# Adiabatic Compressibility of Molten Globules†

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ABSTRACT: We report the measurement of the adiabatic compressibility $\beta$ for "molten globule" states of cytochrome $b_{562}$ and cytochrome *c*. Precise density and sound velocity measurements allow determination of $\beta$ in aqueous solution. Surprisingly, the molten globule apocytochrome $b_{562}$ shows a $\beta$ comparable to the native holocytochrome $b_{562}$. We estimated the maximum difference in the hydration contribution to the compressibility of apocytochrome $b_{562}$ relative to holocytochrome $b_{562}$ to be only $-2.6 \times 10^{-11}$ Pa$^{-1}$. Our results suggest that the intrinsic compressibility and the overall volume fluctuations in the molten globule state differ only slightly from those in the native protein. With cytochrome *c* we investigated the changes in $\beta$ for the transitions from the native to the acid molten globule and expanded premolten globule states. The molten globule at pH 2.2 was found to be somewhat "softer" than the native protein at neutral pH. In contrast, a decrease in $\beta$ of the premolten globule indicates an increased penetration of water into the macromolecule. In both of these intermediates the differences in adiabatic compressibility relative to the native state are small in comparison to the range of adiabatic compressibility observed for different native globular proteins.

"Molten globule" intermediates (Dolgikh *et al.*, 1981; Ohgushi & Wada, 1983; Pfeil *et al.*, 1986) with compact (Gast *et al.*, 1986; Damaschun *et al.*, 1986) but fluid-like three-dimensional structure and a native-like amount of secondary structure (Dolgikh *et al.*, 1981), which have been reported for more than 40 natural proteins, fragments of proteins, apoproteins, and polypeptides, are considered as playing a key role in our further understanding of protein folding, dynamics, and function (Kuwajima, 1989; Ptitsyn *et al.*, 1990; Baldwin, 1990; Bycroft *et al.*, 1990; Goto & Aimoto, 1991; Kuroda *et al.*, 1992; Jaenicke & Buchner, 1993; Chyan *et al.*, 1993; Barrick & Baldwin, 1993). The heat capacity, enthalpy, and free energy of molten globules have been investigated using calorimetry (Pfeil, 1981; Pfeil *et al.*, 1986; Xie *et al.*, 1991). Remarkable differences have been found in the thermodynamics between the molten globule states of different proteins and even between molten globules of the same protein (Pfeil, 1988). Thus the term molten globule does not describe a unique state but rather a class of different states. Goot *et al.* (1991) proposed that the molten globule is an intermediate on the pathway of insertion of the pore-forming colicin A into membranes. In this context Pain (1991) raised the question of "moltenness" or "squidgeability" of the molten globule.

In this paper, we present the measurement of the adiabatic compressibility for molten globule states of cytochrome $b_{562}$ and cytochrome *c*. The adiabatic compressibility $\beta$ of proteins in aqueous solution consists of the intrinsic compressibility of the molecule, the difference in the compressibility in the hydration shell relative to bulk water, and a component which is related to relaxation processes (Gekko & Hasegawa, 1986; Sarvazyan, 1991). The presence of relaxation terms increases the adiabatic compressibility, while increased hydration causes a decrease. For globular proteins the intrinsic compressibility is of the same order of magnitude as the absolute contribution due to hydration (Sarvazyan, 1991; Kharakoz & Sarvazyan, 1993). In the 0.1–10 MHz frequency range, the relaxation component in globular proteins is usually less than 10% of the apparent adiabatic compressibility *K*, which is defined as the change of the adiabatic compressibility of the solution per amount of solute (Sarvazyan, 1991):

$$K = (\beta_s - \beta_{so})/c \quad (1)$$

where $\beta_s$ and $\beta_{so}$ are the adiabatic compressibilities of protein solution and solvent, respectively, and *c* is the concentration of protein. Precise density ($\rho$) and sound velocity ($u$) measurements allow determination of the adiabatic compressibility $\beta_s$ of solutions by using the Laplace formula $1/\beta_s = \rho u^2$ (Sarvazyan, 1991).

Cytochrome $b_{562}$ from *Escherichia coli* is a soluble protein of 12 kdaltons. Its shape is roughly that of a cylinder of 5-nm length and 2.5-nm diameter. The structure of cytochrome $b_{562}$ is the four helix bundle motif of many other globular proteins (Lederer *et al.*, 1981; Robinson & Sligar, 1993). Apocytochrome $b_{562}$ has been shown to be a molten globule by NMR, circular dichroism, and size exclusion chromatography (Feng *et al.*, 1991; Feng & Sligar, 1991; Feng *et al.*, 1993). Apocytochrome $b_{562}$ is an excellent model for our investigation since its average three-dimensional structure can be evaluated from NMR (Feng *et al.*, 1993). However, apocytochrome $b_{562}$ displays a significant number of interhelix NOEs (Feng *et al.*, 1991), which might be indicative for a lower degree of tertiary structure fluctuations than in other molten globules.

Equine cytochrome *c* adopts a molten globule state at high salt concentration and pH 2.2 (Potekhin & Pfeil, 1989; Jeng *et al.*, 1990; Jeng & Englander, 1991; Kuroda *et al.*, 1992). In contrast, at low salt concentration and pH 2.2, an expanded molecule with presumably two times higher diameter has been described, which is considered to be a premolten globule state (Jeng & Englander, 1991).

## MATERIALS AND METHODS

For sound velocity measurement we used the resonator method (Gavish *et al.*, 1983a,b; Nölting & Sligar, 1993). The volume of the acoustic resonator is 0.6 mL. The sound

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frequency was 2 MHz. Density measurements were made using the common mechanical oscillator technique (Kratky *et al.*, 1969). For calibration we used the sound velocity and density increments of sodium chloride solutions (Millero *et al.*, 1977). Holo- and apocytochrome $b_{562}$ were prepared as described by Feng and Sligar (1991). The buffer for measurement was 50 mM potassium phosphate buffer, pH 7. Concentration was determined by the dry weight of the salt free protein. The differences in sound velocity $\Delta u$ and density $\Delta\rho$ between protein solution and buffer were measured and used for the calculation of $\beta$ (Sarvazyan, 1991). Concentration was determined for equine ferric cytochrome *c* (Type VI: Sigma) by diluting a part of the sample 20-fold with 5 mM potassium ferricyanide, 50 mM potassium phosphate buffer, pH 7, and using the extinction coefficient of 91.97 $mM^{-1}$ $cm^{-1}$ at 409 nm (Kuroda *et al.*, 1992), molar mass 12 384 g $mol^{-1}$. Measurements of cytochrome $b_{562}$ and cytochrome *c* were performed at 25.0 °C with 10 g $L^{-1}$ protein. The sound velocity and density of most protein solutions in the concentration range of up to 10 g $L^{-1}$ have been found to be linearly dependent on the concentration (Gekko & Noguchi, 1979; Kharakoz & Mkhitaryan, 1986; Gekko & Hasegawa, 1986). Errors are estimated to be less than ±2% for $\Delta u/c$ and $\Delta\rho/c$ of both cytochrome $b_{562}$ and cytochrome *c* and less than $\pm 1 \times 10^{-11}$ $Pa^{-1}$ for both $\Delta\beta$ of cytochrome *c* and the difference in $\beta$ between holo- and apocytochrome $b_{563}$. Estimates of errors in concentration of protein and of its water content are about 2%, and the error in the sound velocity and density measurements a few 0.1%.

## RESULTS

The compressibility of holocytochrome $b_{562}$, $\beta = 7.3 \times 10^{-11}$ $Pa^{-1}$ (Table I) is found to be in the range of that of other globular proteins in water, which is from $\leq -1 \times 10^{-11}$ $Pa^{-1}$ to $\geq 11 \times 10^{-11}$ $Pa^{-1}$ (Gekko & Hasegawa, 1986). Surprisingly, the difference between the native cytochrome $b_{562}$ and the molten globule, $(0.7 \pm 1) \times 10^{-11}$ $Pa^{-1}$, is small in comparison to the range of compressibility of different globular proteins. Like other globular proteins, the compressibility of apocytochrome $b_{562}$ is much lower than that of typical organic liquids (Gavish *et al.*, 1983a). The similarity in compressibility of apo- and holocytochrome $b_{562}$ indicates that the contact of water to the molten globule is much less than that of oligopeptides and unfolded proteins. Oligopeptides typically display a negative $\beta$ due to an increased hydration shell (Sarvazyan, 1991). Thirty percent unfolded protein displays about $5 \times 10^{-11}$ $Pa^{-1}$ lower $\beta$ than native protein (Kharakoz & Mkhitaryan, 1986). For the pure theoretical case of fully unfolded protein, the compressibility has been calculated to be about $-80 \times 10^{-11}$ $Pa^{-1}$ (Iqbal & Verrall, 1988), corresponding to a sound velocity increment $\Delta u/c = 1.3$ m $s^{-1}$ L $g^{-1}$.

Using equine cytochrome *c* we monitored the sound velocity and density changes for the transitions from the native state at neutral pH to the acid molten globule state at pH 2.2 and high salt concentration (Potekhin & Pfeil, 1989; Jeng *et al.*, 1990; Jeng & Englander, 1991; Kuroda *et al.*, 1992) and to the expanded premolten globule state at low pH and low salt concentration (Jeng & Englander, 1991) and calculated the corresponding changes in the adiabatic compressibility.

As shown in Figure 1 (top), the sound velocity observed at low and high salt is lower at pH 3 than in the neutral region. When the pH is lowered below 3 at high salt, the sound velocity decreases slightly. Conversely, at low salt the sound velocity increases significantly, suggesting an enhanced penetration

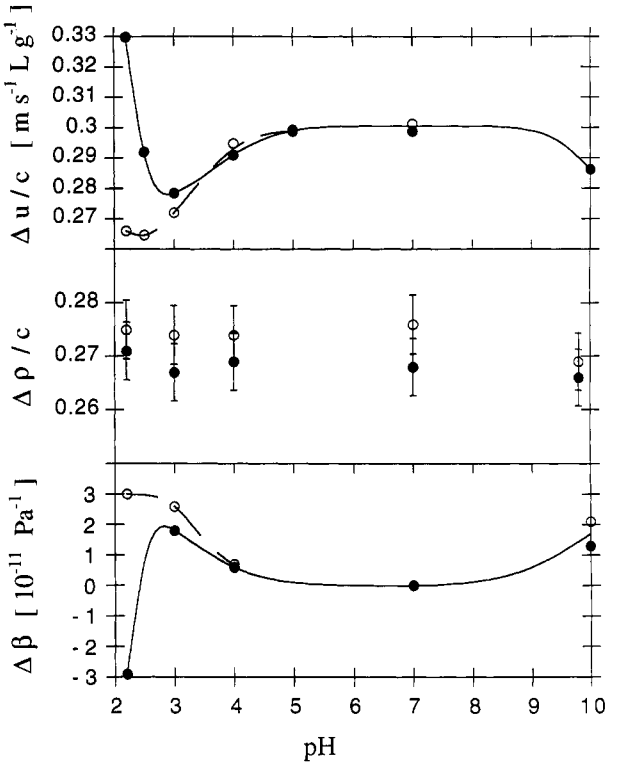


FIGURE 1: pH dependent changes in cytochrome *c* at different sodium chloride concentrations: 10 mM NaCl (—, closed circles) and 200 mM NaCl (– –, open circles); (top) sound velocity increment, $\Delta u/c$; (middle) density increment, $\Delta\rho/c$; (bottom) difference in the adiabatic compressibility relative to pH 7, $\Delta\beta$.

of water into the expanded premolten globule. On the other hand, the sound velocity increment in the premolten globule at pH 2.2 of about 0.33 m $s^{-1}$ L $g^{-1}$ is still roughly two times smaller than that for amino acids and peptides (Sarvazyan, 1991; Chalikian *et al.*, 1992). This was expected from the observation of a large amount of maintained secondary structure and submolecular folding units (Jeng & Englander, 1991). The sound velocity increment of the acid molten globule, and most strikingly also that of the expanded premolten globule state, is in the range of that of natural globular proteins, ≤0.21 m $s^{-1}$ L $g^{-1}$ to ≥0.37 m $s^{-1}$ L $g^{-1}$ (Gekko & Hasegawa, 1986).

The density increment at neutral pH, $\Delta\rho/c = 0.27$, matches former extrapolation to zero concentration (Gekko & Hasegawa, 1986). This is further indication of only small concentration dependency at up to 10 g $L^{-1}$ cytochrome *c*. At low salt as well as at high salt we found no significant changes with the pH in the range from 10 to 2.2. (Figure 1, middle). Intriguingly for different native globular proteins a relative uniform density increment of about 0.27 ± 0.03 has been observed (Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993). That suggests that, for globular proteins, the differences in sound velocity make the major contribution to the differences in adiabatic compressibility. The compressibility changes (Figure 1, bottom) are dominated by the sound velocity changes. The molten globule state at pH 2.2 is by roughly $3 \times 10^{-11}$ $Pa^{-1}$ "softer" than the native cytochrome *c* at neutral pH. In contrast the adiabatic compressibility of the premolten globule decreases by roughly $3 \times 10^{-11}$ $Pa^{-1}$ between pH 7 and 2.2.

## DISCUSSION

The decomposition of the adiabatic compressibility into individual contributions is complicated by the different sign

of its components. Intrinsic compressibility and relaxation make a positive contribution to the overall compressibility, while an increase in the hydration contribution causes a decrease in $\beta$ (Sarvazyan, 1991). Large hydration and low intrinsic compressibility are responsible for the negative $\beta$ of fibrilar proteins. Thus there are different possible explanations for the similarity of the adiabatic compressibility between native cytochrome $b_{562}$ and the molten globule.

(1) Each of the three components of the adiabatic compressibility is not significantly changed in the molten globule. The enhanced side-chain mobility and lower structural order in the molten globule does not lead to a significant change in the intrinsic compressibility. The ambiguous tertiary structure in the molten globule might be caused by slow transitions between substates (Frauenfelder *et al.*, 1991) which do not contribute much to the overall conformational relaxation. These transitions might occur at lower frequency than 2 MHz or they might involve only small volume and sound velocity changes. The tertiary structure fluctuations might be connected with only small changes in the local or temporal average of the hydration, for example, due to a slipping movement of the helices against each other instead of spreading or due to only short periods of time of enlarged exposure to water while undergoing transitions over unfavored energy barriers.

(2) The intrinsic compressibility and hydration shell are significantly changed but contribute to the overall compressibility in the opposite direction. For example the interior of the molecule might be less compact due to less ordered packing which increases the intrinsic compressibility and the hydration shell might be enlarged which causes a decrease of $\beta$.

(3) The small relaxation contribution in the native state is significantly enlarged in the molten globule but compensated by a higher compactness of the interior of the molecule or by an enlarged hydration. An increased relaxation contribution might be caused by increased conformational relaxation or by increased proton exchange (Sarvazyan *et al.*, 1979) due to a higher degree of exposure of side chains to solvent.

*Relaxation Contribution in Cytochrome $b_{562}$.* The relaxation contribution to the compressibility of cytochrome $b_{562}$ can be estimated by monitoring the frequency dependency of the sound velocity. All relaxations with a frequency not significantly lower than the frequency of the sound wave contribute to the sound velocity. Proton exchange relaxation can contribute at frequencies as high as 100 MHz (Kanda *et al.*, 1976). Thus, to determine the amount of relaxation at a certain frequency, a comparison with a measurement at a frequency greater than 100 MHz would be necessary. This frequency range is not possible with our existing equipment. On the other hand, the proton exchange reaction makes a significant contribution to the compressibility only in the pH range where the acid and basic side chains are titrating (Sarvazyan & Hemmes, 1979). The amount of conformational relaxation in molten globules has not been estimated. Changes in volume for very slow conformational transitions can be determined with the repetitive pressure perturbation method (Pryse *et al.*, 1992). We can, however, make an estimate for an upper limit of the relaxation contribution in holocytochrome $b_{562}$. For a protein with $\beta = 7.3 \times 10^{-11}$ Pa$^{-1}$ in water with $\beta_w = 45 \times 10^{-11}$ Pa$^{-1}$ (Gavish *et al.*, 1983a), a relaxation contribution of less than 10% to the apparent adiabatic compressibility $K$ (Sarvazyan, 1991) corresponds to a relaxation contribution to $\beta$ of less than $3.8 \times 10^{-11}$ Pa$^{-1}$.

*Hydration Contribution in Cytochrome $b_{562}$.* In gel filtration chromatography the apocytochrome $b_{562}$ exhibits a 3.3% higher apparent weight which might be due to a more asymmetric shape than the holoprotein or due to an overall expansion of the molecule in the absence of the heme (Feng & Sligar, 1991). Taking into account the enlargement of the outer surface of the protein molecule and the possible penetration of water into the protein molecule, we estimated the changes in the hydration shell contribution. The decrease in the compressibility due to enlarged hydration contribution is roughly $\leq 1 \times 10^{-11}$ Pa$^{-1}$ (see supplementary material).

Table I: Sound Velocity Increment, $\Delta u/c$, Density Increment, $\Delta\rho/c$, and the Adiabatic Compressibility, $\beta$, of Holo- and Apocytochrome $b_{562}$

| protein | $\Delta u/c$ (m s$^{-1}$ L g$^{-1}$) | $\Delta\rho/c$ | $\beta$ ($10^{-11}$ Pa$^{-1}$) |
|---|---|---|---|
| holo-$b_{562}$ | 0.260 | 0.266 | 7.3 |
| apo-$b_{562}$ | 0.252 | 0.266 | 8.0 |

The NMR structure of apocytochrome $b_{562}$ (J. A. Wand, personal communication, 1993; Feng *et al.*, 1993) reveals about 15–20% enlarged linear dimensions of the molten globule relative to the native protein and a deep cleft at the position of the absent heme. Using INSIGHT II and this structure data of holo- and apocytochrome $b_{562}$, we calculated the number of water molecules in the hydration shell as a function of the assumed thickness of the shell and the decrease in the compressibility of apocytochrome $b_{562}$ relative to holocytochrome $b_{562}$ due to increased hydration. We found a decrease by roughly (1.4–2.6) $\times 10^{-11}$ Pa$^{-1}$ (see supplementary material). Thus, a decrease by $2.6 \times 10^{-11}$ Pa$^{-1}$ is considered as an upper limit. This limit might be somewhat decreased due to a higher content of hydrophobic side chains of molten globules which are in contact with water relative to the native protein (Pothekin & Pfeil, 1989; Xie *et al.*, 1991; Kuroda *et al.*, 1992): The absolute value of the hydration contribution per molecular area of solvent exposed hydrophobic side chains is less than that of exposed polar and charged side chains (Kharakoz & Sarvazyan, 1993).

*Intrinsic Compressibility of Cytochrome $b_{562}$.* The compressible void volume generated by the random close packing of the interior of the molecule is considered to be the main source of the intrinsic compressibility in globular proteins (Gekko & Hasegawa, 1986). The molten globule state is proposed to have a higher side-chain mobility and less interlocking between secondary structure elements. Thus the intrinsic compressibility in the molten globule was expected to be significantly enlarged. We found a difference of only $(0.7 \pm 1) \times 10^{-11}$ Pa$^{-1}$ in the adiabatic compressibility $\beta$ between apo- and holocytochrome $b_{562}$ (Table I). As mentioned previously, the relaxation contribution in the molten globule is not significantly decreased, and the hydration contribution is changed by no more than $-2.6 \times 10^{-11}$ Pa$^{-1}$. Thus taking also into account the experimental error, the intrinsic compressibility of apocytochrome $b_{562}$ is less than $4.3 \times 10^{-11}$ Pa$^{-1}$ greater than that of holocytochrome $b_{562}$. On the other hand, it is also unlikely that the intrinsic compressibility is significantly decreased in the molten globule: NMR (Feng *et al.*, 1993) unambiguously reveals a less perfect packing of the interior of apocytochrome $b_{562}$. Consequently, the enlargement of the relaxation contribution in apocytochrome $b_{562}$ relative to holocytochrme $b_{562}$ is probably less than $4.3 \times 10^{-11}$ Pa$^{-1}$. Thus all of the three components of the adiabatic compressibility differ between holo- and apocytochrome $b_{562}$ by less than $\pm 4.3 \times 10^{-11}$ Pa$^{-1}$.

*Volume Fluctuations in Cytochrome $b_{562}$.* The volume fluctuations expressed by $\langle \delta V^2 \rangle$ are related to the isothermal bulk compressibility $\beta_t$ by $\langle \delta V^2 \rangle = k_B T V \beta_t$ (Cooper, 1976; Gekko & Hasegawa, 1986; Kharakoz & Mkhitaryan, 1986),

in which $k_B$ is the Boltzmann's constant, $T$ the absolute temperature, and $V$ the volume of a cytochrome $b_{562}$ molecule. The adiabatic compressibility $\beta$ is connected with the isothermal compressibility $\beta_t$ by (Priev *et al.*, 1990; Gekko & Hasegawa, 1986):

$$\beta_t = \beta + \alpha^2 v^0 T/c_p \quad (2)$$

where $\alpha$ and $c_p$ are the coefficient of thermal expansion and the heat capacity at constant pressure, respectively, and $v^0$ is the partial specific volume of the protein. The difference between $\beta_t$ and $\beta$ has been calculated for 24 globular proteins to be (1–5) $\times 10^{-11}$ Pa$^{-1}$ (Gekko & Hasegawa, 1986). Using $\beta = 7.3 \times 10^{-11}$ Pa$^{-1}$, and the average $\beta_t \approx \beta + 3 \times 10^{-11}$ Pa$^{-1}$, yields for holocytochrome $b_{562}$ $\langle \delta V^2 \rangle / V^2 \approx 3 \times 10^{-5}$. However, this estimate does not account for the protein hydration and presents a lower limit. Applying the estimate of the upper limit for the intrinsic isothermal compressibility of protein of $20 \times 10^{-11}$ Pa$^{-1}$ (Kharakoz & Mkhitaryan, 1986) to holocytochrome $b_{562}$, we find $\langle \delta V \rangle / V^2 \approx 6 \times 10^{-5}$. In the case of isotropic motions, these volume fluctuations correspond roughly to fluctuations in the radius of the molecule (approximation for a spherical shape; Lee, 1983) of 0.03 and 0.06 Å, respectively. The intrinsic compressibility of apo- and holocytochrome $b_{562}$ probably differs by less than $4.3 \times 10^{-11}$ Pa$^{-1}$. Thus for isotropic breathing motions the difference in radial fluctuations is less than 0.02 Å. This is very unexpected and seems to conflict with the assumed fluidity of molten globules. The comparison with unfolded protein illustrates why that small difference is not necessarily in contradiction to the "moltenness" (Pain, 1991) of the molten globule: Unfolded but highly mobile protein has a much lower intrinsic compressibility and consequently much lower volume fluctuations. It is highly deformable but not very compressible.

*Molten Globule Conformation of Cytochrome c.* For the molten globule conformation of cytochrome *c* there are basically the same three possibilities for contributions to the compressibility. The neutralization of carboxyl groups at low pH leads to a decreasing sound velocity and density contribution. For amino acids, the volume change due to neutralization of carboxyl side chains is approximately 7 mL mol$^{-1}$ (Chalikian *et al.*, 1992). Because of the incomplete accessibility of solvent to the carboxyl groups, the volume change is likely to be less than 7 mL mol$^{-1}$ in the molten globule. Thus the titration of all of the 13 carboxyl groups in equine cytochrome *c* would lead to a change in the partial specific volume of the molecule of less than 91 mL mol$^{-1}$. This corresponds to a change in the density increment comparable with or less than the experimental error of 2%. The decrease in the sound velocity increment of proteins due to the neutralization of the carboxyl side chains at low pH can be several percent (Sarvazyan *et al.*, 1979; Sarvazyan & Hemmes, 1979; Chalikian *et al.*, 1992). Thus a significant part of the decrease in sound velocity in cytochrome *c* at low pH, in comparison to the native protein at neutral pH, might be attributed to the neutralization of carboxyl side chains. Also at high pH the neutralization of the amino groups is expected to make the sound velocity decreasing contribution (Chalikian *et al.*, 1992), which might be accounted for the observed slight decrease in $\Delta u/c$ in our experiment. A further source of chemical relaxation and change in the intrinsic compressibility in cytochrome *c* at low and high pH might be the displacement of the ligands of the heme iron, histidine 18, and methionine 80 (Myer *et al.*, 1980; Dyson & Beattie, 1982; Robinson *et al.*, 1983; Potekhin & Pfeil, 1989).

*Expanded Conformation of Cytochrome c.* The thermal unfolding of cytochrome *c* at low salt and low pH has been found by Kuroda *et al.* (1992) to be a two-state process. In contrast to Jeng and Englander (1991), they considered cytochrome *c* under these conditions not as a molten globule. This contradiction reflects the ambiguity in the use of the term molten globule for different intermediates of folding. A more precise nomenclature for protein conformations with different content of secondary and tertiary structure has been described (Dolgikh *et al.*, 1981; Pfeil, 1988). For the expanded conformation at low pH and low ionic strength, with loss of a defined tertiary structure but retention of a high amount of secondary structure, we suggest the term "E-state" for "expanded-state". The decrease in compressbility of the E-state of cytochrome *c*, relative to the native state at neutral pH, indicates significantly enhanced penetration of water into the molecule that one would expect neither for a hydrophobic collapsed molten globule nor for a native protein.

## SUMMARY

For three types of equilibrium intermediates, namely the molten globule state of apoprotein (apostate), an acid molten globule, and an expanded premolten globule (E-state), we found similarities in the adiabatic compressibility in comparison to native proteins. This indicates that the moltenness (Pain, 1991) of these intermediates is not connected with a large overall "softening" of the molecule. For the molten globule apocytochrome $b_{562}$, our results suggest less than $\pm 4.3 \times 10^{-11}$ Pa$^{-1}$ difference in all three contributions to the adiabatic compressibility relative to the native holoprotein. Decreased compressibility of the expanded conformation of cytochrome *c* indicates significant penetration of water into the molecule.

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## SUPPLEMENTARY MATERIAL AVAILABLE

Experimental details of hydration contribution in cytochrome $b_{562}$ and a figure containing calculated number of water molecules in the hydration shell of holo- and apocytochrome $b_{562}$ (6 pages). Ordering information is given on any current masthead page.

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