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Volumetric Characterizations of the Native, Molten Globule and Unfolded States of Cytochrome *c* at Acidic pH

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Cytochrome *c* can exist in a native (N), a molten globule (MG) or an unfolded (U) state depending on solution conditions. We have used high-precision ultrasonic and densimetric techniques to measure volume and compressibility changes accompanying the N to MG, N to U and U to MG transitions of the protein. For the N to MG transition (induced by lowering the pH to 2 in the presence of 200 mM CsCl), we measure a volume increase of 0.014 $cm^3 g^{-1}$ and a compressibility increase of 3.8×10^{-6} cm^3 g^{-1} bar^{-1}. For the N to U transition (induced by lowering the pH to 2 in the absence of salt), we measure a volume increase of 0.010 cm^3 g^{-1} and a compressibility decrease of 2.0×10^{-6} cm^3 g^{-1} bar^{-1}. For the U to MG transition at pH 2 (induced by adding CsCl up to 200 mM), we measure a volume increase of 0.006 cm^3 g^{-1} and a compressibility increase of 6.8×10^{-6} cm^3 g^{-1} bar^{-1}. We interpret these data to reach the following conclusions about the three states of cytochrome *c*. (1) A solvent-inaccessible core is preserved in the molten globule state, with the volume of this core being about 40% of the intrinsic volume of native cytochrome *c*. (2) The coefficient of the adiabatic compressibility of this preserved molten globule core is 61×10^{-6} bar^{-1}, a value that is over four times higher than that of the interior of the native protein. This result is consistent with the interior of the preserved MG core being liquid-like in contrast to the more tightly packed, solid-like interior of the native state. (3) In the unfolded state of cytochrome *c*, only 70 to 80% of the surface area of a fully unfolded conformation is exposed to the solvent, a result that reflects some level of order in the ''denatured'' state. (4) The relative volume fluctuations of the solvent-inaccessible interiors of the native, molten globule and unfolded states are equal to 0.6%, 2.0% and 2.9%, respectively. These data are consistent with the solvent-inaccessible core of the molten globule state being much more loosely packed than the core of the native state. In fact, the fluctuations in the molten globule and unfolded states are so high that one cannot exclude the possibility that formally buried atomic groups transiently contact solvent molecules. To the best of our knowledge, the data reported here provide the first characterizations of the intrinsic volume and compressibility properties of the native, molten globule and unfolded states of a single protein. We discuss in terms of the current protein literature the new insights that can be derived from these data.

Keywords: cytochrome *c*; protein folding; ultrasonics; volume; adiabatic compressibility

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Introduction

To date, the most direct and complete thermodynamic characterizations of the conformational states and transitions of globular proteins have been derived from calorimetric studies (Sturtevant, 1987; Khechinashvili, 1990; Murphy & Freire, 1991; Privalov, 1979, 1989, 1992; Hainie & Freire, 1993). By contrast, volumetric characterizations of protein transitions have been relatively sparse (for reviews, see Zamyatnin, 1984; Durchschlag, 1986; Sarvazyan, 1991; Chalikian *et al*., 1994a). This deficiency is unfortunate since volumetric data are complementary to calorimetric heat capacity data and can provide unique insights into the hydration proper-

Abbreviations used: N, native; MG, molten globule; U, unfolded.

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Table 1. The relative specific sound velocity increment, $[u]$, apparent specific volume, φV, and apparent specific adiabatic compressibility, φK_S, of cytochrome *c* in aqueous solution as a function of CsCl concentration

[CsCl] (mol/l)	$[u]$ ($cm^3\ g^{-1}$)	φV ($cm^3\ g^{-1}$)	φK_S ($10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$)
0	0.207 ± 0.002	0.737 ± 0.002	2.5 ± 0.4
	0.204[a]	0.7308 ± 0.0013[a]	2.99[a]
	0.199 ± 0.006[b]	0.733 ± 0.008[b]	2.9 ± 1.3[b]
	0.207[c]	0.725[c]	0.05[c]
0.1	0.208 ± 0.002		3.0 ± 0.4
0.2	0.210 ± 0.002	0.738 ± 0.002	3.4 ± 0.4
0.3	0.213 ± 0.002	0.738 ± 0.003	3.6 ± 0.5
0.4	0.217 ± 0.003		3.8 ± 0.6
0.5	0.219 ± 0.003		4.1 ± 0.6
0.6	0.221 ± 0.003	0.739 ± 0.003	4.3 ± 0.6
0.7	0.224 ± 0.003		4.5 ± 0.6
0.8	0.227 ± 0.003	0.739 ± 0.003	4.6 ± 0.6

[a] From Eden *et al*. (1982).
[b] From Kharakoz & Mkhitaryan (1986).
[c] From Gekko & Hasegawa (1986).

ties of individual protein states (Chalikian *et al*., 1994a). To address this deficiency, we report here the first in a series of studies devoted to volumetric characterizations of conformational transitions in globular proteins.

The horse cytochrome *c* system studied in this work is a small globular protein (12.4 kDa) composed of a single polypeptide chain of 104 amino acid residues. A survey of the relevant literature reveals that a broad range of experimental techniques have been used to study the pH-induced conformational transitions of this protein (Stellwagen & Babul, 1975; Dyson & Beattie, 1982; Ohgushi & Wada, 1993; Potekhin & Pfeil, 1989; Goto *et al*., 1990a,b, 1983; Myer & Saturno, 1990, 1991; Kuroda *et al*., 1992). Despite these important studies, a number of fundamental questions remain unanswered. (1) What are the hydration changes that accompany the N to MG, N to U and U to MG transitions of cytochrome *c*? (2) What is the extent of unfolding in the U state? (3) What are the differences in the packing of the protein interior in the N and MG states? (4) What is the extent to which the structure and hydration of the N, MG and U states depend on the solution ionic strength? (5) What are the differences in the volume fluctuations exhibited by the N, MG and U states? To address these questions, we have performed high-precision density and sound velocity measurements at 25°C to evaluate the changes in volume and adiabatic compressibility that accompany the acid-induced N to MG, N to U and U to MG transitions of cytochrome *c*. We interpret the resulting data in terms of the hydration properties and intramolecular interactions associated with each state of cytochrome *c*.

Results

Table 1 presents the volumetric properties we have measured for cytochrome *c* as a function of salt concentration, with all parameters being defined in Materials and Methods. Specifically, we list the values of the relative specific sound velocity increment, $[u]$, the apparent specific volume, φV, and the apparent specific adiabatic compressibility, φK_S, of cytochrome *c* at CsCl concentrations ranging from 0 to 0.8 M. For comparative purposes we list the few available corresponding published values. Errors were estimated by taking into account uncertainties due to concentration determinations, temperature drifts and inherent instrument characteristics. For globular proteins (Gekko & Noguchi, 1979; Arakawa & Timasheff, 1982; Durchschlag, 1986; Iqbal & Verrall, 1987b) and, particularly, for cytochrome *c* (Eden *et al*., 1982; Gekko & Hasegawa, 1986), the concentration dependences of the apparent specific volume, φV, and apparent specific adiabatic compressibility, φK_S, are known to be negligible in the protein concentration range between 0 and 5 mg/ml. The data listed in Table 1 were determined at a cytochrome *c* concentration of 3 mg/ml. Within the limits of the experimental uncertainty, our data are consistent with the corresponding partial v° and k_S° values obtained by extrapolation to infinite dilution. For this reason, in the discussion that follows, there is no need to discriminate between partial and apparent values.

Inspection of the data in Table 1 reveals that our results at low ionic strength are consistent with those reported by Eden *et al*. (1982) and Kharakoz & Mkhitaryan (1986) for ferricytochrome *c*. By contrast,

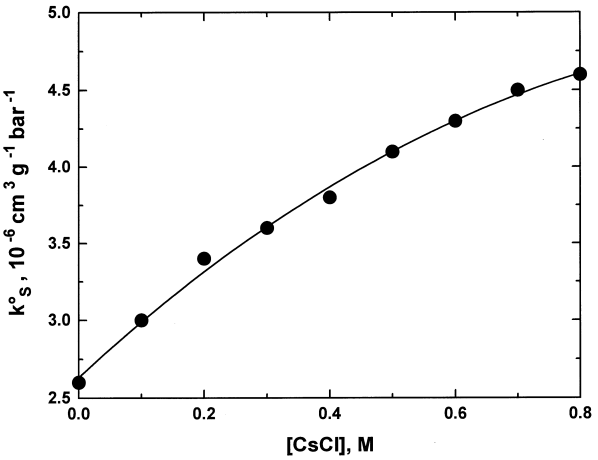


Figure 1. Salt-dependence of the partial specific adiabatic compressibility, k_S°, of native cytochrome *c* at pH ~ 7.

we find poorer agreement between our values for the partial specific volume, v°, and the partial specific adiabatic compressibility, k_S°, and those reported by Gekko & Hasegawa (1986). At present, we offer no explanation for this discrepancy.

Figure 1 graphically displays the salt-dependence of the k_S° data listed in Table 1. Inspection of this Figure reveals that k_S° increases with CsCl concentration, while, within experimental error, v° remains the same (see data in Table 1). Our spectroscopic measurements (CD and optical absorption) do not reveal a salt-induced conformational change from cytochrome *c* up to 1 M CsCl. Consequently, we interpret the observed increase in k_S° as being reflective of salt-induced changes in cytochrome *c* hydration. A decrease in protein hydration with increasing salt concentration can result from a reduction in the amount of free water available for hydration at higher CsCl concentrations where more water molecules are subsumed in the hydration shells of the ions.

Volumetric detection and characterization of the pH-induced N to MG and N to U transitions of cytochrome *c*

Figure 2 presents the pH-dependences (between pH 9 and 1) of (a) $[u]$, (b) v° and (c) k_S°, of cytochrome *c* in the absence of CsCl. There is only one other report in the literature of such pH-dependent measurements (Nölting & Sligar, 1993). Unfortunately, in that work, the accuracy of the densimetric measurements was insufficient to detect changes in v° with a decrease in pH. Consequently, no direct comparison with our volumetric data is possible. Nevertheless, the ultrasonic velocity data reported by Nölting & Sligar (1993) in the pH range 2.2 to 10 qualitatively agree with our results.

Note that the pH-dependence of v° shown in Figure 2(b) manifests only one broad transition within the 6 to 2 pH range. By contrast, inspection of panels Figure 2(a) and (c) reveals that the

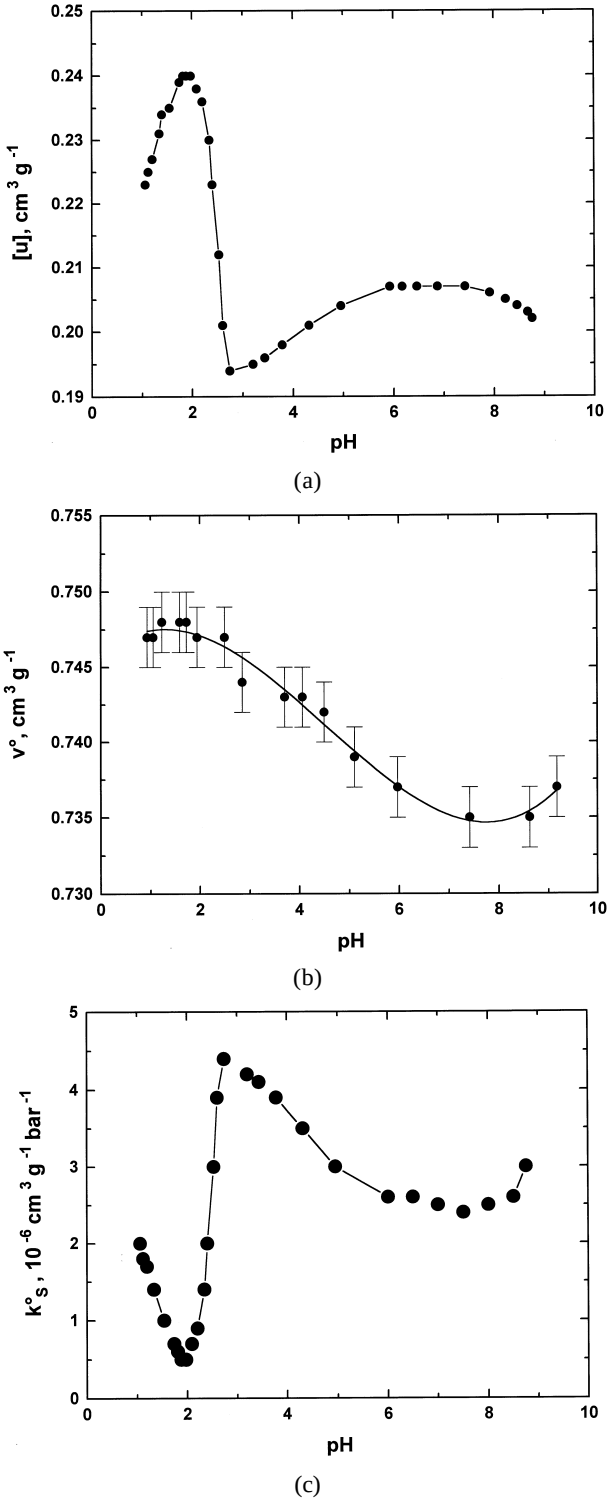


Figure 2. (a) pH-dependence of the relative specific sound velocity increment, $[u]$, for cytochrome *c* in the absence of CsCl; (b) pH-dependence of the partial specific volume, v°, of cytochrome *c* in the absence of CsCl; (c) pH-dependence of the partial specific adiabatic compressibility, k_S°, of cytochrome *c* in the absence of CsCl.

pH-dependence of (a) $[u]$ and (c) of k_S° exhibit three distinguishable transitions, which take place at pH values of about 4, 2.5 and 1. This volumetric-based observation of three transitions is consistent with

previously published spectroscopic studies (Myer & Saturno, 1990; Goto *et al*., 1993). Specifically, at low ionic strength and pH 3.6($\pm$0.3), Myer & Saturno (1990) observe a transition from the native state to an intermediate state (their state IIIa) with a slightly different (possibly more folded) conformation. Our data reveal that this transition is accompanied by an increase in k_S° (Figure 2(c)). Further acidification to pH 2 induces a transition to an unfolded high spin form of the protein (Ohgushi & Wada, 1983; Myer & Saturno, 1990; Goto *et al*., 1990a,b, 1993). Our volumetric measurements also detect this transition and reveal that it is accompanied by a substantial decrease in k_S°. Lowering the pH below 2 by further addition of HCl has been shown to cause refolding of the protein due to a decrease in electrostatic repulsion resulting from specific anion binding (Cl^-) to the positively charged residues (Goto *et al*., 1990a,b, 1993). Once again our volumetric measurements detect this transition with a midpoint of about pH 1, while also revealing that it is accompanied by an increase in k_S°. These results underscore the utility of sound velocity and compressibility measurements for detecting and characterizing protein transitions. By contrast with the compressibility observable (Figure 2(c)), we find that the volume observable (Figure 2(b)) detects only a single, broad pH-induced transition between pH 2 and 6. In general, we find that compressibility is a more sensitive probe of protein conformational states than is volume. Furthermore, as was previously shown (Buckin *et al*., 1989; Sarvazyan, 1991; Chalikian *et al*., 1994a) compressibility is exquisitely sensitive to changes in hydration and, under appropriate conditions, can be interpreted in terms of the relative degrees of hydration of different protein states.

The salt-dependence of the pH-induced transitions of cytochrome *c*

Figure 3(a) shows the influence of CsCl concentration (from 0.2 to 0.7 M) on the pH-dependence of $[u]$ for cytochrome *c*. (Our sound velocity data at 0.2 M CsCl qualitatively agree with those reported for cytochrome *c* at the same concentration of NaCl; Nölting & Sligar, 1993.) Previously it has been shown that at alkali halide concentrations above 100 mM acidification of a cytochrome *c* solution to pH 2 leads to a transition from the native to the molten globule state (Ohgushi & Wada, 1983; Goto *et al*., 1990a,b, 1993). Inspection of Figure 3(a) shows that our volumetric measurements also detect this transition while further revealing that at all ionic strengths studied the transition leads to a decrease in $[u]$. Note, however, that at 200 and 300 mM CsCl, a decrease in pH below 2 causes an additional transition in cytochrome *c* that is accompanied by an increase in $[u]$. At higher CsCl concentrations, this transition becomes less obvious. These observations are in agreement with the results of an earlier optical study reported by Stellwagen & Babul (1975).

Inspection of Figure 3(b) reveals that an increase in [CsCl] causes a linear increase in $[u]$. More

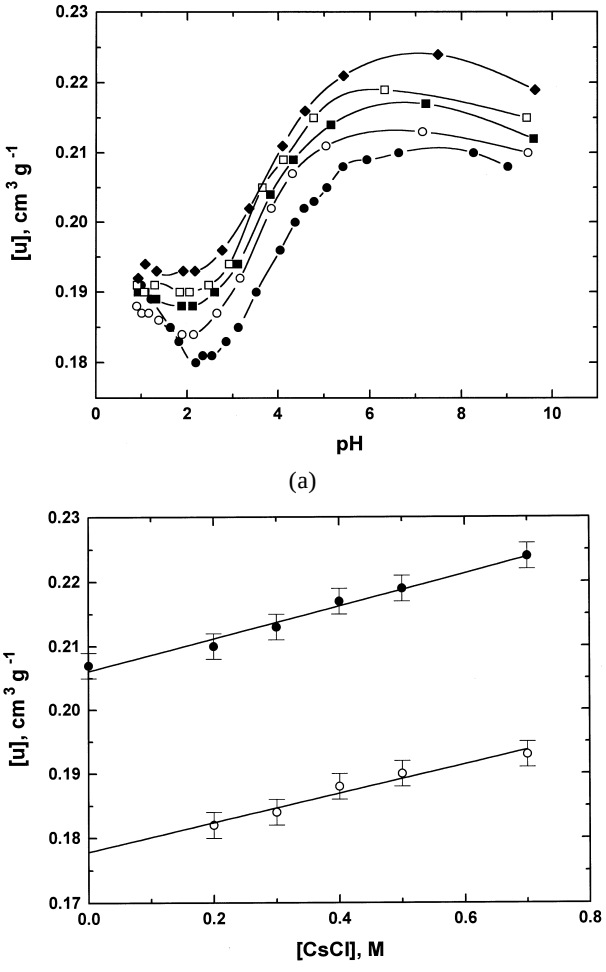


Figure 3. (a) pH-dependence of the relative specific sound velocity increment, $[u]$, of cytochrome *c* as a function of CsCl concentration: (●) 0.2 M; (○) 0.3 M; (■) 0.4 M; (□) 0.5 M; (◆) 0.7 M. (b) Salt-dependences of the relative specific sound velocity increment of cytochrome *c* in the native state at pH 9 (●) and in the molten globule state at pH 2 (○).

significantly, both the native and pH 2 molten globule states exhibit the same slope, $\partial[u]/\partial[\mathrm{CsCl}]$, for this dependence. This similarity in $\partial[u]/\partial[\mathrm{CsCl}]$ means that the difference between the values of $[u]$ that correspond to the native and molten globule states is salt-independent. Consequently, when studying the native to molten globule transition, it is sufficient to investigate the transformation at a single salt concentration. In selecting this salt concentration, it is preferable to choose the lowest possible concentration of CsCl at which the state exists and the transformations can be studied so as to minimize evaporation errors and to facilitate subsequent solution preparation at other salt concentrations. These conditions are met by a CsCl concentration of 200 mM. As shown in Figure 4, we have determined for cytochrome *c* the pH-dependences of (a) v° and (b) k_S° at 200 mM CsCl. Inspection of Figure 4 reveals that the transition from the native to the molten globule states is accompanied by increases in both v° and k_S°.

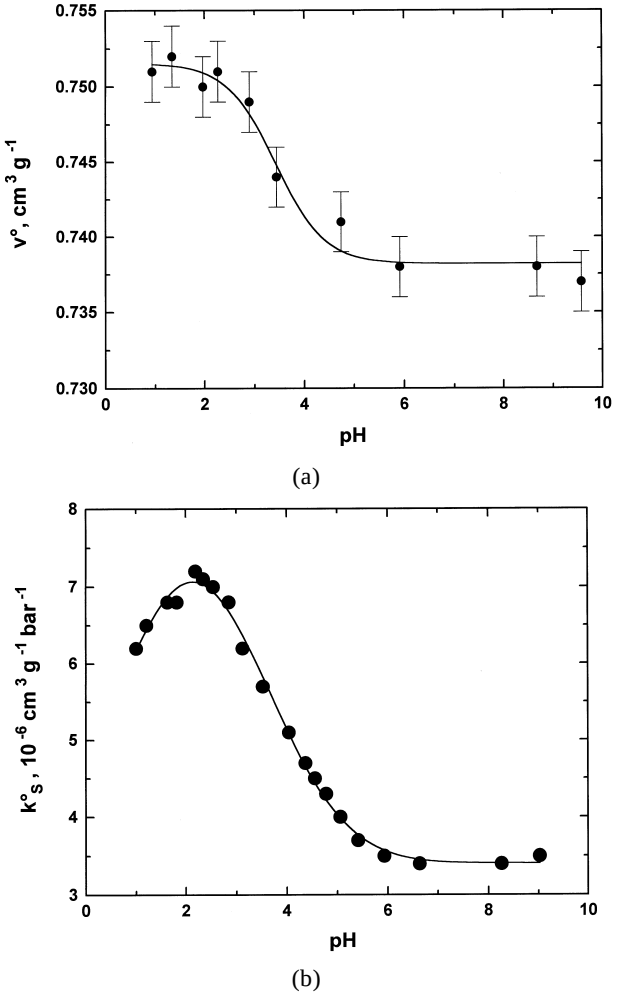


Figure 4. (a) pH-dependence of the partial specific volume, $v°$, of cytochrome *c* at 0.2 M CsCl; (b) pH-dependence of the partial specific adiabatic compressibility, $k_S°$, of cytochrome *c* at 0.2 M CsCl.

Volumetric detection and characterization of the salt-induced U to MG transition of cytochrome *c*

In the preceding section we presented the volumetric data we have measured for two cytochrome *c* transitions; namely, the native to molten globule (N → MG) and the native to unfolded (N → U) transitions. To complete the thermodynamic cycle of cytochrome *c* transitions (N → MG → U → N) we now present the results of our studies on the salt-induced U to MG transition at pH 2, which occurs upon increasing the CsCl concentration. Figure 5 shows the salt-dependences of (a) [*u*], of (b) $v°$ and (c) of $k_S°$ for cytochrome *c* at pH 2. These data reflect the salt-induced transitions of cytochrome *c* from the unfolded (U) to the molten globule state (MG). Note that Figure 5(b) and (c) reveal that the U to MG transition is accompanied by increases in both $v°$ and $k_S°$.

Spectroscopic characterization of the protein states associated with the volumetrically detected and characterized transitions of cytochrome *c*

We also have measured the CD and optical absorption spectra of cytochrome *c* at the relevant pH values and salt concentrations to confirm independently that cytochrome *c*, in fact, exists in its native, molten globule and unfolded states under the experimental conditions investigated. Figure 6 presents the optical absorption spectrum of ferricytochrome *c* in its native state (curve 1; the spectra measured at low ionic strength and at 1 M CsCl coincide), in its pH 2, 0.2 M CsCl molten globule state (curve 2), and in its pH 2, low ionic strength unfolded state (curve 3). Figure 7(a) and (b) show, respectively, the far UV and near UV CD spectra of ferricytochrome *c* in its native state (the spectra measured at low ionic strength and at 1 M CsCl coincide), its molten globule state and its unfolded state. Our spectroscopic measurements are consistent with previously published studies on cytochrome *c* (Babul & Stellwagen, 1972; Stellwagen & Babul, 1975; Dyson & Beattie, 1982; Myer & Saturno, 1990, 1991; Goto & Nishikori, 1991; Goto *et al*., 1990a,b, 1993). Inspection of Figure 6 reveals that the Soret absorption has maxima at 409 nm, 400 nm and 394 nm, which correspond to the N, MG and U states, respectively. These results are in agreement with the corresponding spin states of cytochrome *c* (Dyson & Beattie, 1982). The far UV CD spectra (Figure 7(a)) of the N and MG states show minima at 208 nm and 222 nm, which reflect α-helical content in these states. By contrast, the far UV CD spectra of the U state is characterized by a lack of secondary structure, with a single minimum at 200 nm. The near UV spectra of the MG and U states are distinct from that of the N state. This distinction suggests that the MG and U states do not manifest the tertiary structure present in the native state.

In summary, our spectroscopic measurements reveal the following features. (1) At pH 7, cytochrome *c* exists in its native, low-spin state, regardless of the salt concentration. (2) At pH 2 and ''low'' ionic strength, cytochrome *c* primarily exists in an unfolded high-spin state that lacks secondary structure. (3) At pH 2 and at [CsCl] $\geqslant$ 200 mM, cytochrome *c* exists in a mixed spin molten globule state, with a native-like secondary structure and non-native tertiary structure. As discussed below, these spectroscopic characterizations provide us with a framework within which to interpret our volumetric data.

Discussion

General considerations for molecular interpretations of volume and compressibility data

The partial molar volume, $V°$, of a protein in aqueous solution can be considered to be the sum of

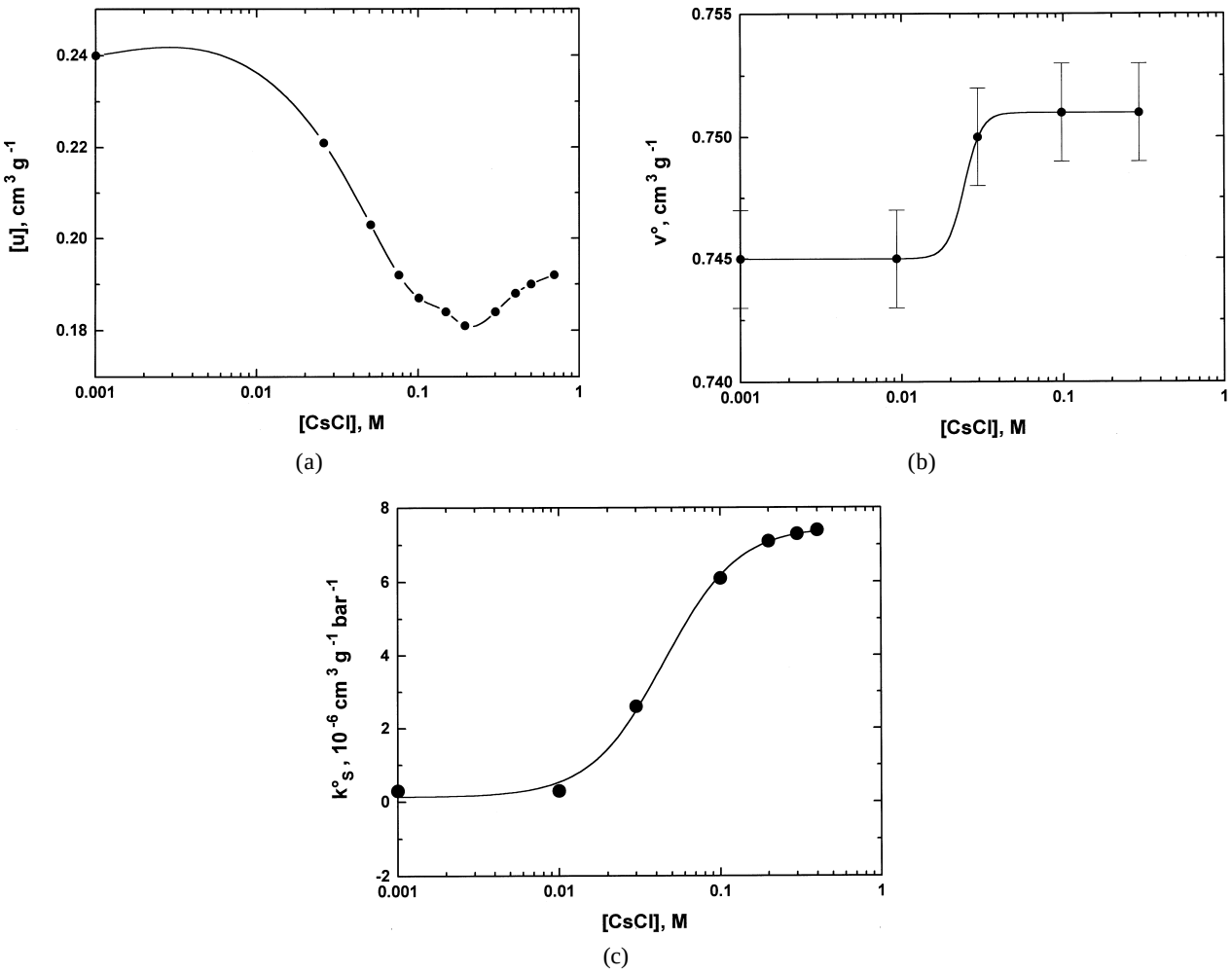


Figure 5. (a) Salt-dependence of the relative specific sound velocity increment of cytochrome *c* at pH 1.95. (b) Salt-dependence of the partial specific volume, $v°$ of cytochrome *c* at pH 1.95. (c) Salt-dependence of the partial specific adiabatic compressibility, k_S°, of cytochrome *c* at pH 1.95.

three terms: (1) the geometric volume, V_M, of the constituent atoms of the polypeptide chain(s); (2) the volume of the voids, V_V, within the protein that result from imperfect packing; and (3) changes in the volume of the solvent that result from interactions of the solvent molecules with the surface-accessible atomic groups of the protein; in other words, changes in the volume of the solvent, ΔV_h, due to solute hydration (Kauzmann, 1959). We can write an expression for the partial molar volume, $V°$, of a protein in terms of the sum of these three terms:

$$V° = V_M + V_V + \Delta V_h \quad (1)$$

The partial specific volume, $v°$, of a protein, in turn, can be expressed as the sum of two terms:

$$v° = v_{int} + \Delta v_h \quad (2)$$

where v_{int} is the specific intrinsic volume, which equals $(V_M + V_V)/M$; and Δv_h is the specific change in the volume of the solvent due to hydration, which by definition is equal to $\Delta V_h/M$, where M is the molecular mass of the protein.

The hydration term, ΔV_h, in equation (1) contributes negatively to the partial molar volume of a globular protein, while the terms V_M and V_V are positive. Interestingly, for native globular proteins in

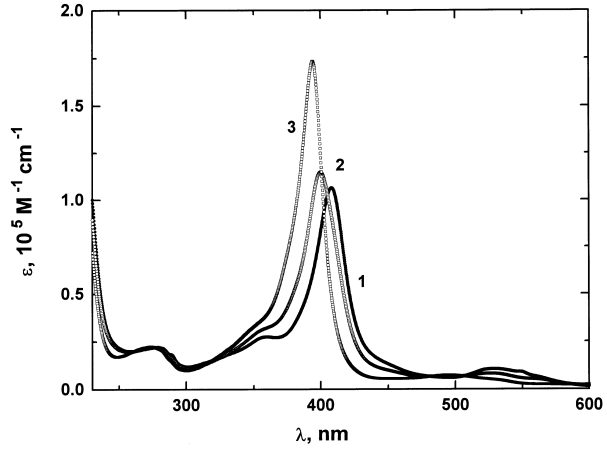
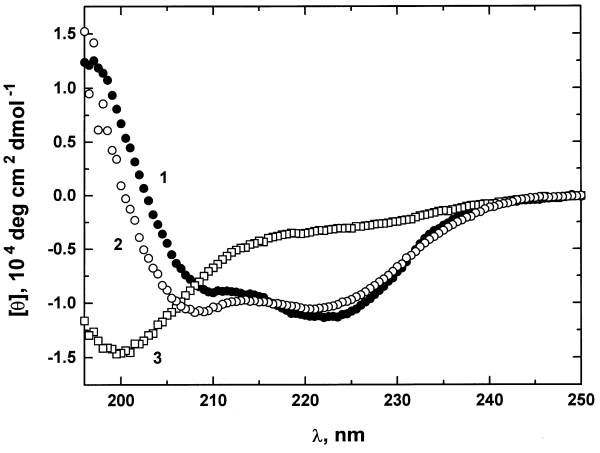
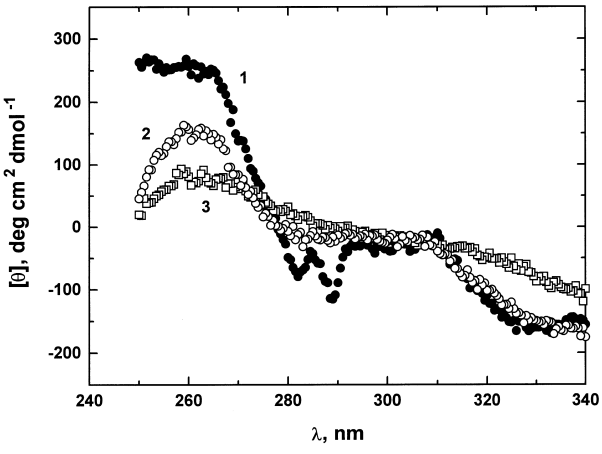


Figure 6. Optical absorbance spectra of cytochrome *c*. Curve 1, pH 7, low ionic strength native state; curve 2, pH 2, 0.2 M CsCl molten globule state; curve 3, pH 2, low ionic strength unfolded state.



(a)



(b)

Figure 7. (a) Far UV spectra of cytochrome *c*. Curve 1, pH 8, low ionic strength native state; curve 2, pH 2, 0.2 M CsCl molten globule state; curve 3, pH 2, low ionic strength unfolded state; (b) near UV spectra of cytochrome *c*. Curve 1, pH 8, low ionic strength native state; curve 2, pH 2, 0.2 M CsCl molten globule state; curve 3, pH 2, low ionic strength unfolded state.

water the hydration term, ΔV_h, and the void volume, V_V, tend to cancel (Kauzmann, 1959). Consequently, for rough estimations, one generally can ignore hydration or imperfect packing and simply calculate the partial volume of a protein from the amino acid composition. Based on X-ray coordinate data for 12 globular proteins, Richards (1977) has estimated the average value of the partial specific intrinsic volume, v_{int}, of a globular protein to be equal to 0.764 $cm^3\ g^{-1}$.

Relationships analogous to those described above for volume can be written for the partial molar and/or specific adiabatic compressibilities of a protein (Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986):

$$K_S^\circ = K_{SM} + K_{SV} + \Delta K_{Sh} \quad (3)$$

$$k_S^\circ = k_{Sint} + \Delta k_{Sh} \quad (4)$$

where K_{SM} is the intrinsic adiabatic compressibility of the constituent polypeptide chain(s); K_{SV} is the adiabatic compressibilities of the ''void'' volume in the protein interior; ΔK_{Sh} is the hydration change in the solvent adiabatic compressibility; k_{Sint} is the partial specific adiabatic compressibility of a protein interior, which is equal to $(K_{SM} + K_{SV})/M$; and $\Delta k_{Sh} = \Delta K_{Sh}/M$.

The K_{SM} term in equation (3) is determined by the compressibilities of the component covalent bonds and by their associated external electron shells. This compressibility is very small and can be neglected. The intrinsic compressibility of a protein is determined predominantly by the compressibility of the void volume due to imperfect packing. The average value of k_{Sint} for globular proteins has been estimated to be about 10×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$ (Gavish *et al*., 1983; Kharakoz & Sarvazyan, 1993). Assuming, the average v_{int} of globular proteins to be 0.764 $cm^3\ g^{-1}$ (Richards, 1977), the coefficient of adiabatic compressibility, β_{Sint}, of a globular protein interior can be estimated to be about 13×10^{-6} bar^{-1}. This value is 3.5 times smaller than the coefficient of adiabatic compressibility of water, 45×10^{-6} bar^{-1}, and characterizes the globular protein interior as a rigid, tightly packed, solid-like substance.

Hydration-induced changes in volume, ΔV_h, and compressibility, ΔK_{Sh}, which occur due to solvent interactions with all the different solvent-accessible protein atomic groups, can be expressed as follows:

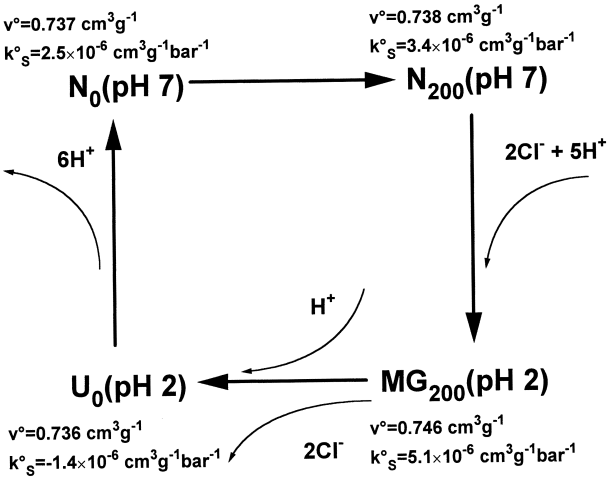
$$\Delta V_h = \sum_i n_{hi}(V^\circ_{hi} - V^\circ_0) \quad (5)$$

$$\Delta K_{Sh} = \sum_i n_{hi}(K^\circ_{Shi} - K^\circ_{S0}) \quad (6)$$

where n_{hi} is the number of water molecules in the hydration shell (the so-called hydration number) of the surface of the *i*th atomic group on a protein surface; V_{hi} and K_{Shi} are the partial molar volume and partial molar adiabatic compressibility of the water solvating the *i*th accessible surface atomic group; V_0° and K_{S0}° are the partial molar volume and partial molar adiabatic compressibility of the bulk solvent.

We and others have shown that the number of water molecules in the first monolayer around an atomic group on the protein surface can be considered as a lower limit for the hydration number, n_{hi}, in equations (5) and (6) (Buckin *et al*., 1989; Sarvazyan, 1991; Chalikian *et al*., 1994a). Therefore, n_{hi} can be defined as the ratio of the solvent-accessible surface area of the atomic group of interest (S_{Ai}) to the effective cross-section of a water molecule (S_W) (Kharakoz & Sarvazyan, 1993; Chalikian *et al*., 1994a). Thus, n_{hi} is equal to S_{Ai}/S_W, where the effective cross-section of a water molecule, S_W, can be taken to be equal to 9 $Å^2$. Furthermore, small molecular mass model compound studies at 25°C reveal that the overall hydration contribution to the partial adiabatic compressibility of a protein, Δk_{Sh}, to be negative (Sarvazyan, 1991; Kharakoz, 1991; Chalikian *et al*., 1992, 1993, 1994a,b).

Based on the preceding discussion, we interpret our compressibility data to draw the following



Scheme 1.

conclusions: (1) the partial adiabatic compressibility of cytochrome *c* (and other globular proteins) is determined by a positive contribution due to imperfect packing of the protein interior and a negative contribution due to hydration; (2) the more atomic groups of cytochrome *c* (or of any protein) that are exposed to the solvent (in other words, the higher the total solvent-accessible surface area) the more negative the hydration contribution to the partial adiabatic compressibility and, consequently, the smaller its value; (3) the more rigid the interior of cytochrome *c* (or any globular protein), the smaller its intrinsic compressibility and, consequently, the smaller the total partial compressibility of the protein.

Volume and compressibility changes associated with the conformational transitions of cytochrome *c*

The transitions of cytochrome *c* that we have spectroscopically detected and volumetrically characterized can be expressed as a thermodynamic cycle (see Scheme 1 in which the curved arrows indicate specific uptake or release by the protein of hydrogen and / or chloride ions, while the values for $v°$ and k_S° corresponding to each state are shown). The cycle consists of four steps: (1) a salt-induced transition from a low-salt native state at pH 7, N_0(pH 7), to a high-salt ($\geqslant$200 mM CsCl) native state at pH 7, N_S(pH 7); (2) a pH-induced transition from the high-salt, neutral pH native state, N_S(pH 7), to a high-salt, low pH (2) molten globule state, MG_S(pH 2); (3) a transition from the high-salt, low pH (2) molten globule state, MG_S(pH 2), to the low-salt, low pH (2) unfolded state, U_0(pH 2); (4) a pH-induced transition from the low-salt, low pH (2) unfolded state, U_0(pH 2), to the low-salt, neutral pH native state, N_0(pH 7).

For the reasons discussed earlier, we can consider the molten globule state of cytochrome *c* at a single CsCl concentration (200 mM). The changes in the relative specific sound velocity increment, $\Delta[u]$, the partial specific volume, $\Delta v°$, and partial specific adiabatic compressibility, Δk_S°, accompanying each of the four transitions described and shown in Scheme 1 are given in Table 2. Note that, within experimental error, the sum of both the volume and compressibility changes for the four steps equals zero, as expected for a cycle. The data presented in Table 2 reveal that the transition from the native N_S(pH 7) to the molten globule state MG_S(pH 2) is accompanied by increases in both volume, 0.014 $cm^3\,g^{-1}$, and compressibility, 3.8×10^{-6} $cm^3\,g^{-1}\,bar^{-1}$, while the transition from the native N_0(pH 7) to the unfolded U_0(pH 2) state is accompanied by an increase in volume, 0.010 $cm^3\,g^{-1}$, and a decrease in compressibility, -2.0×10^{-6} $cm^3\,g^{-1}\,bar^{-1}$. Further note that the transition from the unfolded U_0(pH 2) to the molten globule state MG_S(pH 2) state is accompanied by increases in both volume, 0.006 $cm^3\,g^{-1}$, and compressibility, 6.8×10^{-6} $cm^3\,g^{-1}\,bar^{-1}$.

The influence of an added substance, X, on protein denaturation (hydrogen or chloride ions for our pH-induced and salt-induced transitions, respectively) can be described by the general expression (Tanford, 1970):

$$\partial \ln K / \partial \ln a_X = \Delta\nu_X = \nu_{X,D} - \nu_{X,N} \qquad (7)$$

This expression is valid when the effect arises from high-affinity binding by the added substance to specific sites of the protein. In this equation, K is the equilibrium constant of protein denaturation and equals $\exp(-\Delta G°/RT)$, a_X is the activity of X in solution; $\Delta G°$ is the denaturation free energy of the protein; T is the absolute temperature; R is the universal gas constant; $\nu_{X,D}$ and $\nu_{X,N}$ are the numbers of X molecules bound per molecule of denatured protein and native protein, respectively; $\Delta\nu_X$ is the

Table 2. The changes in the relative specific sound velocity increment, $\Delta[u]$, the partial specific volume, $\Delta v°$, and the partial specific adiabatic compressibility, Δk_S°, accompanying cytochrome *c* conformational transitions

Transition	$\Delta[u]$ ($cm^3\,g^{-1}$)	$\Delta v°$ ($cm^3\,g^{-1}$)	Δk_S° (10^{-6} $cm^3\,g^{-1}\,bar^{-1}$)
N_0(pH 7) → N_{200}(pH 7)	0.003 ± 0.003	0.001 ± 0.003	0.9 ± 0.4
N_{200}(pH 7) → MG_{200}(pH 2)	–0.030 ± 0.003	0.014 ± 0.003	3.8 ± 0.4
MG_{200}(pH 2) → U_0(pH 2)	0.059 ± 0.003	–0.006 ± 0.003	–6.8 ± 0.4
U_0(pH 2) → N_0(pH 9)	–0.033 ± 0.003	–0.010 ± 0.003	2.0 ± 0.4

difference in the number of X molecules bound to the protein in its denatured and native states.

The difference in the number of protons bound to ferricytochrome *c* in its unfolded and native states, $\Delta\nu_H^+$, has been estimated from calorimetric studies to be 5.6 (Potekhin & Pfeil, 1989). Subsequently, Kuroda *et al*. (1992) reported a value of 6.5 based on temperature-dependent optical studies. Thus, for ferricytochrome *c*, on average, six more protons are bound to the unfolded state than to the native state. These and other studies also reveal the native to molten globule transition in cytochrome *c* to be accompanied by an uptake of about five protons ($\Delta\nu_H^+$) and about two chloride ions ($\Delta\nu_{Cl}^-$) from the solvent (Potekhin & Pfeil, 1989; Kuroda *et al*., 1992; Goto *et al*., 1993). Clearly, the differential uptake of protons and / or chloride ions by cytochrome *c* in its different states will make additional contributions to the values of $\Delta v°$ and $\Delta k_S°$ that we measure for the N_0(pH 7) → U_0(pH 2), N_S(pH 7) → MG_S(pH 2) and MH_S(pH 2) → U_0(pH 2) transitions. We take these additional contributions into account when, as described below, we interpret our experimental data in terms of the partial volumes and partial adiabatic compressibilities of cytochrome *c* in its various conformational states.

The native state of cytochrome *c*

At low salt concentrations, cytochrome *c* displays a partial specific volume of 0.737 $cm^3\ g^{-1}$ and a partial specific adiabatic compressibility of 2.5×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$. Both of these values are typical of globular proteins (Kuntz & Kauzmann, 1974; Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993). Recall that the partial specific volume, v_{int}, of a globular protein interior is, on average, 0.764 $cm^3\ g^{-1}$ (Richards, 1977). Using this value in conjunction with our experimental data and equation (2) we calculate the hydration contribution, Δv_h, to the partial specific volume, $v°$, of cytochrome *c* to be equal to –0.027 $cm^3\ g^{-1}$. Thus, the modulus of the hydration contribution, Δv_h, to the partial specific volume, $v°$, of cytochrome *c* is small and does not exceed 4% of the total value of $v°$. This observation is general for globular proteins, which, on average, exhibit partial specific volumes of 0.72($\pm$0.03) $cm^3\ g^{-1}$ at 25°C (Kuntz & Kauzmann, 1974; Gekko & Hasegawa, 1986). The significant point to emphasize is that the absolute value of the average hydration contribution to the partial specific volume, $v°$, of a globular protein is about 3 to 7% of the magnitude of $v°$. Therefore, large volume alterations are not expected for processes that involve hydration changes in globular proteins.

The situation is quite different for the compressibility properties of globular proteins. Applying equation (4) and using the average value of 10×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$ for the intrinsic compressibility, k_{Sint}, of a globular protein (Kharakoz & Sarvazyan, 1993) one can estimate the hydration contribution, Δk_{Sh}, to be -7.5×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$. Thus, the absolute value of the hydration contribution, Δk_{Sh}, is three times as large as the partial specific adiabatic compressibility, $k_S°$, of cytochrome *c*, an observation that contrasts with the corresponding behavior of Δv_h.

The hydration contribution to the partial molar adiabatic compressibility of a protein, as well as the hydration contributions to other partial molar thermodynamic observables, such as hydration enthalpy, hydration entropy or hydration free energy (Oobatake & Ooi, 1993), can be assumed to be roughly proportional to the accessible surface area, A_s, of the protein (Kharakoz & Sarvazyan, 1993). The average value of the accessible surface area, A_s, of a globular protein is equal to 11.12 $M^{2/3}$ Å^2 (Richards, 1977). For cytochrome *c* with a molecular mass of 12.4 kDa, the accessible surface area can be calculated to be 5957 Å^2, which is in good agreement with 5932 Å^2, the value calculated by Oobatake & Ooi (1993). The hydration contribution to the partial specific adiabatic compressibility, Δk_{Sh}, of a solute is proportional to the specific accessible surface area, a_S. The specific accessible surface (per gram of protein) of a globular protein, a_S, can be calculated from the relationship (Richards, 1977):

$$a_S = A_S N_A / M = 11.12 M^{-1/3} N_A \qquad (8)$$

where N_A is Avogadro's number.

For cytochrome *c*, a_S is equal to 2.9×10^7 $cm^2\ g^{-1}$. Thus, from the relationship $\Delta k_{Sh} = \gamma a_S$ we calculate the proportionality coefficient, γ, to be equal to -2.6×10^{-13} cm bar^{-1}. Significantly, this value is very similar to the corresponding coefficients that we have calculated for the X-Gly-Gly and Gly-Gly-X families of tripeptides (-2.8×10^{-13} to -2.1×10^{-13} cm bar^{-1}, where X is an apolar amino acid residue) from the ratio between their partial specific adiabatic compressibilities and their specific accessible surface areas (Chalikian *et al*., 1994c). To appreciate this similarity, it should be noted that γ values for other classes of compounds are very different. For example, using the compressibility data presented by Hoiland (1986) and calculating accessible surface areas according to Bondi (1964), we derive γ values of -4.32×10^{-13} cm bar^{-1} for glycine, -1.44×10^{-13} cm bar^{-1} for D-glucose, 1.33×10^{-13} cm bar^{-1} for acetic acid and 2.01×10^{-13} cm bar^{-1} for ethanol. These values are very different from each other and from the similar values we have determined for cytochrome *c* and the diglycyl tripeptides noted above. Based on this similarity, we propose that water molecules contacting the surface atomic groups of globular proteins experience the same average influence as water molecules that solvate solvent-accessible short oligopeptides. Thus, the value of $k_S°$ for an extended fully unfolded polypeptide chain should be similar to that of a short tripeptide and, therefore, equal to $-18(\pm 3) \times 10^{-6}$ $cm^3\ g^{-1}\ bar^{-1}$ (Chalikian *et al*., 1994c). This value for $k_S°$ is more than three times lower in magnitude (less negative) than that of a fully unfolded protein as calculated by Iqbal & Verrall (1988) from the sum of compressibility contributions of the constituent amino acid and peptide units.

In fact, the value of $-60(\pm 3) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} reported by Iqbal & Verrall (1988) is much more negative than that of the amino acid glycine -35.4×10^{-6} cm^3 g^{-1} bar^{-1} (Millero *et al*., 1978), in which the hydration shell is dictated almost completely by strong electrostatic solute-solvent interactions, while being comparable with the partial specific adiabatic compressibility of ions (e.g. k_S° of KCl is equal to -59.2×10^{-6} cm^3 g^{-1} bar^{-1}; Millero *et al*., 1977). We believe that the discrepancy between our k_S° value for a fully unfolded polypeptide chain and that calculated by Iqbal & Verrall (1988) results from the fact that they used a value of -8.5×10^{-4} cm^3 mol^{-1} bar^{-1} for the compressibility contribution of the peptide unit (—CH_2CONH—). However, we recently have shown the compressibility contribution of the peptide unit to be $-1.1(\pm 0.5) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} at 25°C (Chalikian *et al*., 1994b), a value that is significantly higher (less negative) than that previously reported by Iqbal & Verrall (1987a).

The data in Table 1 and the plot in Figure 1 reveal that an increase in the CsCl concentration leads to an increase in k_S° while causing nearly no change in v°. As mentioned earlier, we interpret the observed increase in k_S° as primarily reflecting an increase in the value of the hydration contribution, Δk_{Sh}, (dehydration of the protein) due to a reduction in solvent activity with increasing salt concentration, rather than resulting from the changes in k_S° due to salt-induced alterations in structure/conformation. Consistent with this interpretation, Feng & Englander (1990) report that two-dimensional NMR does not reveal any substantial ionic strength-dependent structural changes in cytochrome *c*. However, based on small-angle X-ray scattering measurements, Trewhella *et al*. (1988) calculate the radius of gyration, R_g, of horse ferricytochrome *c* at neutral pH and low salt to be 8% higher than at 200 mM NaCl (14.2(±0.2) Å) *versus* 13.1(±0.2) Å). This observation suggests that ferricytochrome *c* becomes more compact (more tightly packed) at higher solution ionic strengths. This conclusion is qualitatively consistent with the proton exchange data presented by Liu *et al*. (1989), who, based on ultraviolet resonance Raman measurements, found the Trp59 indole $^1H/^2H$ exchange rate to decrease by a factor of 7.5 between Na_2SO_4 concentrations of 0.005 and 0.9 M (Liu *et al*., 1989). This decrease suggests that protein fluctuations associated with the Trp59 indole $^1H/^2H$ exchange are inhibited at high ionic strength.

With regard to our cytochrome *c* compressibility data, a ''tightening'' of the protein should result in a decrease in intrinsic compressibility, k_{Sint}, and, consequently, a decrease in the total partial compressibility of the protein. Inspection of Figure 1 reveals that the k_S° of cytochrome *c*, in fact, increases with increasing CsCl concentration. This behavior suggests that the total contribution to the partial compressibility of any structural changes which may or may not accompany an increase in ionic strength is negligible compared to the large contribution from protein dehydration that accompanies the ionic strength-induced decrease in solvent activity.

The unfolded state of cytochrome *c*

Inspection of the data in the final row of Table 2 reveals that the transition from N_0(pH 7), the low-salt, native salt of cytochrome *c* at pH 7, to U_0(pH 2), the unfolded state at pH 2, results in a volume increase of $0.010(\pm 0.003)$ cm^3 g^{-1} and a compressibility decrease of $2.0(\pm 0.4) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}. This N_0(pH 7) → U_0(pH 2) transition conceptually can be divided into two subtransitions. In the first subtransition, the N_0(pH 7) state retains its native conformation upon neutralization of the seven carboxyl groups (the total number of carboxyl groups of cytochrome *c* is 13). We represent this hypothetical subtransition by N_0(pH 7) + $7H^+$ → N_0^*(pH 2) is the hypothetical native form at pH 2, which differs from N_0(pH 7) only by the fact that the seven carboxyl groups are neutralized. In the second subtransition, N_0^*(pH 2) absorbs six more protons from the solvent to form the unfolded U_0(pH 2) state. This second hypothetical subtransition, N_0^*(pH 2) + $6H^+$ → U_0(pH 2), reflects the fact that about six more protons are bound to the unfolded state compared to the native state. Thus, in the N_0(pH 7) → U_0(pH 2) transition, acidification of the solution to pH 2 causes neutralization of all 13 carboxyl groups of cytochrome *c*, an event that contributes positively to the measured changes in both volume and compressibility. For the analysis employed here, we will assume that the changes in volume, ΔV, and compressibility, ΔK_S, that occur upon neutralization of each of the cytochrome *c* carboxyl groups can be approximated by the corresponding values we have measured for the neutralization of the carboxyl group in triglycine (Chalikian *et al*., 1994c), which are $10.5(\pm 0.5)$ cm^3 mol^{-1} and $18.4(\pm 0.9) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1}, respectively. With this assumption, we calculate that the hydration changes associated with neutralization of the 13 carboxyl groups of cytochrome *c* cause an increase in v° of 0.011 cm^3 g^{-1} and an increase in k_S° of 1.9×10^{-6} cm^3 g^{-1} bar^{-1}.

The differences in the v° and the k_S° of the U_0 (pH 2) and N_0^*(pH 2) states can be considered to be equal to the changes in volume, Δv°, and compressibility, Δk_S°, that accompany the pH-independent native to unfolded state transition of cytochrome *c*. We have calculated these values from our experimental data after taking into account the volume and compressibility changes due to the neutralization of the 13 carboxyl groups. Specifically, we find that $\Delta v^\circ = 0.010 - 0.011 = -0.001$ cm^3 g^{-1} and $\Delta k_S^\circ = -2.0 \times 10^{-6} - 1.9 \times 10^{-6} = -3.9 \times 10^{-6}$ cm^3 g^{-1} bar^{-1}. The Δv° value reveals that the unfolding of cytochrome *c* is accompanied by a small decrease in volume of 0.001 cm^3 g^{-1} (10 cm^3 mol^{-1}), which is about 0.1% of the total value of the partial specific (molar) volume. This result is consistent with earlier observations that protein denaturation does

not cause large changes in the partial volume (Durchschlag, 1986). By contrast, the Δk_S° value reflects a decrease in adiabatic compressibility of 3.9×10^{-6} cm^3 g^{-1} bar^{-1} accompanying cytochrome *c* unfolding, a change that is significant relative to the k_S° value of 2.5×10^{-6} cm^3 g^{-1} bar^{-1} for native cytochrome *c*. The k_S° of the unfolded cytochrome *c* can be calculated by subtracting the compressibility decrease upon unfolding, 3.9×10^{-6} cm^3 g^{-1} bar^{-1}, from the k_S° of the native cytochrome *c*, 2.5×10^{-6} cm^3 g^{-1} bar^{-1} to yield a value of -1.4×10^{-6} cm^3 g^{-1} bar^{-1}. This value is much higher than -18×10^{-6} cm^3 g^{-1} bar^{-1}, which is the estimate we made above in the previous section for the k_S° of the fully unfolded protein, assuming all groups are solvent exposed in the denatured state. This discrepancy suggests that not all the buried surface of the cytochrome *c* becomes exposed to the solvent upon unfolding.

To estimate the fraction of the total surface of the protein that is exposed to the solvent in the unfolded state one needs to recall that the k_S° of the unfolded protein should be equal to the sum of the intrinsic specific compressibility, k_{Sint}, of the preserved hydrophobic core and the hydration contribution, Δk_{Sh}. The k_{Sint} of the preserved core is a product of the coefficient of adiabatic compressibility, β_{Sint}, and the partial specific volume of the core, v_{Sint}. The hydration contribution, Δk_{Sh}, is a product of the accessible specific surface area, a_S, and the coefficient γ (-2.6×10^{-13} cm bar^{-1}). Consequently, one can write:

$$k_S^\circ = \beta_{Sint} v_{Sint} + \gamma a_S \qquad (9)$$

The accessible surface area, a_S, is the fraction, α, of the total solvent exposed surface area, a_T, of the extended polypeptide chain. The value of a_T can be approximated by $1.45 \times 10^{-16} N_A$ cm^2 g^{-1} (Richards, 1977). The molecular mass, M_C, of the preserved core is approximately equal to $(1 - \alpha)M$ (where M = 12.4 kDa is the molecular mass of cytochrome *c*). The packing volume (in Å^3) of the preserved core should be close to that of a globular protein with the same molecular mass, 1.27 M_C (Richards, 1977). Consequently, the specific volume of the core, v_{Sint}, in cm^3 per gram of cytochrome *c*, should be equal to $1.27 \times 10^{-24} N_A M_C / M$. With these estimates, equation (9) can be rewritten as:

$$k_S^\circ = 1.27 \times 10^{-24} \beta_{Sint} N_A (1 - \alpha) + 1.45 \times 10^{-16} \gamma N_A \alpha \qquad (10)$$

From equation (10), the fraction, α, of the total protein surface area can be calculated if the coefficient of adiabatic compressibility, β_{Sint}, of the core is known. It is reasonable to expect the majority of the solvent-inaccessible core amino acid residues to be apolar. Therefore, β_{Sint} can be taken to be between that of a liposome interior, 60×10^{-6} bar^{-1} (Buckin *et al*., 1979), and that of a liquid hydrocarbon, $\sim 100 \times 10^{-6}$ bar^{-1} (Weast, 1968). Incorporating these values into equation (10) yields α values equal to 0.7 to 0.8, which means that only 70 to 80% of the total surface of cytochrome *c* is exposed to the solvent in the unfolded state. This conclusion is in good agreement with Lee (1991), who estimated that the exposure of a protein to solvent in the unfolded state is about two-thirds the value that is expected for a fully extended chain. In general, our results are consistent with the suggestion of Richards (1992), who states that ''the entropic penalty of opening the chain to the extent necessary for full accessibility is simply too large''.

The molten globule state of cytochrome *c*

Inspection of the data in the second row of Table 2 reveals that the acid-induced transition from native state cytochrome *c* at 200 mM CsCl, N_S(pH 7), to the molten globule state, MG_S(pH 2), results in increases in both the volume (0.014 ± 0.003 cm^3 g^{-1}) and the compressibility (3.8×10^{-6} cm^3 g^{-1} bar^{-1}). As noted above, the native to molten globule transition is accompanied by an uptake from solvent of about five protons and two chloride ions (Potekhin & Pfeil, 1989; Kuroda *et al*., 1992; Goto *et al*., 1993). Consequently, like the N to U transition, the N_{200}(pH 7) to MG_{200}(pH 2) transition conceptually can be divided into two hypothetical subtransitions. In the first subtransition, the N_{200}(pH 7) protein retains its native conformation upon neutralization of the seven carboxyl groups. We can represent this hypothetical subtransition by N_{200}(pH 7) + $7H^+ \rightarrow N^*_{200}$(pH 2), where N^*_{200}(pH 2) is the hypothetical native form existing at pH 2 and 200 mM CsCl, which differs from N_{200}(pH 7) only by the fact that the seven carboxyl groups are neutralized. In the second subtransition, N^*_{200}(pH 2) absorbs from the solvent five more protons and two chloride ions to form the molten globule MG_{200}(pH 2) state. This second hypothetical subtransition can be written as N^*_{200}(pH 2) + $5H^+$ + $2Cl^- \rightarrow MG_{200}$(pH 2).

Strictly speaking, neutralization of the carboxyl groups at 200 mM CsCl should result in smaller values of Δv° and Δk_S° compared with those that occur at zero ionic strength. However, since these salt-dependent differences are small and fall within the experimental uncertainties, we will use the zero ionic strength values to estimate the volume and compressibility changes associated with the first subtransition. The values Δk_S° and Δv° for neutralization of 12 carboxyl groups can be calculated to be 0.010 cm^3 g^{-1} and 1.8×10^{-6} cm^3 g^{-1} bar^{-1}, respectively. With regard to the effect of chloride ion binding, one should recall that the partial molar volume of a chloride ion is equal to 22.9 cm^3 mol^{-1} (Takenaka & Arakawa, 1989) and the partial molar adiabatic compressibility is equal to -17×10^{-4} cm^3 mol^{-1} bar^{-1} (Mathieson & Conway, 1974). Thus, the uptake of two chloride ions from solvent in the second subtransition causes a volume decrease of 0.004 cm^3 g^{-1} and a compressibility increase of 0.3×10^{-6} cm^3 g^{-1} bar^{-1}.

The differences in v° and k_S° for the MG_{200}(pH 2) and N^*_{200}(pH 2) states of cytochrome *c* can be considered to be the changes in volume, Δv°, and

compressibility, Δk_S°, that accompany the pH-independent native to molten globule transition. Thus, we calculate that the native to molten globule transition causes an increase in v° of 0.008 $cm^3\ g^{-1}$ (0.014 – 0.010 + 0.004) and an increase in k_S° of 1.7×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$ ($3.8 \times 10^{-6} - 1.8 \times 10^{-6} - 0.3 \times 10^{-6}$). Correspondingly, k_S° of cytochrome *c* in the molten globule state is equal to 5.1×10^{-6} $cm^3\ g^{-1}\ bar^{-1}$ ($3.4 \times 10^{-6} + 1.7 \times 10^{-6}$).

Note that the volume increase of 0.008 $cm^3\ g^{-1}$ (100 $cm^3\ mol^{-1}$) that accompanies the native to molten globule transition is only about 1% of the partial specific (molar) volume of the native cytochrome *c*. However, for this transition, it has been shown that the hydrodynamic volume increases by about 60% (Goto *et al*., 1990a). This apparent discrepancy between the changes in the partial and hydrodynamic volumes suggests penetration of water inside the protein in its molten globule state, a penetration that results in compensation of the observed increase in hydrodynamic volume. Thus, 60% of the intrinsic volume of the protein in the molten globule state becomes accessible to the solvent. By extension, one can assume that roughly 60% of the total surface area of cytochrome *c* is exposed to the solvent. Applying equation (10) one can calculate the coefficient of adiabatic compressibility, β_C, of the solvent-inaccessible core using $\alpha = 0.6$. This calculation yields a β_{Sint} value equal to 61×10^{-6} bar^{-1}, which is typical for liquids. Thus, based on our data we conclude that, while the interior of the native state is solid-like, the interior of the preserved core of the molten globule state is more liquid-like. This conclusion is consistent with theoretical studies on globular proteins, which model the N to MG transformation as a solid to liquid transition (Karplus & Shakhnovich, 1992). As far as we are aware, the results presented here represent the first thermodynamic characterization and comparison of the interiors of the molten globule and native states of a protein.

Protein dynamics in different conformational states

Recall that the mean-square volume fluctuation, δV^2, is directly proportional to the coefficient of isothermal compressibility, β_{Tint}, of a protein interior (Cooper, 1976):

$$\delta V^2 = k_B T V_{int} \beta_{Tint} \qquad (11)$$

where k_B is Boltzmann's constant, T is the absolute temperature and V_{int} is the intrinsic volume of the protein. Consequently, the larger intrinsic compressibility that we have determined for the protein in the molten globule *versus* the native state implies higher volume fluctuations in the former state.

In applying equation (11) one can ignore any difference between the coefficients of isothermal, β_{Tint}, and adiabatic, β_{Sint}, compressibilities, since any such difference would fall within the experimental uncertainty. Thus, we use β_{Tint} values at 13×10^{-6} bar^{-1} and 61×10^{-6} bar^{-1} for the native and molten globule states, respectively. The intrinsic volume, V_{int}, (in Å^3) can be taken to be equal to 1.27 M, which yields 15,730 Å^3 for the native state (Richards, 1977). For the molten globule state, V_{int} can be taken to be 40% (6300 Å^3) of the value for the native state. Use of these data in equation (11) yields δV values of 90 Å^3 and 125 Å^3 for the native and molten globule states, respectively. Thus, the relative amplitude of volume fluctuations, $\delta V / V_{int}$, is equal to 0.6% for the native state and 2.0% for the molten globule state. These calculations reveal that the relative volume fluctuations in the molten globule state are more than three times higher than in the native state.

Similar calculations can be made for unfolded cytochrome *c* assuming V_{int} to be equal to 20 to 30% of the intrinsic volume of the native state cytochrome *c* (between 3150 Å^3 and 4700 Å^3), and β_{Tint} to be between 100×10^{-6} bar^{-1} and 60×10^{-6} bar^{-1}, respectively. Use of these values in equation (11) yields volume fluctuations, δV, of 110 Å^3 for the preserved core of unfolded cytochrome *c*, which corresponds to a relative amplitude of volume fluctuations, $\delta V / V_{int}$, equal to 2.3 to 3.5%. This value for the unfolded state is 50($\pm$30)% higher than that of the molten globule state. Such large volume fluctuations suggest loose packing of the interior of the preserved hydrophobic core of cytochrome *c* in the unfolded state.

Retrospective overview

In general, our results suggest the following features of the states associated with the salt and pH-induced conformational transitions of cytochrome *c* at 25°C. (1) The transition from the native to the molten globule state is accompanied by exposure to solvent of additional protein surface, with the total accessible surface area in the molten globule state being equal to 60% of that of a fully unfolded polypeptide chain. (2) The preserved core within the molten globule state retains secondary structural elements but is not as tightly packed as in the native state, since its coefficient of adiabatic compressibility is more than four times higher than that of the native state. (3) The relative volume fluctuations of the molten globule state increase threefold compared with the native state. (It should be noted here that Xie & Freire (1994) have suggested that the proportion of buried apolar surface in the folded region of the molten globule state may be higher than in the native state.) (4) The transition from the molten globule to the unfolded state results in further solvent exposure of the buried residues, with the total accessible surface reaching 70 to 80% (but not 100%) of the surface of an extended polypeptide chain. Thus, even in the unfolded state, a ''core'' is still preserved. (It is reasonable to expect that the majority of the buried amino acid residues in this core are apolar.) (5) No secondary structural elements remain in the unfolded state (perhaps, only hydrophobic

interactions are responsible for the formation of this core.) (6) The relative volume fluctuations of the preserved hydrophobic core of the unfolded state are about 50% higher compared with those of the molten globule and four to six times higher than those of the native state. In light of these large volume fluctuations, one cannot exclude the possibility that formally buried atomic groups may transiently contact solvent molecules.

Concluding Remarks

We have determined the pH-dependencies of the relative specific sound velocity increment, $[u]$, the partial specific volume, $v°$, and the partial specific adiabatic compressibility, $k_S°$, of cytochrome *c* over a range of CsCl concentrations (0 to 800 mM). We used the resulting data to evaluate the volume and compressibility changes that accompany the salt-induced and pH-induced transitions of cytochrome *c* at 25°C. Specifically, we detected and characterized the native state (N) to unfolded state (U) transition that occurs upon acidification to pH 2 in the absence of salt; the native to the molten globule state (MG) transition that occurs upon acidification to pH 2 in the presence of 200 mM CsCl; and the unfolded to the molten globule state transition that occurs upon addition of CsCl (up to 200 mM) at pH 2. For the N to MG transition, we determined a volume increase of 0.014 $cm^3\ g^{-1}$ and a compressibility increase of $3.8 \times 10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$. For the N to U transition, we determined a volume increase of 0.010 $cm^3\ g^{-1}$ and a compressibility decrease of $2.0 \times 10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$. For the U to MG transition, we determined a volume increase 0.006 $cm^3\ g^{-1}$ and a compressibility increase $6.8 \times 10^{-6}\ cm^3\ g^{-1}\ bar^{-1}$.

We interpreted these data to reach the following general conclusions. (1) In the MG state, a solvent-inaccessible core is preserved, with the volume of the core being about 40% of the intrinsic volume of the native cytochrome *c*. (2) In the MG state, the coefficient of the adiabatic compressibility of the preserved core is $61 \times 10^{-6}\ bar^{-1}$, a value over four times higher than that of the interior of the native protein. Thus, we concluded that the interior of the preserved MG core is liquid-like in contrast to the solid-like interior of the native core. (3) In the unfolded state, only 70 to 80% of the surface area of the fully unfolded conformation is exposed to the solvent. Thus, the ''denatured'' state is not fully disordered in so far as solvent exposure is concerned. (4) The relative volume fluctuations of the solvent-inaccessible interiors of cytochrome *c* in the native, molten globule and unfolded states are 0.6%, 2.0% and 2.9%, respectively. Given the magnitude of these fluctuations in the MG and U states, it is not unreasonable to envision the possibility of transient interchange of solvent-exposed and solvent-inaccessible residues. To the best of our knowledge, this work provides the first characterization and comparison of the intrinsic volume and compressibility properties of the native, molten globule and unfolded states of a single protein.

Materials and Methods

Materials

Horse heart cytochrome *c* of the highest commercially available purity was purchased from two sources, Sigma Chemical (St. Louis, MO) and Fluka (Rokonkoma, NY) and used without further purification. The UV and CD spectra as well as the ultrasonic and densimetric measurements on samples obtained from these two sources were, within experimental error, indistinguishable. Cesium chloride and hydrochloric acid were purchased from J. T. Baker Inc. (Phillipsburg, NJ).

The optical absorption spectrum (200 to 600 nm) of the protein coincided with the spectrum previously reported for the oxidized ferri-form of cytochrome *c* (Margoliash & Schejter, 1966). Addition of the strong oxidizing agent $K_3Fe(CN)_6$ up to a concentration of 0.5 mM did not produce any detectable changes in the shape of the optical absorption spectrum. Consequently, all results and conclusions presented in this paper refer to ferricytochrome *c*. This point is relevant since, based on one report (Eden *et al*., 1982), the oxidation state of cytochrome *c* has a strong impact on the partial compressibility of the protein. However, it also should be noted that two subsequent studies found that a change in the oxidation state of the protein did not induce a significant change in its partial compressibility (Kharakoz & Mkhitaryan, 1986; Cruanes *et al*., 1992).

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 CsCl. The concentrations of cytochrome *c* were determined spectrophotometrically using the following extinction coefficients: $\epsilon_{409} = 106\ mM^{-1}\ cm^{-1}$ for the native state and $\epsilon_{395} = 172\ mM^{-1}\ cm^{-1}$ for the unfolded state at pH 2 and low ionic strength (Robinson *et al*., 1983). We independently verified both of these published ϵ values by dry weight analysis. We also found ϵ_{409} for the native ferricytochrome *c* to be salt-independent up to at least 1 M CsCl concentrations. For all of the densimetric and ultrasonic velocimetric measurements reported here, cytochrome *c* concentrations were about 250 μM. For the UV and far UV CD spectral measurements, the protein concentration was about 15 μM, while for the near UV CD measurements the protein concentration was about 500 μM. Significantly, we did not observe aggregation of the protein in any of its states or under any of the salt or pH conditions investigated, even at the highest concentrations studied.

Methods

Ultrasonic velocimetry

Solution sound velocity measurements were performed at 7.5 MHz using the resonator method as described (Eggers & Funck, 1973; Sarvazyan, 1982; Eggers, 1992). We used an ultrasonic resonator cell with lithium niobate piezo transducers and a minimum sample volume of 0.8 cm^3 (Sarvazyan, 1982). For this type of acoustic resonator, the relative precision of the sound velocity measurements at frequencies near 7.5 MHz is at least $\pm 1 \times 10^{-4}$% (Sarvazyan *et al*., 1988; Sarvazyan & Chalikian, 1991).

The characteristic of a solute directly derived from ultrasonic measurements is the relative specific sound velocity increment, $[u]$, which is equal to $(U - U_0)/(U_0 c)$. In this expression, c is the specific concentration of a solute (which is the solute molar concentration, C, divided by the solute molecular mass, M); U and U_0 are the sound velocities in the solution and the solvent, respectively. Acoustic titration experiments were performed by adding to both the sample and the reference cells (which each contain exactly 0.80 cm^3 of the protein solution and the water, respectively) equal aliquots of a 1 M HCl solution (for pH titrations) or a 4 to 5 M CsCl solution (for salt titrations). The initial 0.80 cm^3 were delivered to each cell using a 1 ml Hamilton syringe, while titrant additions were made using 5 µl, 10 µl, 25 µl and 100 µl Hamilton syringes (Hamilton Co., Reno, NV). Each syringe was equipped with a Chaney adapter that allowed a relative delivery accuracy of $\pm 0.01\%$. When calculating the relative specific sound velocity increment, $[u]$, we took into account changes in the sound velocity in the solvent, U_0, and in the specific concentration of the solute, c, which result from addition of the titrant.

Densimetry

All densities were measured at 25°C with a precision of $\pm 1.5 \times 10^{-6}$ g cm^{-3} using a vibrating tube densimeter (DMA-60, Anton Paar, Austria). We calculated the apparent specific volume, φV, of the cytochrome using the well-known relationship (Kupke, 1973):

$$\varphi V = 1/\rho_0 - (\rho - \rho_0)/(\rho_0 c) \qquad (12)$$

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

Densimetric titrations were done in a specially designed vial (with the total volume 4 cm^3) equipped with a stirring bar. The inlet and outlet of the vial were connected to the two ends of a U-shaped densimetric cell *via* Tygon tubing, with one of the tubes passing through a peristaltic pump. Two consecutive titrations were done for the solution and the solvent, using the same initial volume of 3 cm^3 and the same added aliquots of the titrant. Thus, at each point in the titration, one knows the densities of both the protein solution with the titrant added and the corresponding solvent. In calculating the apparent specific volume, φV, by means of equation (12), we took into account the changes in the solvent density and the concentration of the solute that result from addition of the titrant.

Determination of the apparent specific adiabatic compressibility

The relative specific sound velocity increments, $[u]$, determined as described above were used in conjunction with the measured apparent specific volume data, φV, to calculate the apparent specific adiabatic compressibility, φK_S, of cytochrome *c* using the following relationship (Barnartt, 1952; Owen & Simons, 1957):

$$\varphi K_S = \beta_{SO}(2\varphi V - 2[u] - 1/\rho_0) \qquad (13)$$

where β_{SO} is the coefficient of adiabatic compressibility of the solvent.

Each densimetric or ultrasonic velocimetric titration experiment was repeated three to five times, with the average values of $[u]$ and φV being used to calculate φK_S values from equation (13).

Optical spectroscopy

Circular dichroism spectra were recorded at 25°C using an AVIV model 60 DS spectropolarimeter (Aviv Associates, Lakewood, NJ). Optical absorption spectra were measured using a Perkin-Elmer Lambda 4C spectrophotometer (Perkin-Elmer, Norwalk, CT). Optical absorption and CD titration profiles were measured by incrementally adding aliquots of HCl or CsCl to an optical cell containing a known amount of cytochrome *c*. Optical absorption measurements were performed using a 5 mm cell, while a 1 mm cell was used for the CD measurements.

pH measurements

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 unit.

References

Arakawa, T. & Timasheff, S. N. (1982). Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry*, **21**, 6545–6552.

Babul, J. & Stellwagen, E. (1972). Participation of the protein ligands in the folding of cytochrome *c*. *Biochemistry*, **11**, 1195–1200.

Barnartt, S. (1952). The velocity of sound in electrolytic solutions. *J. Chem. Phys.* **20**, 278–279.

Bondi, A. (1964). van der Waals volumes and radii. *J. Phys. Chem.* **68**, 441–451.

Buckin, V. A., Sarvazyan, A. P. & Pasechnik, V. I. (1979). Investigation of vesicular lipid membranes by the ultrasound method. *Biofizika*, **24**, 62–68.

Buckin, V. A., Sarvazyan, A. P. & Kharakoz, D. P. (1989). Water around biological molecules. In *Water in Disperse Systems* (Deryagin, B. V., Churayev, N. V. & Ovcharenko, F. D., eds), pp. 45–63, ''Chemistry'', Moscow (in Russian).

Chalikian, T. V., Kharakoz, D. P., Sarvazyan, A. P., Cain, C. A., McGough, R. J., Pogosova, I. V. & Gareginian, T. N. (1992). Ultrasonic study of proton-transfer reactions in aqueous solutions of amino acids. *J. Phys. Chem.* **96**, 876–883.

Chalikian, T. V., Sarvazyan, A. P. & Breslauer, K. J. (1993). Partial molar volumes, expansibilities, and compressibilities of α,ω-aminocarboxylic acids in aqueous solutions between 18 and 55°C. *J. Phys. Chem.* **97**, 13017–13026.

Chalikian, T. V., Sarvazyan, A. P. & Breslauer, K. J. (1994a). Hydration and partial compressibility of biological compounds. *Biophys. Chem.* **51**, 89–109.

Chalikian, T. V., Sarvazyan, A. P., Funck, Th. & Breslauer, K. J. (1994b). Partial molar volumes, expansibilities, and compressibilities of oligoglycines in aqueous solutions at 18–55°C. *Biopolymers*, **34**, 541–553.

Chalikian, T. V., Sarvazyan, A., Gindikin, V. S. & Breslauer, K. J. (1994c). Hydration of charged atomic groups of biological molecules. *Biophys. J.* **66**, A65.

Cooper, A. (1976). Thermodynamic fluctuations in protein molecules. *Proc. Natl Acad. Sci. USA*, **73**, 2740–2741.

Cruanes, M. T., Rodgers, K. K. & Sligar, S. G. (1992). Protein electrochemistry at high pressure. *J. Am. Chem. Soc.* **114**, 9660–9661.

Durchschlag, H. (1986). Specific volumes of biological macromolecules and some other molecules of biological interest. In *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., ed.),

pp. 45–128, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
Dyson, H. J. & Beattie, J. K. (1982). Spin state and unfolding of ferricytochrome *c* in acidic solutions. *J. Biol. Chem.* **257**, 2267–2273.
Eden, D., Matthew, J. B., Rosa, J. J. & Richards, F. M. (1982). Increase in apparent compressibility of cytochrome *c* upon oxidation. *Proc. Natl Acad. Sci. USA*, **79**, 815–819.
Eggers, F. (1992). Ultrasonic velocity and attenuation measurements in liquids with resonators, extending the MHz frequency range. *Acustica*, **76**, 231–240.
Eggers, F. & Funck, Th. (1973). Ultrasonic measurements with milliliter liquid samples in the 0.5–100 MHz range. *Rev. Sci. Instrum.* **44**, 969–978.
Feng, Y. & Englander, Y. (1990). Salt-dependent structure change and ion binding in cytochrome *c* studied by two-dimensional proton NMR. *Biochemistry*, **29**, 3505–3509.
Gavish, B., Gratton, E. & Hardy, C. J. (1983). Adiabatic compressibility of globular proteins. *Proc. Natl Acad. Sci. USA*, **80**, 750–754.
Gekko, K. & Hasegawa, Y. (1986). Compressibility-structure relationship of globular proteins. *Biochemistry*, **25**, 6563–6571.
Gekko, K. & Noguchi, H. (1979). Compressibility of globular proteins in water at 25°C. *J. Phys. Chem.* **83**, 2706–2714.
Goto, Y. & Nishikori, S. (1991). Role of electrostatic repulsion in the acidic molten globule of cytochrome *c*. *J. Mol. Biol.* **222**, 679–686.
Goto, Y., Calciano, L. J. & Fink, A. L. (1990a). Acid-induced folding of proteins. *Proc. Natl Acad. Sci. USA*, **87**, 573–577.
Goto, Y., Takahashi, N. & Fink, A. L. (1990b). Mechanism of acid-induced folding of proteins. *Biochemistry*, **29**, 3480–3488.
Goto, Y., Hagihara, Y., Hamada, D., Hoshino, M. & Nishii, I. (1993). Acid-induced unfolding and refolding transitions of cytochrome *c*: a three-state mechanism in H_2O and D_2O. *Biochemistry*, **32**, 11878–11885.
Hainie, D. T. & Freire, E. (1993). Structural energetics of the molten globule state. *Proteins: Struct. Funct. Genet.* **16**, 115–140.
Hoiland, H. (1986). Partial molar compressibilities of organic solutes in water. In *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., ed.), pp. 129–147, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
Iqbal, M. & Verrall, R. E. (1987a). Partial molar volumes and adiabatic compressibilities of glycyl peptides at 25°C. *J. Phys. Chem.* **91**, 967–971.
Iqbal, M. & Verrall, R. E. (1987b). Volumetric properties of aqueous solutions of bovine serum albumin, human serum albumin, and human hemoglobin. *J. Phys. Chem.* **91**, 1935–1941.
Iqbal, M. & Verrall, R. E. (1988). Implications of protein folding. Additivity schemes for volumes and compressibilities. *J. Biol. Chem.* **263**, 4159–4165.
Karplus, M. & Shakhnovich, E. (1992). Protein folding: theoretical studies of thermodynamics and dynamics. In *Protein Folding* (Creighton, T. E., ed.), pp. 127–195, W. H. Freeman & Co., New York.
Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. *Advan. Protein Chem.* **14**, 1–63.
Kharakoz, D. P. (1991). Volumetric properties of proteins and their analogues in diluted water solutions. 2. Partial adiabatic compressibilities of amino acids at 15–70°C. *J. Phys. Chem.* **95**, 5634–5642.
Kharakoz, D. P. & Mkhitaryan, A. G. (1986). Change in compressibility of cytochrome *c* during oxidation-reduction transition. *Mol. Biol.* **20**, 312–321 (English translation from Russian).
Kharakoz, D. P. & Sarvazyan, A. P. (1993). Hydrational and intrinsic compressibilities of globular proteins. *Biopolymers*, **33**, 11–26.
Khechinashvili, N. N. (1990). Thermodynamic properties of globular proteins and the principle of stabilization of their native structure. *Biochim. Biophys. Acta*, **1040**, 346–354.
Kuntz, I. D. & Kauzmann, W. (1974). Hydration of proteins and polypeptides. *Advan. Protein Chem.* **28**, 239–345.
Kupke, D. W. (1973). Density and volume change measurements. In *Physical Principles and Techniques of Protein Chemistry.* (Leach, S. J., ed.), *Part C*, pp. 1–75, Academic Press, New York and London.
Kuroda, Y., Kidokoro, S. & Wada, A. (1992). Thermodynamic characterization of cytochrome *c* at low pH. Observation of the molten globule state and of the cold denaturation process. *J. Mol. Biol.* **223**, 1139–1153.
Lee, B. (1991). Isoenthalpic and isoentropic temperatures and the thermodynamics of protein denaturation. *Proc. Natl Acad. Sci. USA*, **88**, 5154–5158.
Liu, G., Grygon, C. A. & Spiro, T. G. (1989). Ionic strength dependence of cytochrome *c* structure and Trp-59 H/D exchange from ultraviolet resonance Raman spectroscopy. *Biochemistry*, **28**, 5046–5050.
Margoliash, E. & Schejter, A. (1966). Cytochrome *c*. *Advan. Protein Chem.* **21**, 113–286.
Mathieson, J. G. & Conway, B. E. (1974). Partial molal compressibilities of salts in aqueous solution and assignment of ionic contributions. *J. Solution Chem.* **3**, 455–477.
Millero, F. J., Ward, G. K. & Chetirkin, P. V. (1977). Relative sound velocities of sea salts at 25°C. *J. Acoust. Soc. Am.* **61**, 1492–1498.
Millero, F. J., Lo Surdo, A. & Shin, C. (1978). The apparent molal volumes and adiabatic compressibilities of aqueous amino acids at 25°C. *J. Phys. Chem.* **82**, 784–792.
Murphy, K. P. & Freire, E. (1991). Thermodynamics of structural stability and cooperative folding behavior in proteins. *Advan. Protein Chem.* **43**, 313–361.
Myer, Y. P. & Saturno, A. P. (1990). Horse heart ferricytochrome *c*: conformation and hem configuration of low ionic strength acidic forms. *J. Protein Chem.* **9**, 379–387.
Myer, Y. P. & Saturno, A. P. (1991). Horse heart ferricytochrome *c*: conformation and hem configuration of high ionic strength acidic forms. *J. Protein Chem.* **10**, 481–494.
Nölting, B. & Sligar, S. G. (1993). Adiabatic compressibility of molten globules. *Biochemistry*, **32**, 12319–12323.
Ohgushi, M. & Wada, A. (1983). 'Molten globule': a compact form of globular proteins with mobile side-chains. *FEBS Letters*, **164**, 21–24.
Oobatake, M. & Ooi, T. (1993). Hydration and heat stability effects on protein unfolding. *Prog. Biophys. Mol. Biol.* **59**, 237–284.
Owen, B. B. & Simons, H. L. (1957). Standard partial molal compressibilities by ultrasonics. 1. Sodium chloride and potassium chloride at 25°C. *J. Phys. Chem.* **61**, 479–482.
Potekhin, S. & Pfeil, W. (1989). Microcalorimetric studies of conformational transitions of ferricytochrome *c* in acidic solution. *Biophys. Chem.* **34**, 55–62.
Privalov, P. L. (1979). Stability of proteins. Small globular proteins. *Advan. Protein Chem.* **33**, 167–241.

Privalov, P. L. (1989). Thermodynamic problems of protein structure. *Annu. Rev. Biophys. Biophys. Chem.* **18**, 47–69.

Privalov, P. L. (1992). Physical basis of the stability of the folded conformations of proteins. In *Protein Folding* (Creighton, T. E., ed.), pp. 83–126, W. H. Freeman & Co., New York.

Richards, F. M. (1977). Areas, volumes, packing, and protein structure. *Annu. Rev. Biophys. Bioeng.* **6**, 151–176.

Richards, F. M. (1992). Folded and unfolded proteins: an introduction. In *Protein Folding* (Creighton, T. E., ed.), pp. 1–58, W. H. Freeman & Co., New York.

Robinson, J. B., Jr, Strottmann, J. M. & Stellwagen, E. (1983). A globular high spin form of ferricytochrome *c*. *J. Biol. Chem.* **258**, 6772–6776.

Sarvazyan, A. P. (1982). Development of methods of precise ultrasonic measurements in small volumes of liquids. *Ultrasonics*, **20**, 151–154.

Sarvazyan, A. P. (1991). Ultrasonic velocimetry of biological compounds. *Annu. Rev. Biophys. Biophys. Chem.* **20**, 321–342.

Sarvazyan, A. P. & Chalikian, T. V. (1991). Theoretical analysis of an ultrasonic interferometer for precise measurements at high pressures. *Ultrasonics*, **29**, 119–124.

Sarvazyan, A. P., Selkov, E. E. & Chalikian, T. V. (1988). Constant-path acoustic interferometer with transition layers for precision measurements in small liquid volumes. *Sov. Phys. Acoust.* **34**, 631–634.

Stellwagen, E. & Babul, J. (1975). Stabilization of the globular structure of ferricytochrome *c* by chloride in acidic solvents. *Biochemistry*, **14**, 5135–5140.

Sturtevant, J. M. (1987). Biochemical applications of differential scanning calorimetry. *Annu. Rev. Phys. Chem.* **38**, 463–488.

Takenaka, N. & Arakawa, K. (1989). Determination of individual partial molar volumes of ions in water from measurements of ultrasonic vibration potentials. *Bull. Chem. Soc. Jpn*, **62** 2880–2884.

Tanford, C. (1970). Protein denaturation. Part C. Theoretical models for the mechanism of denaturation. *Advan. Protein Chem.* **24**, 1–95.

Trewhella, J., Carlson, V. A. P., Curtis, E. H. & Heidorn, D. B. (1988). Differences in the solution structures of oxidized and reduced cytochrome *c* measured by small-angle X-ray scattering. *Biochemistry*, **27**, 1121–1125.

Weast, R. C. (1968). *Handbook of Chemistry and Physics*, 49th edit., pp. F-12–F-14, The Chemical Rubber Co., Cleveland.

Xie, D. & Freire, E. (1994). Structure-based prediction of protein folding intermediates. *J. Mol. Biol.* **242**, 62–80.

Zamyatnin, A. A. (1984). Amino acid, peptide, and protein volume in solution. *Annu. Rev. Biophys. Bioeng.* **13**, 145-165.

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