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Universal Convergence of the Specific Volume Changes of Globular Proteins upon Unfolding[†]

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ABSTRACT: Both pressure and temperature are important environmental variables, and to obtain a complete understanding of the mechanisms of protein folding, it is necessary to determine how protein stability is dependent on these fundamental thermodynamic parameters. Although the temperature dependence of protein stability has been widely explored, the dependence of protein stability on pressure is not as well studied. In this paper, we report the results of the direct thermodynamic determination of the change in specific volume ($\Delta V/V$) upon protein unfolding, which defines the pressure dependence of protein stability, for five model proteins (ubiquitin, eglin c, ribonuclease A, lysozyme, and cytochrome *c*). We have shown that the specific volumetric changes upon unfolding for four of the proteins (ubiquitin, eglin c, ribonuclease A, and lysozyme) appear to converge to a common value at high temperatures. Analysis of various contributions to the change in volume upon protein unfolding allowed us to put forth the hypothesis that the change in volume due to hydration is very close to zero at this temperature, such that $\Delta V/V$ is defined largely by the total volume of cavities and voids within a protein, and that this is a universal property of all small globular proteins without prosthetic groups. To test this hypothesis, additional experiments were performed with variants of eglin c that had site-directed substitutions at two buried positions, to create an additional cavity in the protein core. The results of these experiments, coupled with the structural analysis of cytochrome *c* showing a lower packing density compared to those of the other four proteins, provided further support for the hypothesis. Finally, we have shown that the deviation of the high-temperature ΔV value of a given protein from the convergence value can be used to determine the size of the excess cavities in globular proteins.

Pressure and temperature are both fundamental thermodynamic variables. Pressure is also an important environmental variable that plays an essential role in biological adaptation for many extremophilic organisms, in particular those that live in the deep sea (*1*). Changes in pressure, much like changes in temperature, can lead to protein unfolding. The pressure dependence of the equilibrium constant of protein unfolding is defined by the difference in volume between the unfolded and native states of a protein. The changes in volume can originate from different factors, including the size of internal cavities in the native state and the hydration of internal protein groups that become exposed to solvent upon unfolding (*2*). Until recently, researchers performed experimental measurements of the volumetric changes upon protein unfolding either by taking the first derivative of the pressure dependence of the equilibrium constant (*3*−*6*) or by doubly integrating the temperature dependence of the density of protein solutions (*3*, *7*). The recent introduction of new instrumentation, pressure perturbation calorimetry (PPC), has made it possible to directly measure the volumetric changes that accompany protein unfolding (*8*−*11*). We have used PPC to measure volume changes as a function of transition temperature for five different proteins: ubiquitin, eglin c, cytochrome *c*, ribonuclease A, and lysozyme. In this paper, we show that the changes in volume upon unfolding are strongly dependent on temperature and, for some of the proteins, show peculiar behavior. Namely, the specific volume changes upon protein unfolding show universal convergence at high temperatures. We present a hypothesis to explain this observation and provide further experimental tests of the hypothesis by measuring the thermodynamic parameters for cavity-creating single-site variants of eglin c. Finally, we show that, within the proposed formalism, one can use PPC to experimentally determine the excess cavity volume for globular proteins.

MATERIALS AND METHODS

Bovine ubiquitin (Ubq, catalog no. U6253), horse heart cytochrome *c* (Cyt, catalog no. C-7752), bovine pancreatic ribonuclease A (Rns, catalog no. R5500), and hen egg white lyzosyme (Lyz, catalog no. L-6876) were purchased from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. The eglin c (Egl) variants were prepared as described previously (*12*). Protein concentrations for all experiments were determined spectrophotometrically (*13*), using the following molar extinction coefficients: $\varepsilon_{280} = 10008\ M^{-1}\ cm^{-1}$ for Rns, $\varepsilon_{280} = 38460\ M^{-1}\ cm^{-1}$ for Lyz, $\varepsilon_{280} = 1280\ M^{-1}\ cm^{-1}$ for Ubq, $\varepsilon_{590} = 11220\ M^{-1}\ cm^{-1}$ for Cyt, and $\varepsilon_{280} = 14440\ M^{-1}\ cm^{-1}$ for the Egl variants.

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The PPC experiments were performed on a MicroCal VP-DSC instrument with a PPC attachment (MicroCal, LLC, Northampton, MA). The protein solutions were dialyzed extensively against the corresponding buffers (20−50 mM glycine at pH 2.2−3.6) at 25 °C using Spectrapor3 dialysis membranes with a 3.5 kDa molecular mass cutoff. Samples were centrifuged at 13000 rpm in an Eppendorf 5417R microcentrifuge for 20−30 min at 25 °C to remove insoluble material present in the solution after dialysis. The experiments were performed using protein concentrations between 0.8 and 4.0 mg/mL. The partial specific volumes of the proteins were calculated as previously described (*14*). The following values were used: 0.721 cm^3/g for Rns, 0.729 cm^3/g for Lyz, 0.783 cm^3/g for Cyt, 0.747 cm^3/g for Ubq, and 0.734 cm^3/g for the Egl variants.

The linear thermal expansion coefficient of a protein (α_{pr}) at a given temperature, T, is related to the thermal expansion of the water (α_{H_2O}) and the change in the heat of the calorimetric cell during a water/buffer scan ($\Delta Q_{H_2O/buf}$) and a protein/buffer scan ($\Delta Q_{pr/buf}$) due to a change in the pressure (ΔP) of the system as (*8*)

$$\alpha_{pr} = \alpha_{H_2O} - \frac{\Delta Q_{H_2O/buf}}{T\Delta P v_{cell}} - \frac{\Delta Q_{pr/buf}}{T\Delta P v_{cell} c_{pr} \overline{V}_{pr}} \quad (1)$$

$$\alpha_{pr} = \alpha_{H_2O} - \frac{1}{T\Delta P v_{cell}} \left(\Delta Q_{H_2O/buf} \frac{\Delta Q_{pr/buf}}{c_{pr} \overline{V}_{pr}} \right)$$

where v_{cell} is the volume of the calorimetric cell, c_{pr} is the concentration of protein in the calorimetric cell, and $\overline{V}_{pr}$ is the partial specific volume of protein.

The temperature dependencies of the linear thermal expansion coefficient of a protein, α_{pr} (obtained from the PPC experiments), and of the partial molar heat capacity, $C_{p,pr}$ (obtained from the DSC experiments), were fitted simultaneously so that the enthalpy of protein unfolding, ΔH, explicitly determined in the DSC experiment, could be used for the PPC data analysis (*15*, *16*):

$$\alpha_{pr}(T) = \frac{1}{1+K_{eq}(T)} \alpha_N(T) + \frac{K_{eq}(T)}{1+K_{eq}(T)} \alpha_U(T) + \frac{K_{eq}(T)}{[1+K_{eq}(T)]^2} \frac{\Delta H}{RT^2} \frac{\Delta V_{pr}}{V_{pr}} \quad (2)$$

$$C_{p,pr}(T) = \frac{1}{1+K_{eq}(T)} C_{p,N}(T) + \frac{K_{eq}(T)}{1+K_{eq}(T)} C_{p,U}(T) + \frac{K_{eq}(T)}{[1+K_{eq}(T)]^2} \frac{\Delta H^2}{RT^2} \quad (3)$$

where $K_{eq}(T)$ is the equilibrium constant for a two-state process, $C_{p,N}(T)$ and $C_{p,U}(T)$ are the temperature dependences of the partial molar heat capacities of the native and unfolded state, respectively, represented by a first-order polynomial of temperature, and $\alpha_N(T)$ and $\alpha_U(T)$ are the temperature dependencies of the linear expansion coefficients for the native and unfolded state, respectively, represented by a third-order polynomial of temperature (*15*). It is important to use the values of ΔH determined from DSC experiments when fitting the PPC data because the errors associated with determining ΔH from PPC alone can significantly affect the interpretation of the PPC results (*15*). All nonlinear regression fits were performed using scripts for NLREG (*17*).

The volumes of the cavities created as a result of Val-to-Ala substitutions in eglin c were calculated in the following manner (*18*). The structures for the Egl-V14A and Egl-V54A variants were generated using homology modeling [Modeler 7v7 (*19*)] using Protein Data Bank (PDB) entry 1CSE as the template of the wild-type structure. To calculate the molar volume of each protein, constant-pressure molecular dynamics simulations were performed on the structures for each of the Egl variants at 298 K and 1 atm using the GROMOS96 53a5 force field (*20*) in explicit solvent [SPC water model (*21*)] for 2 ns, and the trajectory was analyzed on snapshots that were saved every 1 ps. The simulations were run using Nose−Hoover temperature coupling (τ_T = 1.0) (*22*, *23*) and Parrinello−Rahman (τ_p = 1.0 and $\beta = 4.5 \times 10^{-5}$) pressure coupling (*24*). The volume of the simulation box for each frame was divided into complementary shell and bulk regions (*25*). The shell region is defined by a distance ($R < 1$ nm) to any heavy atom of the protein. To calculate the sizes of the shell volume and bulk volume, the volume of the box is divided by a grid of equal-sized volume elements, with a lattice spacing of 0.5 Å. For each frame of the trajectory, the shell (V_{shell}) and bulk (V_{bulk}) volumes are determined by counting the number of corresponding volume elements in the grid. The number of water molecules belonging to either the shell or the bulk is also determined, where the position of the oxygen atom of the water molecule is used to decide whether a given molecule belongs in the shell or in the bulk. Finally, the molar volume of the protein (V_{pr}) is determined by subtracting the volume occupied by the water molecules in the shell region from the total volume of the shell (V_{shell}), where the volume of water molecules is approximated as the volume per molecule of bulk water (*25*):

$$V_{pr} = V_{shell} - N_{W,shell} \frac{V_{W,bulk}}{N_{W,bulk}} \quad (4)$$

where $N_{W,shell}$ is the number of waters within the shell region, $V_{W,bulk}$ is the partial volume of water in the bulk solution, and $N_{W,bulk}$ is the number of water molecules in the bulk region. All partial molar volumes are obtained by averaging the results of these calculations over all 2000 structures in the MD trajectory of each protein. The volumes of the Egl variants determined by this structure-based method were 9570 $Å^3$ for Egl-WT, 9470 $Å^3$ for Egl-V14A, and 9520 $Å^3$ for Egl-V54A.

RESULTS

Figure 1 shows the temperature-induced unfolding of two different proteins, ubiquitin and cytochrome *c*, monitored by measuring either the partial molar heat capacity, $C_p(T)$, using differential scanning calorimetry (DSC), or the linear thermal expansion coefficient, $\alpha(T)$, using pressure perturbation calorimetry (PPC). Since both methods monitor the same unfolding process, the data can be fitted simultaneously to a common set of thermodynamic parameters. As discussed in Materials and Methods, the analysis of PPC data is more robust if done simultaneously with the analysis of the DSC profile, because the former requires accurate knowledge of the enthalpy of protein unfolding. Integration of the $\alpha(T)$ function gives direct access to the volume change upon protein unfolding $\Delta V_{pr}/V_{pr}$.

Figure 2 shows the dependence of $\Delta V_{pr}/V_{pr}$ on transition temperature (T_m) for five different proteins: ubiquitin (Ubq), eglin c (Egl), cytochrome *c* (Cyt), ribonuclease A (Rns), and lysozyme (Lyz). These proteins differ in size (76, 78, 104, 124, and 129 amino acid residues, respectively) and in topologies of

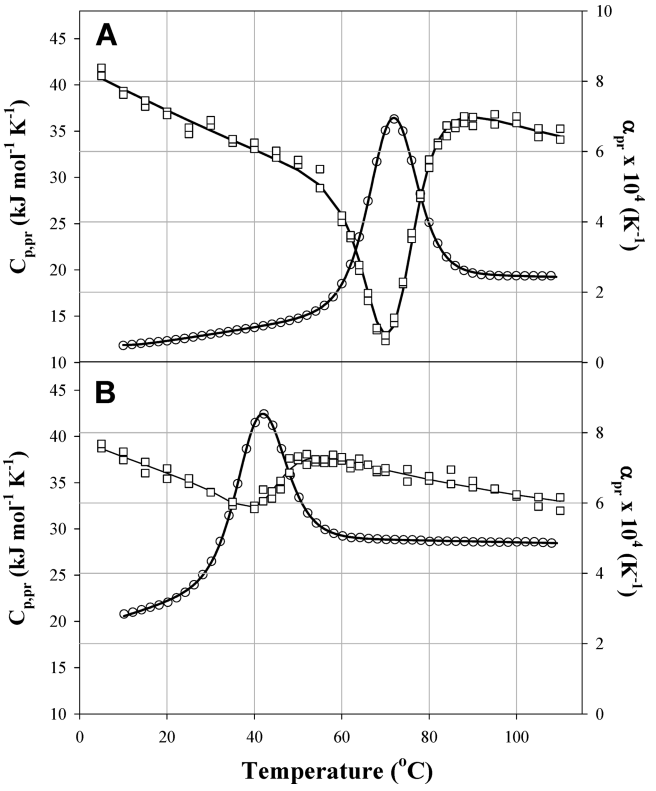

FIGURE 1: Comparison of denaturation profiles obtained from DSC and PPC experiments for (A) ubiquitin in 50 mM glycine buffer (pH 3.0) and (B) cytochrome *c* in 50 mM glycine buffer (pH 3.0). In both panels, the symbols represent the experimental data: (○) DSC data and (□) PPC data. The solid lines represent the fits of the experimental data to a two-state model of unfolding using equations described in Materials and Methods.

tertiary structure. Several trends can be observed from the data presented in Figure 2.

First, the values of $\Delta V_{pr}/V_{pr}$ are relatively small yet very different for different proteins. For most proteins, $\Delta V_{pr}/V_{pr}$ is negative, suggesting that the volume of the unfolded protein is smaller than that of the native protein. This effect was noted first by Kauzmann (*26*, *27*) and more recently by Royer, Winter, and Chalikian (*2*, *5*, *9*, *10*). Cyt is the only protein studied here for which positive values of $\Delta V_{pr}/V_{pr}$ are observed. This observation is consistent with previous predictions (*9*), based on several different sets of experimental information about thermodynamic properties of staphylococcal nuclease, that there should be a switch in the sign of $\Delta V_{pr}/V_{pr}$. However, this has never been observed experimentally; thus, the Cyt data presented here provide a unique and direct confirmation of this prediction.

Second, for all studied proteins, $\Delta V_{pr}/V_{pr}$ increases with the increase in transition temperature. By definition, this suggests that the change in the linear thermal expansion coefficient upon protein unfolding $\{\Delta\alpha = \alpha_U - \alpha_N = [\partial(\Delta V_{pr}/V_{pr})/\partial T]\}$ has positive value. The values of $\Delta\alpha$ are different for different proteins, and there is no apparent trend to suggest that the value of $\Delta\alpha$ is dependent on protein thermostability (defined by the transition temperature, T_m), protein stability (defined by the change in the Gibbs free energy upon unfolding, ΔG), the magnitude of volumetric changes upon unfolding, protein size, or any other obvious structural or thermodynamic parameter.

Finally, the linear extrapolation of the $\Delta V_{pr}/V_{pr}$ versus T_m profiles of four of the five proteins studied (Egl, Lyz, Rns, and Ubq) shows that the $\Delta V_{pr}/V_{pr}(T)$ functions intersect at a similar

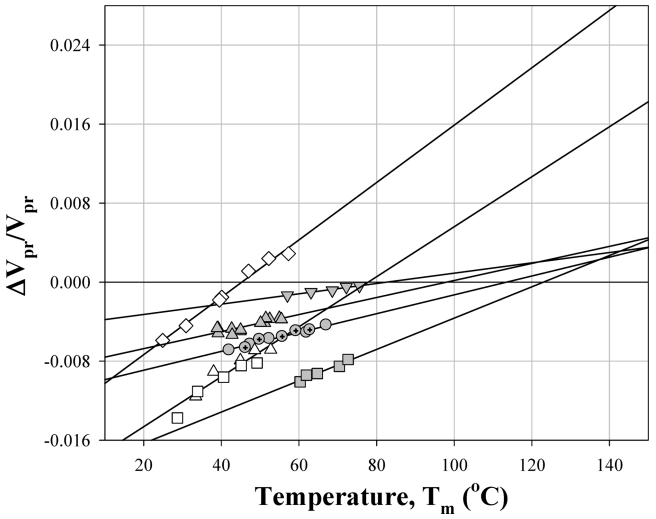

FIGURE 2: Temperature dependence of $\Delta V_{pr}/V_{pr}$ for five model proteins: RNaseA (gray upward-pointing triangles), Lys (gray downward-pointing triangles), Ubq (gray squares), CytC (◇), EgC (gray circles and crossed gray circles are for two independent data sets measured for the wild-type protein 6 months apart), Egl- V14A (△), and Egl-V54A (□). The solid lines represent the linear regressions of the data. The slopes of these lines represent the change in linear thermal expansion coefficient upon protein unfolding, $\Delta\alpha$.

"convergence" value of 0.003 ± 0.001 at the "convergence temperature" of 140 °C (413 K). For the fifth protein, Cyt, the dependence of $\Delta V_{pr}/V_{pr}$ on temperature extrapolates to a much larger value of 0.027 ± 0.002. This raises the question of whether the high-temperature convergence of the volumetric changes upon unfolding of four of the proteins is a coincidence or whether the anomalous behavior of Cyt can be explained by the structural properties of this protein. Below we will put forward a hypothesis to explain these observations and then will provide additional experimental tests that further support it.

DISCUSSION

According to Chalikian (*2*), the volumetric changes of proteins upon unfolding can be described by three terms:

$$\Delta V_{pr} = \Delta V_{hyd} + \Delta V_{cavt} + \Delta V_{ther} \quad (5)$$

where ΔV_{pr} is the net change in a protein volume upon unfolding at a given temperature, T; ΔV_{hyd} is the change in the hydration volume upon protein unfolding at T; ΔV_{cavt} is the volume of internal cavities in the native state of a protein at T; and ΔV_{ther} is the change in the volume at T, resulting from molecular vibrations in the solvent induced by the molecular vibrations in the protein.

The hydration volume is essentially related to the difference in the volume of the water molecules in a hydration shell around the protein relative to the volume in bulk water. The change in the hydration volume, ΔV_{hyd}, is defined as the difference between hydration volumes in the unfolded and native states of a protein; i.e., $\Delta V_{hyd} = V^U_{hyd} - V^N_{hyd}$. Because the buried surface of the five proteins studied here is different (as can be seen from the difference in the ΔC_p of unfolding for these proteins), the absolute value of $\Delta V_{hyd}/V_{pr}$ will depend on the protein (*28*). Furthermore, the data for the linear expansion coefficient, α, of compounds modeling protein groups (Figure 3) suggest that [keeping in mind $(1/V)(\partial V/\partial T) = \alpha$], the temperature dependencies of $\Delta V_{hyd}/V_{pr}$ will also be different because different

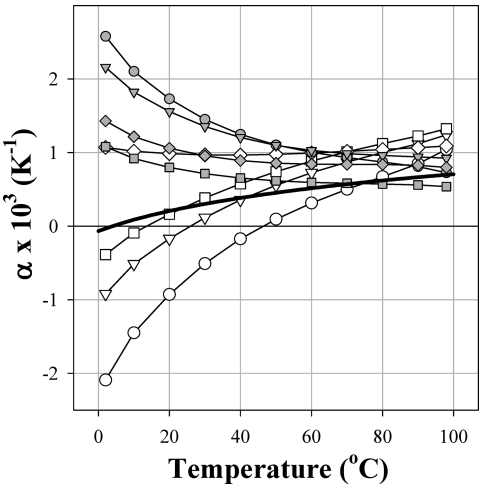

FIGURE 3: Temperature dependence of the linear thermal expansion coefficient, α, for several amino acid side chains, both nonpolar [Ala (○), Val (▽), Leu (□), and Trp (◇)] and polar [Asn (gray circles), Glu (gray triangles), Ser (gray squares), and His (gray diamonds)]. The temperature dependence of α for pure water is shown as a thick solid line. The experimental data for model compounds and for water were taken from ref *8*, and all the data were assembled as suggested by Mitra et al. (*9*).

protein groups display very different and steep temperature dependencies of the linear thermal expansion coefficient. Importantly, at high temperatures, the values of α for model compounds converge to a common value, which is very similar to the thermal expansion coefficient of water (see Figure 3 and ref *9*). This convergence of α is probably a consequence of the fact that there is little difference in the volume of water molecules in the hydration shell and in bulk water at high temperatures (*9*). Moreover, this effect is independent of the polarity of the solute. In other words, the effects of hydration do not significantly contribute to the high-temperature value of the volumetric changes, resulting in high-temperature values of ΔV_{hyd} and $\Delta V_{hyd}/V_{pr}$ that are close to zero.

The $\Delta V_{cavt}/V_{pr}$ values for Egl, Lyz, Rns, and Ubq are also likely to be very similar. This assumption is based on the early analysis of native protein structures (*29*−*33*). For example, Gerstein et al. (*34*) calculated the packing density as a ratio of the van der Waals volume to the occupied volume of amino acid residues from three-dimensional structures. They showed that most globular proteins have very similar packing densities. The molecule of Cyt might have a somewhat lower packing density because it is difficult to pack the bulky and rigid heme group. Indeed, the calculations (www.molmovdb.org/cgi-bin/voronoi.cgi) suggest that Egl (PDB entry 1ACB), Lyz (PDB entry 4LYZ), Rns (PDB entry 7RSA), and Ubq (PDB entry 1UBQ) have very similar packing efficiencies. However, these four proteins have, on average, 7−10% higher overall packing density than the molecule of Cyt (PDB entry 1AKK). Therefore, we can conclude that $\Delta V_{cavt}/V_{pr}$ values will be similar for Egl, Lyz, Rns, and Ubq and different for Cyt. Although there are insufficient data to predict the temperature dependence of $\Delta V_{cavt}/V_{pr}$, some estimates suggest that this term will have a relatively weak dependence on temperature (*2*).

ΔV_{ther} is defined as the difference between thermal volumes in the unfolded and native proteins; i.e., $\Delta V_{ther} = V^{U}_{ther} - V^{N}_{ther}$. Since V^{U}_{ther} is actually directly proportional to the isothermal compressibility of the solvent (*2*, *35*), all of the proteins studied here, including Cyt, should have similar values for V^{U}_{ther}. The proteins studied herein also have a very similar composition of surface residues in the native state and, thus, should also have very similar V^{N}_{ther} values. Consequently, the term ΔV_{ther} is expected to be rather similar for all of the proteins studied in this paper. Since there are a larger number of groups in contact with solvent in the unfolded state than in the native state, the thermal volume of the unfolded state will be larger than the thermal volume of the native state. Therefore, ΔV_{ther} will be positive and will have a positive (increasing) temperature dependence. Correspondingly, the specific value $\Delta V_{ther}/V_{pr}$ will also have a very small positive temperature dependence.

These observations allow us to formulate the following hypothesis: the $\Delta V_{pr}/V_{pr}$ functions for four proteins (Egl, Lyz, Rns, and Ubq) converge to a common value of 0.003 ± 0.001 at 140 °C (413 K) because they have very similar values of $\Delta V_{cavt}/V_{pr}$ and $\Delta V_{ther}/V_{pr}$, while at this temperature, $\Delta V_{hyd}/V_{pr}$ is very small; the $\Delta V_{pr}/V_{pr}$ function for Cyt does not converge to the same value at 140 °C because it has larger cavities and, thus, a different $\Delta V_{cavt}/V_{pr}$ term.

One way of experimentally testing this hypothesis is to create a cavity in a protein for which the value of $\Delta V_{pr}/V_{pr}$ converges to the common value at 140 °C. If the hypothesis is correct, a protein with cavity-creating amino acid substitutions will have a different temperature dependence of its $\Delta V_{pr}/V_{pr}$ function and, importantly, will have a different (higher) value of $\Delta V_{pr}/V_{pr}$ at the convergence temperature. To test our hypothesis, we studied two variants of Egl that have valine to alanine substitutions in the buried core of this protein (Egl-V14A and Egl-V54A) (*12*), which should create a cavity in the Egl core. Depending on the estimates (*30*, *36*), the side chain of Ala has a van der Waals volume that is ~23−38 Å^3 smaller than that of the side chain of Val. Furthermore, the volume that Ala occupies in proteins is typically 40−50 Å^3 smaller than that of Val (*30*). This means that the Val-to-Ala substitution creates a cavity of at least 40−50 Å^3 in the protein interior.

Figure 2 shows the experimentally measured values of $\Delta V_{pr}/V_{pr}$ for the two cavity-creating variants of eglin c, Egl-V14A and Egl-V54A. Several notable features can be observed. First, the $\Delta V_{pr}/V_{pr}$ values for the Egl-V14A and Egl-V54A variants are very similar and have similar temperature dependencies. This is expected because similar substitutions in the core should create cavities of approximately the same size.

Second, the temperature dependence of $\Delta V_{pr}/V_{pr}$ for the Egl-V14A and Egl-V54A variants is steeper than that for Egl-WT [Figure 3; $\Delta\alpha_{WT} = (10 \pm 1) \times 10^{-5}$ K^{-1}, and $\Delta\alpha_{V\rightarrow A} = (27 \pm 3) \times 10^{-5}$ K^{-1}]. This result is expected on the basis of the temperature dependence of the thermal expansion coefficient, α, of model compounds. The slope of $\Delta V_{pr}/V_{pr}$ versus T_m is the change in α upon protein unfolding; i.e., $\Delta\alpha = \alpha_U - \alpha_N$. Consequently, Δα includes a contribution for the changes in α due to the hydration of protein groups that are exposed to solvent upon unfolding. To a first approximation, the difference in Δα for single-site Val-to-Ala variants, relative to that of the wild type, is due to the difference in α between these two side chains. Figure 3 shows that the absolute value of α for Ala is larger than for Val in the temperature range where $\Delta V_{pr}/V_{pr}$ values are experimentally determined which is consistent with a larger value of Δα for Egl-V14A and Egl-V54A, relative to that of wild-type Egl.

Finally, the values of $\Delta V_{pr}/V_{pr}$ for Egl-V14A and Egl-V54A extrapolated to 140 °C are 0.016 ± 0.002 (Figure 2), which is larger than the convergence values of $\Delta V_{pr}/V_{pr}$ at this temperature for Egl, Lyz, Rns, and Ubq by 0.013 ± 0.004. This

observation supports our hypothesis that, at the convergence temperature (~140 °C), the volume change upon hydration, $\Delta V_{hyd}/V_{pr}$, is close to zero, and therefore, the value of $\Delta V_{pr}/V_{pr}$ at the convergence temperature is defined by the sum of $\Delta V_{cavt}/V_{pr}$ and $\Delta V_{ther}/V_{pr}$, which are very similar for Egl, Lyz, Rns, and Ubq. In particular, the substitutions created in the Egl-V14A and Egl-V54A variants created cavities that affected the $\Delta V_{cavt}/V_{pr}$ term, because additional or larger cavities should increase the value of $\Delta V_{cavt}/V_{pr}$.

The hypothesis can be further tested by comparing structural and thermodynamic data. Structurally, we can calculate the expected changes in the volume of internal cavities in a molecule of eglin C due to the Val-to-Ala substitutions. The thermodynamic data shown in Figure 2 can also be converted to the changes in the internal cavity volumes of the Egl-V14A or Egl-V54A variant relative to the wild type by converting the difference in specific values of $\Delta\Delta V_{cavt}/V_{pr}$ at the convergence temperature (140 °C) for the WT and variants to the absolute values, i.e., $\Delta\Delta V_{cavt}$(Val−Ala). This can be done by multiplying the $[\Delta\Delta V_{cavt}(\text{Val}-\text{Ala})]/V_{pr}$ value of 0.013 by the partial molar volume of eglin C (6760 cm^3/mol or 11230 $Å^3$), which gives a $\Delta\Delta V_{cavt}$(Val−Ala) value of 150 ± 40 $Å^3$ for the size of the cavity created by the Val-to-Ala substitutions. This value of $\Delta\Delta V_{cavt}$(Val−Ala) can be compared with the value of $\Delta\Delta V_{cavt}$(Val−Ala) obtained from the structure-based modeling, which gives a value between 50 $Å^3$ (V54A) and 100 $Å^3$ (V14A). The good correspondence between the experimentally estimated thermodynamic values of $\Delta\Delta V_{cavt}$(Val−Ala) and those calculated from geometrical structure-based principals further supports the hypothesis that the high-temperature convergence of the volumetric changes upon unfolding of several proteins is primarily due to the sizes of the internal cavities of these proteins.

Since it appears that the high-temperature volumetric changes are primarily defined by differences in the sizes of internal cavities in proteins, it should be possible to use this approach to estimate the sizes of cavities in proteins that are in excess of the average packing defects found in typical globular proteins such as Egl, Lyz, Rns, and Ubq. For example, the convergence value of $\Delta V_{pr}/V_{pr}$ for Cyt is 0.027 ± 0.002, which corresponds to 0.024 ± 0.004 of excess cavity volume. Considering that the partial molecular volume of Cyt is 16110 $Å^3$, this results in an excess cavity volume of 390 ± 60 $Å^3$ for this protein, which is comparable to the estimates of net cavity size in this protein based on structural modeling. Indeed, the geometrical structure-based molecular volume of the holoprotein is 12110 $Å^3$, and the molecular volume of apocytochrome *c* (heme removed from the structure of the holo form) is 11210 $Å^3$; the volume of the heme itself is 420 $Å^3$. This means that the incorporation of the heme group into holocytochrome *c* results in an excess cavity volume of 480 $Å^3$ (obtained as 12110 $Å^3$ − 11210 $Å^3$ − 420 $Å^3$), which is in good agreement with the experimental estimate of 390 ± 60 $Å^3$ determined from the high-temperature values of $\Delta V_{pr}/V_{pr}$.

CONCLUDING REMARKS

Although there are several different computational approaches for estimating the sizes of internal cavities or voids in proteins, there are no experimental approaches available to confirm the outcome of these calculations. The results presented in this paper highlight the potential of using PPC to determine the total volume of internal voids and cavities in proteins. The high-temperature convergence of the volumetric changes upon unfolding for four of five of the proteins studied here suggests that most globular proteins are packed similarly well, and the value of $\Delta V_{pr}/V_{pr}$ at 140 °C is primarily determined by the volume of internal voids and cavities in the protein (V_{cavt}). The larger values of $\Delta V_{pr}/V_{pr}$ for Cyt and the Egl-V14A and Egl-V54A variants support this hypothesis. In fact, the differences in volume based on the high-temperature estimates for these proteins are in excellent agreement with the values obtained with structure-based calculations, providing further support to the idea that it is possible to use PPC to measure the total volume of cavities and voids within globular proteins. Finally, it is interesting that the globular proteins appear to have evolved such a universal temperature dependence of the volumetric change upon unfolding. Does this reflect the possible evolutionary history or benefits, or is it merely a consequence of the tight packing of the protein core and properties of water? More data will be required to formulate a plausible hypothesis regarding this universal specific volume convergence upon unfolding of globular proteins.

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