_{pH2}$(NaCl) transition, may have been affected by protein aggregation at pH 2 and 200 m*M* NaCl.

Further inspection of Figure 3 reveals that the compressibility change of $-(8.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} calculated for the N-to-U transition of apomyoglobin corresponds to a degree of unfolding, σ, of 0.76 ± 0.02. Thus, 76% of the amino residues of apomyoglobin are effectively unfolded in the acid-induced unfolded conformation. Based on this estimate, the acid-induced unfolded conformation of apomyoglobin should be classified as partially unfolded which retains fluctuating water-inaccessible core involving ~24% amino acid residues. This characterization is consistent with NMR results from the Wright group, which suggest that the acid-induced unfolded state of apomyoglobin is not random coil-like but samples transient compact states with native-like contacts between the N- and C-terminal domains.[8,10,20] Moreover, Mohana-Borges et al., based on residual dipolar coupling measurements, determined that the helical population of the acid-induced unfolded state of apomyoglobin involves 29 residues (19%), in good agreement with our estimate.[10]

Comparison Between the Molten Globule States of Apomyoglobin

Our data enable us to perform a volumetric comparison between the various molten globule states of the protein. Using compressibility as a criterion for compactness and global structure, the most highly structured molten globule intermediate of apomyoglobin is the MG_{pH2}(NaTCA) state with the value of Δk_S for the N-to-MG_{pH2}(NaTCA) transition being equal to $(0.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} and the degree of unfolding, σ, of 0.51 $\pm$ 0.02. The MG_{pH2}(NaTCA) state is followed by the much less structured MG_{pH4}(NaTCA) and MG_{pH4}(NaCl) molten globule forms of apomyoglobin. The value of Δk_S for the N-to-MG_{pH4}(NaTCA) transition is equal to $-(2.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, while the degree of unfolding, σ, for the MG_{pH4}(NaTCA) state is 0.61 $\pm$ 0.02. The N-to-MG_{pH4}(NaCl) transition is characterized by the value of Δk_S of $-(3.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} with a degree of unfolding, σ, of the MG_{pH4}(NaCl) state of 0.64 $\pm$ 0.02. Finally, within the limits of uncertainty caused by significant aggregation of the protein at pH 2 and 200 m*M* NaCl, the lowest in this hierarchy is the MG_{pH2}(NaCl) state exhibiting the value of Δk_S for the N-to-MG_{pH2}(NaCl) transition of $-(4.9 \pm 1.1) \times 110^{-6}$ cm^3 g^{-1} bar^{-1} and the degree of unfolding, σ, of 0.68 $\pm$ 0.03. It is difficult to judge at this point if the observed volumetric difference between the MG_{pH4}(NaCl) and MG_{pH4}(NaTCA) is statistically significant. However, our compressibility-based observation that the MG_{pH4}(NaTCA) state is slightly more highly structured than the MG_{pH4}(NaCl) state is consistent with the CD and NMR results of Baldwin and collaborators, which have suggested that, in the former, the A, G, and H helices become more stable with the fourth helix—the B helix—being incorporated into the molten globule.[19]

In the aggregate, our results reveal disparities between the different equilibrium molten globule states of apomyoglobin. Independent of the veracity of any microscopic rationalizations, the volumetric differences between the molten globule states of apomyoglobin represent their independent thermodynamic signatures that can be used for identifying and characterizing each of these states. The observed hierarchy between the volumetric properties of the equilibrium intermediate states may ultimately prove useful in developing a better understanding of the pathways, intermediates, and kinetics of apomyoglobin folding. As a working hypothesis, one can propose that apomyoglobin folding may proceed from unfolded to the folded states sequentially via the MG_{pH2}(NaCl), MG_{pH4}(NaCl), MG_{pH4}(NaTCA), and MG_{pH2}(NaTCA) intermediates. Alternatively, in light of the funnel theory,[60,61] these molten globule states may represent local minima of the folding energy landscape of apomyoglobin that do not necessarily correspond to a consecutive folding pathway. In this scenario, apomyoglobin exhibits multiple folding pathways, a notion consistent with results of relaxation kinetics studies reported by Callender and collaborators.[62,63]

CONCLUDING REMARKS

We employed high-precision densimetric and acoustic measurements to determine changes in ultrasonic velocity, volume, and adiabatic compressibility accompanying transitions between the native (N), molten globule (MG), and unfolded (U) conformations of apomyoglobin. The molten globule states that were studied in this work include the MG_{pH4}(NaCl) state observed at pH 4 and 20 m*M* NaCl, the MG_{pH4}(NaTCA) state observed at pH 4 and 20 m*M* sodium trichloracetate (NaTCA), the MG_{pH2}(NaCl) state observed at pH 2 and 200 m*M* NaCl, and the MG_{pH2}(NaTCA) state observed at pH 2 and 20 m*M* NaTCA. We find that the N-to-MG_{pH4}(NaCl) and N-to-MG_{pH4}(NaTCA) transitions are accompanied by decreases in compressibility of $-(3.0 \pm 0.6) \times 10^{-6}$ and $-(2.0 \pm 0.6) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively. The N-to-MG_{pH2}(NaCl) and N-to-MG_{pH2}(NaTCA) transitions are accompanied by compressibility changes of $-(4.9 \pm 1.1) \times 10^{-6}$ and $(0.7 \pm 0.9) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively.

We used our previously developed model to interpret these compressibility results in terms of ''the degree of unfolding'', σ, of the MG and U states of apomyoglobin. Degree of unfolding is defined as the ratio of the number of amino acid residues in the unfolded domains of the protein to the total number of amino acids, thereby representing an effective measure of the interior packing and solvent exposure of protein groups. For the MG_{pH2}(NaTCA), MG_{pH4}(NaTCA), MG_{pH4}(NaCl), MG_{pH2}(NaCl), and U states of apomyoglobin, we estimate the values of σ equal to 0.51 $\pm$ 0.02, 61 $\pm$ 0.02, 64 $\pm$ 0.02, 0.68 $\pm$ 0.03, and 0.76 $\pm$ 0.02, respectively. In general, our results provide volumetric insights that are useful in developing a better understanding of the pathways, intermediates, and kinetics of apomyoglobin folding.

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