Articles

Volume and Compressibility Changes Accompanying Thermally-Induced Native-to-Unfolded and Molten Globule-to-Unfolded Transitions of Cytochrome *c*: A High Pressure Study†

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ABSTRACT: We have measured the transition temperatures, T_M, and van't Hoff enthalpies, ΔH_M, of the thermally induced native-to-unfolded (N-to-U) and molten globule-to-unfolded (MG-to-U) transitions of cytochrome *c* at pressures between 50 and 2200 bar. We have used the pressure dependence of T_M to evaluate the changes in volume, Δv, accompanying each protein transition event as a function of temperature and pressure. From analysis of the temperature and pressure dependences of Δv, we have additionally calculated the changes in expansibility, Δe, and isothermal compressibility, Δk_T, associated with the thermally induced conformational transitions of cytochrome *c*. Specifically, if extrapolated to 25 °C, the native-to-unfolded (N-to-U) transition is accompanied by changes in volume, Δv, expansibility, Δe, and isothermal compressibility, Δk_T, of $-(5 \pm 3) \times 10^{-3}$ cm^3 g^{-1}, $(1.8 \pm 0.3) \times 10^{-4}$ cm^3 g^{-1} K^{-1}, and ~ 0 cm^3 g^{-1} bar^{-1}, respectively. The molten globule-to-unfolded (MG-to-U) transition is accompanied by changes in volume, Δv, and isothermal compressibility, Δk_T, of $-(2.9 \pm 0.3) \times 10^{-3}$ cm^3 g^{-1} at 40 °C and $-(1.9 \pm 0.3) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} at 35 °C, respectively. By comparing the volumetric properties of the N-to-U and N-to-MG transitions of cytochrome *c*, we have estimated the properties of the native-to-molten globule (N-to-MG) transition. For the latter transition, the changes in volume, Δv, and isothermal compressibility, Δk_T, are ~ 0 cm^3 g^{-1} at 40 °C and 1.9 cm^3 g^{-1} bar^{-1} at 35 °C, respectively. Our estimate for the change in expansibility, Δe, upon the N-to-MG is negative and equal to $-(5 \pm 3) \times 10^{-4}$ cm^3 g^{-1} K^{-1}. This finding contrasts with the results of previous studies all of which report positive changes in expansibility associated with protein denaturation. In general, our volumetric data permit us to assess the combined effect of temperature and pressure on the stability of various conformational states of cytochrome *c*.

Understanding the molecular basis of protein stability starts with the thermodynamic characterization of the temperature-, pressure-, and solvent-induced transitions of proteins between their native and their denatured conformations. From the thermodynamic viewpoint, pressure-related characteristics, such as volume, compressibility, and expansibility, provide macroscopic information that is complementary to that provided by more routinely studied temperature-related characteristics, such as enthalpy, entropy, and heat capacity. This complementarity stems from fundamental thermodynamic relationships that exist between the pressure-related and the temperature-related parameters. Combined determination of the behavior of the system with respect to both temperature and pressure generally allows one to develop a more comprehensive thermodynamic description of protein stability than more conventional temperature-dependent studies (*1*−*5*).

Volume and compressibility, which respectively represent the first and second pressure derivatives of free energy, have been employed and proven useful for characterizing protein transitions (*6*−*12*). The microscopic interpretation of these macroscopic parameters in terms of molecular events enables one to gain insight into hydration, intrinsic packing, and dynamics of the protein as a function of its conformational state (*6*−*12*). It is significant that the changes in volume and compressibility accompanying protein denaturation correlate to the nature of the denatured state (*11*−*15*).

We have previously applied volumetric measurements to studying conformational transitions of a number of globular proteins, including the pH-induced denaturation of cytochrome *c* (*16*, *17*). In this work, we expand these studies to thermally induced conformational transitions of cytochrome *c*. Cytochrome *c* is a small, single-chain globular protein

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(12.4 kDa) that may adopt the native (N),[1] molten globule (MG), or unfolded (U) conformation depending on solution temperature, pH, and ionic strength (*18*−*22*). At room temperature and acidic pH (around pH 2), the conformational state of cytochrome *c* is determined by the solution ionic strength. At low salt, the protein is unfolded, while at high salt it adopts the MG conformation (*18*−*22*).

In contrast to many other proteins that are capable of forming molten globules, cytochrome *c* exhibits a cooperative, thermally induced MG-to-U transition (*23*). In this work, we employ UV/Vis light absorption spectroscopy to monitor the thermally induced N-to-U and MG-to-U transitions of cytochrome *c* as a function of pressure. We analyze the pressure dependences of the denaturation temperature, T_M, to evaluate the changes in volume, ΔV, expansibility, ΔE, and isothermal compressibility, ΔK_T, accompanying the thermally induced N-to-U and MG-to-U transitions of cytochrome *c*. We compare the volumetric characteristics of the thermally induced conformational alterations of cytochrome *c* with similar data previously obtained for pH-induced N-to-U and N-to-MG transitions of the protein at room temperature (*16*, *17*). On the basis of comparison between the two sets of data, we interpret our results in terms of pressure- and temperature-induced changes in the stability of the native state of cytochrome *c* relative to its denatured conformations.

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c* of the highest commercially available purity was purchased from Sigma-Aldrich Canada (Mississauga, ON, Canada) and used without further purification. NaCl and HCl were purchased from EM Science (Gibbstown, NJ) and BDH Inc. (Toronto, ON, Canada), respectively. Acetic acid and glycine were obtained from Sigma-Aldrich Canada (Mississauga, ON, Canada). The protein was dissolved in either 10 mM glycine-hydrochloric acid or 10 mM acetic acid-sodium acetate buffer. All measurements reported here were performed at 200 mM NaCl. The pH of the glycine buffer was 2.0, while the pH of the acetic acid buffer was adjusted to 3.4, 3.6, 4.0, or 4.3. The concentrations of cytochrome *c* were determined spectrophotometrically using the following extinction coefficients: $\epsilon_{409} = 106\ mM^{-1}\ cm^{-1}$ for the native state at pH 3.4−4.3 and $\epsilon_{400} = 115\ mM^{-1}\ cm^{-1}$ for the molten globule state at pH 2 (*16*).

Optical Spectroscopy. Optical absorbance and circular dichroism spectra were recorded using, respectively, an AVIV model 14 DS spectrophotometer and model 62 DS spectropolarimeter (Aviv Associates, Lakewood, NJ). A 10-mm path length cuvette was used for all optical absorbance measurements and for the near UV CD measurements. A cuvette with a path length of 1 mm was used for the far UV CD measurements. Fluorescence intensity measurements were performed in a 10-mm path length cuvette using an Aviv model ATF 105 spectrofluorometer (Aviv Associates, Lakewood, NJ). In temperature-dependent fluorescence intensity measurements, the solutions of cytochrome *c* were excited at 293 nm, and the intensity of emitted light was recorded through a monochromator at 325 nm. For the far and near UV CD spectroscopic experiments, the protein concentration was on the order of ∼0.3 mg/mL, while for the temperature-dependent fluorescence intensity measurements the concentration was roughly ∼0.2 mg/mL.

High-Pressure Optical Melting. The light absorption melting profiles for cytochrome *c*, as monitored by the temperature dependence of the extinction coefficient at 396 nm (for MG-to-U transitions) and 418 nm (for U-to-MG transitions), were recorded at pressures between 1 and 2200 bar in a high-pressure optical cell as previously described (*24*). Both wavelengths are within the Soret absorption region. Recall that Soret absorption reflects the spin state of the heme iron that, in turn, depends on the conformational state of the protein (*25*). The temperature scanning experiments were performed with a rate of 0.5 °C/min with a point collected every minute.

The optical cell is filled with silicon oil as the pressure-transmitting medium. Within the optical cell, the samples are contained in a 5-mm path length quartz cuvette designed to allow for pressure equilibration between the exterior and the interior of the cuvette. In the measurements at high pressure, the cytochrome *c* concentrations were between 0.3 and 0.4 mg/mL.

RESULTS

We have measured the CD and optical absorption spectra of cytochrome *c* at the relevant pH values and temperatures to confirm independently that cytochrome *c*, in fact, exists in its native, molten globule, and unfolded states under the experimental conditions investigated. Figure 1 presents the near (panel A) and far (panel B) UV CD spectra of cytochrome *c* at different temperatures and pH. Specifically, Figure 1 presents the CD spectra of the protein at 25 °C at pH 2.0 (○) and 4.3 (●) and at 95 °C at pH 2.0 (□) and 4.3 (■). We have measured similar CD spectra of the protein at pH 3.4, 3.6, and 4.0 (not shown). These spectra coincide with the CD spectra measured at pH 4.3. Figure 2 presents the Soret optical absorption spectra of cytochrome *c* at 25 °C at pH 2.0 (○) and 4.3 (●) and at 95 °C at pH 2.0 (□) and 4.3 (■). The Soret absorption spectra of cytochrome *c* measured at pH 3.4, 3.6, and 4.0 coincide with the spectrum measured at pH 4.3.

Figure 3A shows a representative denaturation profile of the thermally induced N-to-U transition of cytochrome *c* at pH 4.3 and 50 bar as monitored by light absorption at 418 nm. Figure 3B shows a representative profile of the thermally induced MG-to-U transition of the protein *c* at pH 2.0 and 50 bar as monitored by light absorption at 396 nm. We have measured similar profiles for the N-to-U transitions of the protein at pH 3.4, 3.6, 4.0, and 4.3 (at 418 nm) and MG-to-U transition at pH 2.0 (at 396 nm) at the pressures of 50, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, and 2200 bar (not shown). Each denaturation profile has been analyzed using the two-state approximation to obtain the transition temperature, T_M, and van't Hoff enthalpy, ΔH_M (*26*).

To assess reversibility of cytochrome *c* denaturation, we have carried out additional experiments at atmospheric pressure on the protein at pH 2 and 4. Specifically, we have repetitively heated and cooled cytochrome *c* samples between 10 and 90 °C while monitoring protein denaturation by Soret

[1] Abbreviations: CD, circular dichroism; N, native; MG, molten globule; U, unfolded; T_M, transition temperature.

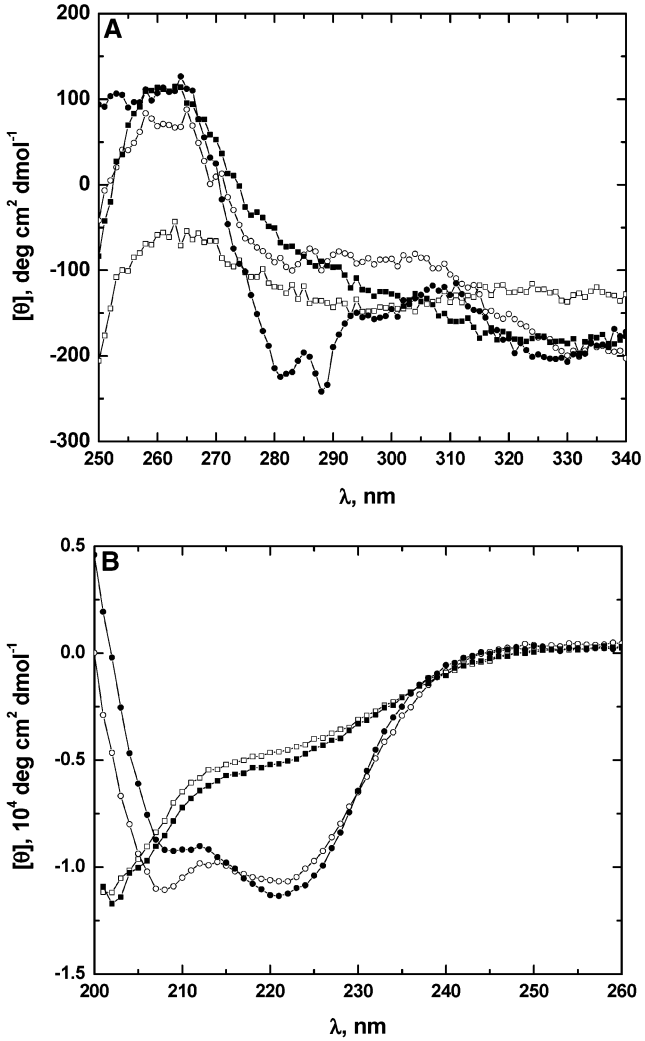

FIGURE 1: CD spectra of cytochrome *c* at 25 °C and pH 2.0 (○); at 25 °C and pH 4.3 (●); at 95 °C and pH 2.0 (□); and at 95 °C and pH 4.3 (■). (A) Near UV region and (B) far UV region.

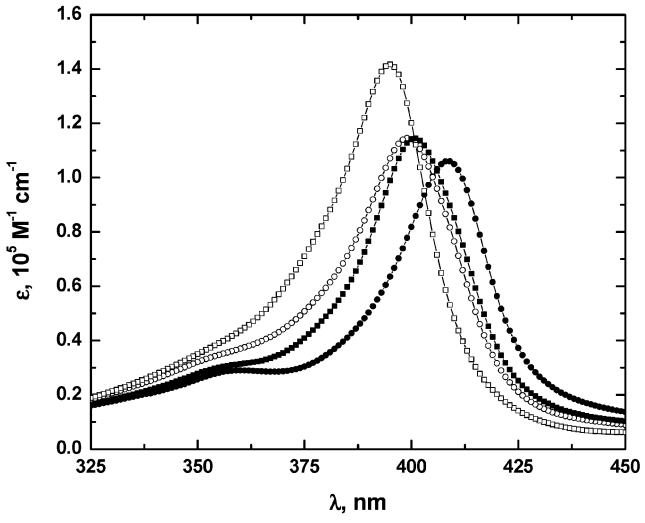

FIGURE 2: Optical absorption spectra of cytochrome *c* in the native state at pH 4.3 and 25 °C (●), the heat-induced unfolded state at pH 4.3 and 95 °C (■), the molten globule state at pH 2.0 and 25 °C (○), and the heat-induced unfolded state at pH 2.0 and 95 °C (□).

absorption and fluorescence intensity (not shown). On the basis of the initial value of light absorption and fluorescence

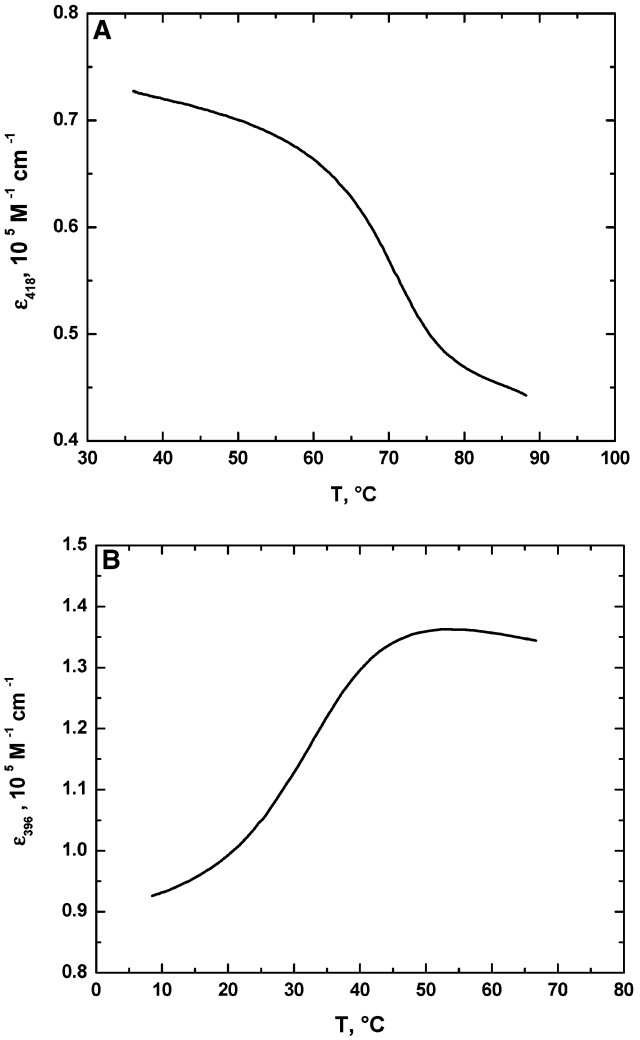

FIGURE 3: (A) Denaturation profile of the thermally induced N-to-U transition of cytochrome *c* at pH 4.3 and 50 bar as monitored by light absorption at 418 nm. (B) Denaturation profile of the thermally induced MG-to-U transition of cytochrome *c* at pH 2.0 and 50 bar as monitored by light absorption at 396 nm.

Table 1: Thermodynamic Parameters for the N-to-U Transition of Cytochrome *c* at pH 3.4

P (bar)	T_M (°C)	ΔH_M (kcal mol^{-1})	ΔV (cm^3 mol^{-1})
50	62.8 ± 0.3	46 ± 5	15 ± 2
250	65.8 ± 0.3	45 ± 5	16 ± 2
500	63.1 ± 0.3	51 ± 5	16 ± 2
750	64.5 ± 0.3	56 ± 5	17 ± 2
1000	64.6 ± 0.3	59 ± 5	17 ± 2
1250	65.2 ± 0.3	59 ± 5	18 ± 2
1500	66.7 ± 0.3	59 ± 5	18 ± 2
1750	66.9 ± 0.3	60 ± 5	19 ± 2
2000	69.4 ± 0.3	61 ± 5	20 ± 2
2200	68.8 ± 0.3	62 ± 5	20 ± 2

intensity measured upon returning to the starting temperature of 10 °C after the second scan, the reversibility was between 85 and 90% both for both the native (pH 4.0) and the molten globule (pH 2.0) conformations. However, the transition temperatures, T_M, and van't Hoff enthalpies, ΔH_M, calculated from the first and second light absorption and fluorescence-monitored scans coincided within experimental error.

Tables 1−4 present the transition temperatures, T_M, and enthalpies, ΔH_M, for the thermally induced N-to-U transition

Table 2: Thermodynamic Parameters for the N-to-U Transition of Cytochrome *c* at pH 3.6

P (bar)	T_M (°C)	ΔH_M (kcal mol^{-1})	ΔV (cm^3 mol^{-1})
50	62.3 ± 0.3	43 ± 5	24 ± 3
250	67.1 ± 0.3	40 ± 5	25 ± 3
500	64.5 ± 0.3	52 ± 5	26 ± 3
750	67.4 ± 0.3	52 ± 5	26 ± 3
1000	64.5 ± 0.3	52 ± 5	28 ± 3
1250	66.9 ± 0.3	59 ± 5	29 ± 3
1500	68.9 ± 0.3	63 ± 5	30 ± 3
1750	69.3 ± 0.3	63 ± 5	31 ± 3
2000	71.3 ± 0.3	65 ± 5	31 ± 3
2200	74.8 ± 0.3	48 ± 5	32 ± 3

Table 3: Thermodynamic Parameters for the N-to-U Transition of Cytochrome *c* at pH 4.0

P (bar)	T_M (°C)	ΔH_M (kcal mol^{-1})	ΔV (cm^3 mol^{-1})
50	69.2 ± 0.3	50 ± 5	34 ± 4
250	68.7 ± 0.3	60 ± 5	34 ± 4
500	70.0 ± 0.3	56 ± 5	35 ± 4
750	71.0 ± 0.3	66 ± 5	36 ± 4
1000	72.8 ± 0.3	66 ± 5	37 ± 4
1250	73.7 ± 0.3	70 ± 5	38 ± 4
1500	75.0 ± 0.3	67 ± 5	38 ± 4
1750	76.5 ± 0.3	71 ± 5	40 ± 4
2000	78.1 ± 0.3	69 ± 5	40 ± 4
2200	78.4 ± 0.3	64 ± 5	41 ± 4

Table 4: Thermodynamic Parameters for the N-to-U Transition of Cytochrome *c* at pH 4.3

P (bar)	T_M (°C)	ΔH_M (kcal mol^{-1})	ΔV (cm^3 mol^{-1})
50	69.5 ± 0.3	59 ± 6	48 ± 6
250	71.4 ± 0.3	68 ± 6	48 ± 6
500	73.1 ± 0.3	70 ± 6	51 ± 6
750	74.9 ± 0.3	82 ± 6	52 ± 6
1000	76.3 ± 0.3	78 ± 6	54 ± 6
1250	78.2 ± 0.3	80 ± 6	56 ± 6
1500	79.1 ± 0.3	80 ± 6	57 ± 6
1750	80.2 ± 0.3	92 ± 6	59 ± 6
2000	81.6 ± 0.3	79 ± 6	61 ± 6
2200	82.5 ± 0.3	89 ± 6	62 ± 6

of cytochrome *c* at pH 3.4, 3.6, 4.0, and 4.3, respectively, at each pressure point applied in this work. Table 5 presents similar data for the thermally induced MG-to-U transitions of the protein at pH 2.0. The pressure dependent T_M data from Tables 1−5 are graphically presented in Figure 4.

DISCUSSION

Conformational States of Cytochrome c. We have used the CD spectra of cytochrome *c* to identify the conformational states of the protein at atmospheric pressure. Judging by the nativelike near and far UV CD spectra (Figure 1A,B, respectively), cytochrome *c* is in the native state at room temperature between pH 3.4 and 4.3. However, at room temperature and pH 2.0, the protein adopts the molten globule conformation as assessed by the presence of secondary (Figure 1B) and absence of tertiary (Figure 1A) structures. A similar conclusion can be drawn based on the Soret absorption spectra of the protein at 25 °C presented in Figure 2. Specifically, at room temperature, the Soret absorption spectra of the protein at pH 4.3 and 2.0 (Figure 2) coincide respectively with the spectrum of the native state at pH 7.0 and low ionic strength and the spectrum of the molten globule state at pH 2.0 and 200 mM CsCl (see ref *16*). These observations are consistent with the ionic strength-versus-pH phase diagrams of cytochrome *c* in the literature (*18*−*21*).

Table 5: Thermodynamic Parameters for the MG-to-U Transition of Cytochrome *c* at pH 2.0

P (bar)	T_M (°C)	ΔH_M (kcal mol^{-1})	ΔV (cm^3 mol^{-1})
50	39.4 ± 0.3	31 ± 3	−36 ± 3
250	37.9 ± 0.3	32 ± 3	−30 ± 3
500	36.0 ± 0.3	28 ± 3	−22 ± 3
750	34.6 ± 0.3	28 ± 3	−14 ± 3
1000	34.1 ± 0.3	26 ± 3	−8 ± 3
1250	34.0 ± 0.3	26 ± 3	−2 ± 3
1500	33.9 ± 0.3	25 ± 3	3 ± 3
1750	34.1 ± 0.3	25 ± 3	9 ± 3
2000	35.2 ± 0.3	26 ± 3	15 ± 3
2200	35.9 ± 0.3	27 ± 3	21 ± 3

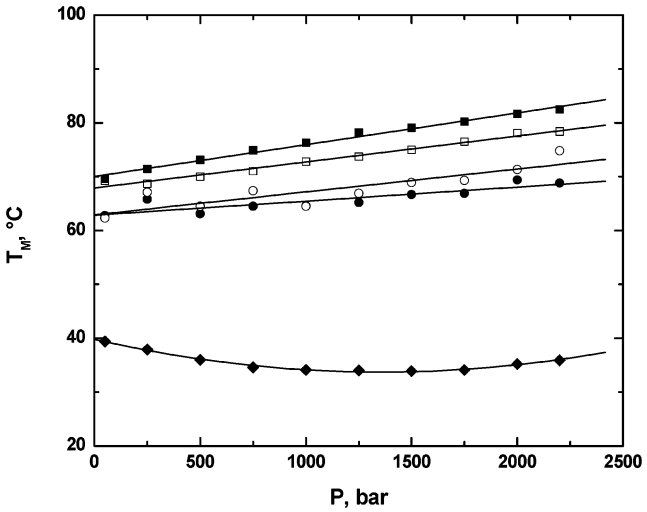

FIGURE 4: Pressure dependence of the denaturation temperatures, T_M, of the thermally induced N-to-U transitions of cytochrome *c* at pH 3.4 (●), 3.6 (○), 4.0 (□), and 4.3 (■) and the MG-to-U transition at pH 2.0 (♦).

At elevated temperatures, the thermally induced denatured states of cytochrome *c* at pH 2.0 and 3.4−4.3 exhibit a lack of tertiary structure (Figure 1A). However, judging by the ellipticity at 222 nm, the protein retains roughly 40% of its native secondary structure (Figure 1B). Thus, the thermally induced unfolded states of cytochrome *c* at either pH 2.0 or 3.4−4.3 are not fully unfolded and retain a significant amount of secondary structural elements. This observation contrasts with the pH-induced unfolded states of cytochrome *c* that do not exhibit significant α-helical content (*16*, *17*). Thus, the thermally induced unfolded conformations of cytochrome *c* at 200 mM NaCl and pH 2.0 and 3.4−4.3 are structurally distinct from the acid- and base-induced unfolded conformations at room temperature in the absence of salt.

Pressure Stabilizes the Native Conformation But Destabilizes the Molten Globule Conformation. Inspection of Figure 4 reveals that the denaturation temperatures, T_M, for the N-to-U transitions at pH 3.4 (●), 3.6 (○), 4.0 (□), and 4.3 (■) linearly increase with pressure. In other words, the slope $\Delta T_M/\Delta P$ is positive over the entire range of pressures studied in this work. This observation implies that pressure stabilizes the native conformation of cytochrome *c* relative

to the ensemble of its thermally induced unfolded conformations. Strictly speaking, protein stability at an arbitrary temperature, T, refers to ΔG, the free energy difference between the native and the unfolded conformations at that temperature and not to the T_M. However, it can be shown that, provided that the transition heat capacity, ΔC_P, remains constant, the values of ΔG and T_M correlate (*27*).

Figure 4 also reveals that the T_M for the MG-to-U transition at pH 2.0 (♦) exhibits a parabolic temperature dependence. The value of T_M decreases with pressure increasing from 1 to 1500 bar ($\Delta T_M/\Delta P$ is negative). Upon a further increase in pressure, the slope, $\Delta T_M/\Delta P$, changes its sign, and T_M increases above 1500 bar. This observation suggests that pressure initially destabilizes the molten globule conformation of cytochrome *c* over the ensemble of its thermally induced unfolded conformations. However, at elevated pressures (above 1500 bar), the molten globule conformation becomes stabilized by pressure. In the section that follows, we use the slopes $\Delta T_M/\Delta P$ to determine the changes in volume, ΔV, accompanying the thermally induced N-to-U and N-to-MG transitions of cytochrome *c*.

Volume Changes Accompanying the Thermally Induced N-to-U and MG-to-U Transitions of Cytochrome c. Our data on the pressure dependence of T_M and ΔH_M (Tables 1−5) were used in conjunction with the Clayperon equation to calculate the volume change, $\Delta V(T_M,P)$, accompanying the heat-induced denaturation of the protein at each experimental pressure, P, and transition temperature, T_M:

$$\Delta V(T_M,P) = (\Delta T_M/\Delta P)\Delta H_M/T_M \qquad (1)$$

For these calculations, the pressure dependence of T_M for the MG-to-U transition at pH 2.0 was approximated by a second-order polynomial, while the pressure dependences of T_M for the N-to-U transitions at pH 3.4, 3.6, 4.0, and 4.3 have been approximated by linear functions (Figure 4). Subsequently, the slopes, $(\Delta T_M/\Delta P)$, in eq 1 were calculated for each pressure point by analytical differentiation of the functions approximating the pressure dependences of T_M. The resulting transition volumes, ΔV, are given in Tables 1−5.

Inspection of the volume data in Tables 1−5 reveals two important observations. First, the volume changes, ΔV_M, accompanying the thermally induced N-to-U transitions of cytochrome *c* are positive under all experimental conditions (pH, T_M, P) employed in this work. At atmospheric pressure (more precisely, at 50 bar), the values of ΔV for the N-to-U transition range from 15 ± 2 cm^3 mol^{-1} (at pH 3.4 and T_M of 62.8 ± 0.3 °C) to 48 ± 6 cm^3 mol^{-1} (at pH 4.3 and T_M of 69.5 ± 0.3 °C). Second, for the MG-to-U transition at pH 2.0, the value of ΔV is negative at low pressures (at 50 bar, ΔV equals -36 ± 3 cm^3 mol^{-1}) but changes its sign and becomes positive at elevated pressures (at 2200 bar, ΔV equals 21 ± 3 cm^3 mol^{-1}).

If expressed in specific units (normalized per gram rather than per mole of protein; $\Delta v = \Delta V/M$, where M is the molecular weight of the protein), the values of Δv for the thermally induced N-to-U and MG-to-U transitions of cytochrome *c* are between -4×10^{-3} and 3×10^{-3} cm^3 g^{-1}. In absolute value, our measured values of Δv are small, an observation consistent with previous reports on changes in volume upon protein denaturation (*12*, *14*, *15*, *28*−*32*). For example, specific changes in volume accompanying the thermally induced denaturation of chymotrypsinogen at pH 2.4, pepsinogen at pH 6.4, hen egg white lysozyme at pH 2.5, bovine pancreatic trypsin inhibitor at pH 4.0, ribonuclease A at pH 5.5, and T4 lysozyme a pH 3.6 are equal to 1.6×10^{-3}, ~0, -0.6×10^{-3}, -0.4×10^{-3}, -2.0×10^{-3}, and -1.6×10^{-3} cm^3 g^{-1}, respectively (*31*). As discussed in our recent publication, small values of Δv represent a general property of the denaturation of globular proteins and result from compensation between the transition-induced changes in intrinsic, thermal, and interaction (hydration) contributions (*15*).

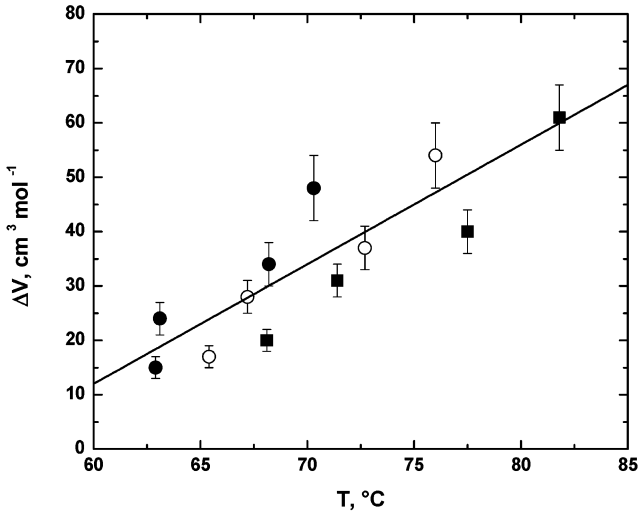

FIGURE 5: Transition volumes, ΔV, for the thermally induced N-to-U transitions of cytochrome *c* plotted against the denaturation temperatures, T_M, at 50 (●), 1000 (○), and 2000 (■) bar.

It is instructive to compare our measured changes in volume, ΔV, accompanying the thermally induced N-to-U and MG-to-U transitions of cytochrome *c* with similar data we previously reported for the pH-induced N-to-U and MG-to-U transitions of this protein (*16*, *17*). The changes in volume associated with the acid-induced N-to-U, N-to-MG, and MG-to-U transitions of cytochrome *c* at 25 °C have been also reported by Foygel et al. (*29*) with their primary experimental data being in good agreement with our data from ref *16*. However, in the analysis below, we will use only our pH-dependent data reported in refs *16* and *17*. Direct comparison of the two sets of data (our previous data and the data presented in this paper) would be invalid since, in this work, the ΔV values have been obtained at elevated temperatures, while the data for the pH-induced transitions were measured at 25 °C. Thus, the transition volumes evaluated in this paper at elevated temperatures must be extrapolated to 25 °C and then compared with the data for the pH-induced transitions (*16*, *17*). Such extrapolations can be performed after taking into account the temperature dependences of ΔV.

N-to-U Transition of Cytochrome c. Figure 5 presents the transition volumes, ΔV, for the thermally induced N-to-U transitions of cytochrome *c* plotted against the denaturation temperature, T_M, at 50 (●), 1000 (○), and 2000 (■) bar. These dependences can be approximated reasonably well by the single linear function $\Delta V = (-120 \pm 28) + (2.2 \pm 0.4)T$. If extrapolated to 25 °C, the transition volume, ΔV, equals -65 ± 38 cm^3 mol^{-1}. In specific units, the transition volume Δv at 25 °C is $-(5 \pm 3) \times 10^{-3}$ cm^3 g^{-1}.

Table 6: Volumetric Properties of the N-to-U, MG-to-U, and N-to-MG Transitions of Cytochrome c

	N-to-U	MG-to-U	N-to-MG
Δv (10^{-3} cm^3 g^{-1})	-5 ± 3^a	-2.9 ± 0.3^b	0^b
Δe (10^{-4} cm^3 g^{-1} K^{-1})	1.8 ± 0.3^c	not determined	$-5 \pm 3^{c,d}$
Δk_T (10^{-6} cm^3 g^{-1} bar^{-1})	0^a	-1.9 ± 0.3^e	1.9^e

[a] Extrapolated to 25 °C. [b] At ~40 °C. [c] Considered to be temperature independent. [d] Needs further verification. [e] At ~35 °C.

From the temperature slope of ΔV, one can determine the temperature- and pressure-independent transition expansibility [$\Delta E = (\partial \Delta V/\partial T)_P$] of 2.2 ± 0.4 cm^3 mol^{-1} K^{-1}. In specific units, the change in expansibility, $\Delta e = \Delta E/M$, is (1.8 ± 0.3) × 10^{-4} cm^3 g^{-1} K^{-1}. This value is within the range of reported expansibility changes associated with protein denaturation, 0.5 × 10^{-4} to 1.5 × 10^{-4} cm^3 g^{-1} K^{-1} (*12*, *31*−*36*). For example, Lin et al. have recently used pressure perturbation calorimetry to measure Δe for the thermally induced denaturation of a number of globular proteins (*31*). The Δe values for chymotrypsinogen at pH 2.4, pepsinogen at pH 6.4, hen egg white lysozyme at pH 2.5, bovine pancreatic trypsin inhibitor at pH 4.0, ribonuclease A at pH 5.5, and T4 lysozyme a pH 3.6 were found to be 0.8 × 10^{-4}, 0.7 × 10^{-4}, 1.0 × 10^{-4}, 0.4 × 10^{-4}, 1.1 × 10^{-4}, and 0.4 × 10^{-4} cm^3 g^{-1} K^{-1}, respectively (*31*). As we have previously discussed (*12*), the net change in expansibility, Δe, associated with a conformational transition of a globular protein results from the changes in the intrinsic, e_M, and hydration, Δe_h, contributions. The values of e_M and Δe_h for a native globular protein are both positive (*12*). Upon unfolding, e_M is expected to decrease because of the disruption of expandable internal voids, while Δe_h should increase because of enhanced hydration of the unfolded state. The overall positive sign of the change in expansibility, Δe, upon the N-to-U transition of a globular protein suggests that the increase in Δe_h prevails over the reduction in e_M (*12*).

The observation that ΔV varies linearly with temperature at 50, 1000, and 2000 bar suggests that, within the range of 50−2200 bar, the ΔV of the thermally induced N-to-U transitions of cytochrome *c* depends on temperature and is practically insensitive to pressure. This important observation further suggests that, within the limit of the uncertainties of our analyses, the change in isothermal compressibility, $\Delta K_T = -(\partial \Delta V/\partial P)_T$, accompanying the thermally induced N-to-U transition of the protein is close to zero. This estimate is apparently independent of (or very slightly dependent on) pressure and temperature within the range of pressures and temperatures employed in this work. Therefore, it is reasonable to assume that the value of ΔK_T will still be approximately zero if extrapolated to 25 °C and atmospheric pressure.

Table 6 presents the volumetric properties of the N-to-U transition of cytochrome *c*. The value of Δv for the thermally induced N-to-U transition extrapolated to 25 °C is equal to $-(5 \pm 3) \times 10^{-3}$ cm^3 g^{-1}. The negative sign of Δv at 25 °C, in contrast to the positive sign at elevated temperatures, suggests that, at room temperature, pressure destabilizes the native conformation of cytochrome *c* relative to its unfolded conformation. The Δv value of $-(5 \pm 3) \times 10^{-3}$ cm^3 g^{-1} is within the range of the values observed for the acid- and base-induced N-to-U transitions of the protein that are equal to -1×10^{-3} and -14×10^{-3} cm^3 g^{-1}, respectively (*16*, *17*). As noted above, the change in volume accompanying protein denaturation, Δv, is a result of strong compensation between the changes in the intrinsic, thermal, and interaction contributions (*15*). It is important to note that, in absolute value, these changes can be an order of magnitude larger than Δv (*15*). Consequently, very small differences in the structure and/or hydration of the pH-induced and thermally induced unfolded states of cytochrome *c* may easily result in the observed differences in Δv (-1×10^{-3}, -5×10^{-3}, and -14×10^{-3} cm^3 g^{-1}). In this connection, recall that our CD data suggest that the thermally induced unfolded state of the protein is structurally distinct from the acid- and base-induced unfolded conformations; insofar as it exhibits a larger α-helical content than the either of the pH-induced unfolded states.

The acid- and base-induced N-to-U transitions of cytochrome *c* at 25 °C are accompanied by changes in adiabatic compressibility, Δk_S, of -3.9×10^{-6} and -3.8×10^{-6} cm^3 g^{-1} bar^{-1}, respectively (*16*, *17*). These values are smaller than zero, our estimate for the change in isothermal compressibility, Δk_T, associated with the thermally induced N-to-U transition of the protein. In this comparison, the difference between the adiabatic and the isothermal quantities can be ignored because of the large heat capacity and small expansibility of water (*9*, *11*, *12*). The disparity between the values of Δk_S for the pH-induced N-to-U transitions and the value of Δk_T for the thermally induced N-to-U transition may originate from structural differences between the unfolded states.

The change in compressibility associated with protein denaturation predominantly depends on the type of transition and is relatively insensitive to the specific protein (*11*, *13*). Analysis of available experimental data revealed that native-to-compact intermediate transitions are accompanied by positive changes in compressibility ranging from 1×10^{-6} to 4×10^{-6} cm^3 g^{-1} bar^{-1}. Native-to-partially unfolded transitions are accompanied by small negative changes in compressibility of between -3×10^{-6} to -7×10^{-6} cm^3 g^{-1} bar^{-1}, while native-to-fully unfolded transitions are accompanied by large negative changes in compressibility of -18×10^{-6} to -20×10^{-6} cm^3 g^{-1} bar^{-1} (*13*). Our recent simulations performed for proteins ranging in molecular weight from 10 to 80 kDa have produced similar results: native-to-compact intermediate transitions are accompanied by the changes in compressibility of -3×10^{-6} to 6×10^{-6} cm^3 g^{-1} bar^{-1}, native-to-partially unfolded transitions are accompanied by relatively small negative changes in compressibility of -2×10^{-6} to -12×10^{-6} cm^3 g^{-1} bar^{-1}, while native-to-fully unfolded transitions result in large negative changes in compressibility of -16×10^{-6} to -25×10^{-6} cm^3 g^{-1} bar^{-1} (*11*). On the basis of this classification, the thermally induced N-to-U transition of cytochrome *c* with its zero value of Δk_T is the native-to-compact intermediate transition. This notion is consistent with the large α-helical content (~40%) exhibited by the thermally induced unfolded state of cytochrome *c*. It should be noted that cytochome *c* is not the first protein that, based on the compressibility criterion, exhibits a thermally induced native-to-compact intermediate transition. Ribonuclease A and α-chymotrypsonogen A both exhibit an increase in

compressibility upon their heat-induced denaturation that thus can be classified as native-to-compact intermediate transitions (*32*, *37*). For example, the heat induced denaturation of α-chymotrypsinogen A at pH 2.0 with a T_M of 41 °C is accompanied by an increase in adiabatic compressibility of 3.6×10^{-6} cm^3 g^{-1} bar^{-1} (*32*).

MG-to-U Transition of Cytochrome c. The volume change, ΔV, accompanying the thermally induced MG-to-U transition of cytochrome *c* at a T_M of 39.4 °C and 50 bar is negative and equal to -36 ± 3 cm^3 mol^{-1} [$\Delta v = -(2.9 \pm 0.3) \times 10^{-3}$ cm^3 g^{-1}] (Table 5). However, an increase in pressure causes a very significant increase in ΔV that, at ~1500 bar, changes its sign and becomes positive. At the maximum pressure of our experiments, 2200 bar, the value of ΔV is 21 ± 3 cm^3 mol^{-1} [$\Delta v = (1.7 \pm 0.3) \times 10^{-3}$ cm^3 g^{-1}]. Such an extensive change in ΔV cannot be attributed to the pressure-induced change in T_M since, at pressures where the T_M values are nearly identical, the values of ΔV are still significantly and consistently different. For example, at 500 bar with a T_M of 36.0 ± 0.3 °C and 2200 bar with a T_M of 35.9 ± 0.3 °C, the values of ΔV are -22 ± 3 and 21 ± 3 cm^3 mol^{-1}, respectively. We propose that the observed increase in ΔV with pressure is related to a nonzero change in isothermal compressibility, ΔK_T, accompanying the thermally induced MG-to-U transition of cytochrome *c*. To estimate the value of ΔK_T, one can compare the ΔV values obtained at different pressures but exhibiting similar values of T_M. At any given T_M, the value of ΔK_T can be evaluated as the ratio of the differential volume to the pressure difference ($\Delta K_T \approx -\Delta\Delta V/\Delta P$). As is seen from Table 5, we can carry out this calculation by using the data at 500 and 2200 bar ($T_M \approx 36$ °C), 750 and 2000 bar ($T_M \approx 35$ °C), and 1000 and 1750 bar ($T_M \approx 34$ °C). Our estimates reveal that, for the narrow temperature range of 35 ± 1 °C, the value of ΔK_T for the thermally induced MG-to-U transition of cytochrome *c* is $-(25 \pm 4) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} [$\Delta k_T = \Delta K_T/M = -(1.9 \pm 0.3) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}]. The volumetric properties of the thermally induced MG-to-U transition are tabulated in Table 6.

The negative value of Δk_T indicates that the molten globule conformation of cytochrome *c* exhibits a higher partial compressibility than its thermally induced unfolded conformation. To compare the partial compressibilities of the native and molten globule conformations of cytochrome *c*, one needs to treat the thermally induced unfolded states of the protein at pH 2 and 4 as volumetrically equivalent. In fact, the volumetric equivalence implies that the thermally induced unfolded conformations of cytochrome *c* are similar in terms of their structure and their hydration at pH ~2 and ~4. We feel that this is a reasonable assumption that can be used in our analysis below; however, further studies are required to verify it.

Thus, assuming that the thermally induced unfolded conformations of cytochrome *c* are equivalent at pH ~2 and ~4 and taking into account the fact that Δk_T of the thermally induced N-to-U transition is zero, one arrives at the conclusion that the N-to-MG transition is associated with the positive value of Δk_T of 1.9×10^{-6} cm^3 g^{-1} bar^{-1}. This result is in close agreement with $(1.7 \pm 0.3) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, the change in adiabatic compressibility caused by the acid-induced N-to-MG transition at 25 °C (*16*). In the absence of fortuitous compensation, this agreement suggests that the temperature dependence of the magnitude of the change in isothermal or adiabatic compressibility associated with the N-to-MG transition of cytochrome *c* is rather small within the temperature range of 25−35 °C. Our estimated volumetric properties of the N-to-MG transition of cytochrome *c* are listed in Table 6.

As noted above, at atmospheric pressure (the difference between the pressure of 50 bar and the atmospheric pressure can be neglected) and ~40 °C, the volume change, ΔV, accompanying the thermally induced MG-to-U transition is -36 ± 3 cm^3 mol^{-1} [$\Delta v = -(2.9 \pm 0.3) \times 10^{-3}$ cm^3 g^{-1}]. To determine the ΔV of the N-to-MG transition, one needs to compare the ΔV values of the N-to-U and MG-to-U transitions (the unfolded states of cytochrome *c* at pH 2 and 4 are treated as equivalent). We estimated the ΔV for the N-to-U transition of cytochrome *c* at 40 °C by extrapolating the data shown in Figure 5 to 40 °C: $(-120 \pm 28) + (2.2 \pm 0.4) \times 40 = -32 \pm 44$ cm^3 mol^{-1}. On the basis of comparison of this value with -36 ± 3 cm^3 mol^{-1}, the value of ΔV for the MG-to-U transition, we conclude that the volume change associated with the N-to-MG transition at 40 °C is practically zero [$\Delta V = 4 \pm 47$ cm^3 mol^{-1} or $\Delta v = (0 \pm 4) \times 10^{-3}$ cm^3 g^{-1}]. At 25 °C, the volume change, Δv, accompanying the acid-induced N-to-MG transition is 8×10^{-3} cm^3 g^{-1} (*16*). Comparison of these two values (0 at 40 °C and 8×10^{-3} cm^3 g^{-1} at 25 °C) suggests that the expansibility change, Δe, accompanying the N-to-MG transition is negative and equals $-(5 \pm 3) \times 10^{-4}$ cm^3 g^{-1} K^{-1}. If true, this would appear to be a remarkable result since all previously reported expansibility changes, Δe, for protein denaturation are positive (*12*).

In principle, the negative change in expansibility, Δe, accompanying the N-to-MG transition of cytochrome *c* can be accounted for in terms of the intrinsic, Δe_M, and hydration, $\Delta\Delta e_h$, contributions. Disruption of expandable intraglobular voids in the unfolded or molten globule conformation should result in a decrease in the intrinsic compressibility ($\Delta e_M < 0$) (*12*). This decrease in e_M is counterbalanced by an increase in the positive hydration contribution, Δe_h, because of enhanced solvation of protein groups in the denatured (molten globule or unfolded) states ($\Delta\Delta e_h > 0$) (*12*). In the unfolded conformation, the extent of protein hydration increases very significantly relative to the native state (*12*). The increase in Δe_h prevails over the decrease in e_M, and consequently, the net change in expansibility, Δe, upon an N-to-U transition becomes positive. In the molten globule state, protein hydration is more extensive relative to the native conformation but less so as compared to the unfolded state. The increase in Δe_h is not sufficient to overcome the negative change in e_M. Consequently, the net change in expansibility, Δe, associated with the N-to-MG transition may exhibit a negative value. Clearly, further studies are required to assess the validity of our estimate of Δe for the N-to-MG transition of cytochrome *c* and assess the veracity of the proposed interpretation.

CONCLUSION

We report the transition temperatures, T_M, and van't Hoff enthalpies, ΔH_M, of the thermally induced native-to-unfolded (N-to-U) and molten globule-to-unfolded (MG-to-U) transitions of cytochrome *c* determined at pressures between 50

and 2200 bar. The denaturation temperatures, T_M, for the N-to-U transitions of cytochrome at pH 3.4, 3.6, 4.0, and 4.3 linearly increase with pressure, an observation that implies that pressure stabilizes the native conformation of cytochrome *c*. The temperature dependence of T_M for the MG-to-U transition at pH 2.0 is parabolic. The T_M initially decreases when pressure increases from 1 to 1500 bar (the slope $\Delta T_M/\Delta P$ is negative); a further increase in pressure brings about a change in the sign of $\Delta T_M/\Delta P$. This observation suggests that pressure destabilizes the molten globule conformation of cytochrome *c* at low pressures, while at elevated pressures (above 1500 bar), the molten globule conformation becomes stabilized by pressure relative to the thermally induced unfolded conformation.

We have used the pressure dependences of T_M in conjunction with the Clayperon equation to calculate the changes in volume, Δv, accompanying the N-to-U and MG-to-U transitions of cytochrome *c* as a function of temperature and pressure. Our analysis of the temperature and pressure dependences of Δv has enabled us to determine the changes in expansibility, Δe, and isothermal compressibility, Δk_T, associated with the thermally induced transitions of cytochrome *c*. If extrapolated to 25 °C, the N-to-U transition of the protein is accompanied by changes in volume, Δv, expansibility, Δe, and isothermal compressibility, Δk_T, of $-(5 \pm 3) \times 10^{-3}$ cm^3 g^{-1}, $(1.8 \pm 0.3) \times 10^{-4}$ cm^3 g^{-1} K^{-1}, and $\sim$0 cm^3 g^{-1} bar^{-1}, respectively. The MG-to-U transition is accompanied by changes in volume, Δv, and isothermal compressibility, Δk_T, of $-(2.9 \pm 0.3) \times 10^{-3}$ cm^3 g^{-1} at 40 °C and $-(1.9 \pm 0.3) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} at 35 °C, respectively.

On the basis of comparison of the volumetric properties of the N-to-U and N-to-MG transitions of cytochrome *c*, we have determined the volumetric properties of the N-to-MG transition. For the latter transition, the changes in volume, Δv, and isothermal compressibility, Δk_T, are $\sim$0 cm^3 g^{-1} at 40 °C and 1.9 cm^3 g^{-1} bar^{-1} at 35 °C, respectively. Our estimate for the change in expansibility, Δe, upon the N-to-MG is negative and equal to $-(5 \pm 3) \times 10^{-4}$ cm^3 g^{-1} K^{-1}. This result contrasts with previous studies all of which report positive changes in expansibility associated with protein denaturation. A possible explanation may be related to the fact that the molten globule conformation of cytochrome *c* is less hydrated than its unfolded conformation. Consequently, an increase in the hydration contribution, Δe_h, upon the N-to-MG transition may not be sufficient to overcome a decrease in the intrinsic contribution, e_M, because of disruption of expandable intraglobular voids. As a result, the net change in exapansibility, Δe, accompanying the N-to-MG transition of the protein may be negative. In general, our volumetric data permit one to assess the combined effect of temperature and pressure on the stability of various conformational states of cytochrome *c* and ultimately construct a pressure-versus-temperature phase diagram of stability for this protein.

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