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Probing the Physical Determinants of Thermal Expansion of Folded Proteins

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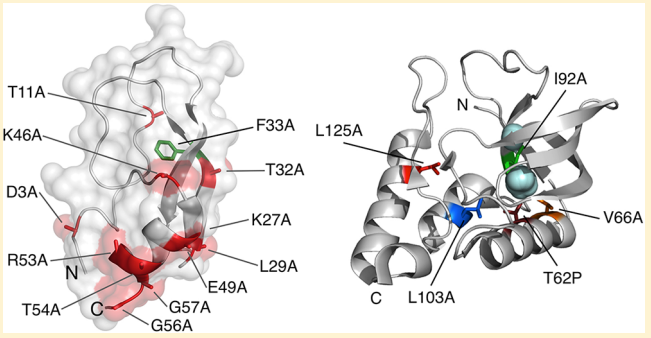
Supporting Information

ABSTRACT: The magnitude and sign of the volume change upon protein unfolding are strongly dependent on temperature. This temperature dependence reflects differences in the thermal expansivity of the folded and unfolded states. The factors that determine protein molar expansivities and the large differences in thermal expansivity for proteins of similar molar volume are not well understood. Model compound studies have suggested that a major contribution is made by differences in the molar volume of water molecules as they transfer from the protein surface to the bulk upon heating. The expansion of internal solvent-excluded voids upon heating is another possible contributing factor. Here, the contribution from hydration density to the molar thermal expansivity of a protein was examined by comparing bovine pancreatic trypsin inhibitor and variants with alanine substitutions at or near the protein−water interface. Variants of two of these proteins with an additional mutation that unfolded them under native conditions were also examined. A modest decrease in thermal expansivity was observed in both the folded and unfolded states for the alanine variants compared with the parent protein, revealing that large changes can be made to the external polarity of a protein without causing large ensuing changes in thermal expansivity. This modest effect is not surprising, given the small molar volume of the alanine residue. Contributions of the expansion of the internal void volume were probed by measuring the thermal expansion for cavity-containing variants of a highly stable form of staphylococcal nuclease. Significantly larger (2−3-fold) molar expansivities were found for these cavity-containing proteins relative to the reference protein. Taken together, these results suggest that a key determinant of the thermal expansivities of folded proteins lies in the expansion of internal solvent-excluded voids.



INTRODUCTION

The effects of temperature on protein stability are relatively well understood.[1−4] In contrast, the physical basis for the effects of pressure on protein folding has remained elusive. Most proteins can be unfolded by pressures of a few hundred MPa because the volume of the unfolded state is smaller than that of the folded state.[5] Although it is large enough to be responsible for pressure induced unfolding, the volume change between the folded and unfolded state (ΔV_u) is quite small, <1% of the molar volume of proteins. Recently, we reported that, unlike the change in heat capacity, ΔC_p, the magnitude of ΔV_u is not correlated with the size of the protein or with the nature (polar or apolar) of the surface area exposed to solvent upon unfolding.[6] Instead, it is the volume of solvent-excluded cavities within a protein's interior that determines the value of ΔV_u.[7] Despite this qualitative level of understanding, it is not yet possible to predict from sequence or from structure the volume difference between folded and unfolded proteins. In part, this is because the value of ΔV_u is a very strong function of temperature. The temperature dependence of ΔV_u arises because the difference in the thermal expansion between the folded and unfolded states, ΔE_u, is significant.

The phase diagram for two-state protein folding in the temperature−pressure plane can be approximated by the expansion of the expression for the free energy change upon unfolding, ΔG_0, around a reference temperature, T_0 and pressure, P_0, as follows:[8−10]

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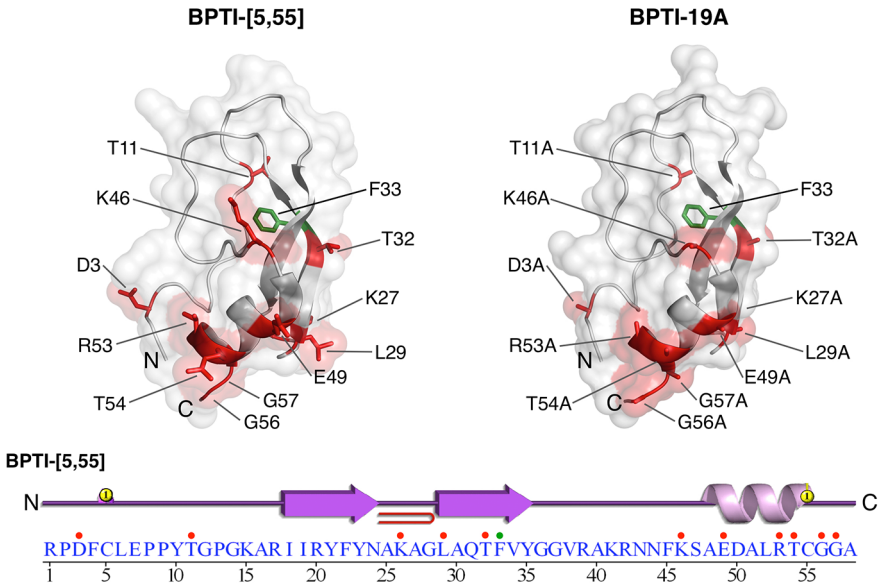

Figure 1. Representation of the structures of BPTI-[5−55] and the simplified variant BPTI-19A. BPTI-19 (ID: 3AUB), containing 19 alanines, corresponds to BTPI-[5,55] (ID: 2ZVX) with 11 residues mutated to alanine. The 11 residues mutated to alanine are colored red and labeled with sticks on both structures and signaled with dots on the protein sequence of BPTI-[5−55].[31] The position of F33 is labeled green and represented with sticks.

$$\Delta G(p, T) = \Delta G_0 - \Delta S_0(T - T_0) + \Delta C_p [T(\ln(T/T_0) - 1) + T_0] + \Delta V_0(P - P_0) + (\Delta\beta'/2)(P - P_0)^2 + \Delta E(p - p_0)(T - T_0) \quad (1)$$

This equation takes the form of an ellipse, in accordance with experimental observation, if the inequality, $-\Delta E^2 > \Delta C_p \Delta\beta'/T_0$, holds. The thermodynamic standard parameters, standard entropy (ΔS_0), enthalpy (ΔH_0), and volume (ΔV_0) changes between the folded and unfolded states, determine the position of the ellipse in the $P-T$ plane, and are given with respect to a reference temperature (T_0) and atmospheric pressure (p_0). The shape of the ellipse is determined by the higher order terms, ΔC_p (the change in heat capacity), $\Delta\beta'$ (the change in isothermal compressibility ($\beta' = (\partial V/\partial p)_T$), and ΔE (where the molar expansivity is $E = V\alpha$, and the coefficient of thermal expansion is $\alpha = 1/V(\partial V/\partial T)_P = E/V$). Note that because the change in volume upon unfolding of a protein is less than 1% of the total molar volume, one can make the approximation that $\Delta\alpha_u = (1/V)(\Delta E)$. Since the value of the molar expansivity, E ($= V\alpha$), for the unfolded state is larger than that of the folded state over most of the accessible temperature range, even proteins that exhibit large and negative ΔV_u values at low temperature can have positive ΔV_u values at higher temperatures. A true understanding and comprehensive exploitation of pressure effects on proteins will require detailed knowledge of the factors governing the magnitude of ΔE_u.

Pressure perturbation calorimetry (PPC) measures the heat change upon small isothermal pressure jumps (5 bar), from which one can calculate the thermal expansion coefficient of the partial volume of a solution (α).[11] The temperature dependence of α for solutions of representative amino acids was experimentally determined using individual amino acids and tripeptides.[11,12] The large and positive thermal expansion coefficients of polar side chains at low temperature were interpreted as arising from electrostriction of solvent, leading to a decrease in density, that is, an increase in volume ($\alpha > 0$) upon heating, as the interactions between solvent and solute are diminished. In contrast, the large and negative expansion coefficients of apolar side chains also at low temperature was suggested to arise from the ice-like structures (of lower density) formed by water around these groups at low temperature, that essentially "melt" upon heating, leading to a decrease in volume ($\alpha < 0$). These observations are generally accepted in the field, although we note that recent PPC studies of hydrophobic compounds have called into question even the sign of the hydrophobic hydration contribution to α.[13]

Unfolded proteins present rather similar α-vs-T profiles and relatively high α values at 10 °C ($\alpha_{(10\ °C)}$ = 1.5−2.0 0 × 10^{-3} K^{-1}). Recently, the temperature dependence of α for unfolded proteins has been parametrized as a linear volume-weighted and volume-normalized sum of the $\alpha(T)$ values from the constitutive amino acids.[14] In this model, for unfolded proteins of varying sequence composition, it is assumed that the molar expansivities, E ($= V\alpha$), of the constitutive amino acids are additive. Moreover, the experimental expansion coefficient and molar volume values for glycine were used to approximate the contribution of the peptide backbone. In contrast to the rather similar α-vs-T profiles for unfolded proteins, those observed for folded proteins vary widely, from 0.4 to 2.0 × 10^{-3} K^{-1} at 10 °C, with strong to no temperature dependence, depending on the protein and despite similar molar volumes for the different proteins tested.[11,15−20] For example, the coefficient of thermal expansion of the folded form of a highly stable variant of staphylococcal nuclease is significantly smaller than that of the true wild type (WT) nuclease, and unlike the WT, totally temperature-independent, and this despite the nearly identical structures and external polarity of the two proteins and only a modest difference in molar volume.[19] Moreover, single amino acid substitutions in this SNase variant with internal ionizable residues also significantly altered the α-vs-T profiles compared with the reference protein of nearly identical molar volume.[19] Here, we used a systematic mutational analysis on two model protein systems, variants of BPTI and SNase, to assess the

contributions of hydration and solvent-excluded internal void volume to the thermal expansion of proteins, the temperature dependence of the thermal expansion, and the differences in thermal expansion of folded and unfolded proteins.

MATERIALS AND METHODS

Proteins. The background protein, BPTI-[5,55]A14GA38 V, is a variant of BPTI-[5,55],[21] with the two stabilizing substitutions at residues A14G and A38 V.[22] In this study, it is referred as [5,55]. BPTI-19 (containing 19 alanines and referred to as 19A in this study) corresponds to BTPI-[5,55]A14GA38 V with 11 residues mutated to alanine (Figure 1).[23] [5,55]-F33A and 19A-F33A correspond to the previously described variants with the additional single alanine substitution at position F33. Proteins were produced, expressed, and purified as described previously.[23] Cysteines were air-oxidized in denaturant conditions at room temperature to form the intramolecular disufide bond between C5 and C55 residues. The protein fusion partner, a His-tagged TrpΔLE leader, was cleaved by cyanogen bromide treatment and removed by dialysis in 20 mM phosphate buffer, pH 6. After reversed phase HPLC, purification proteins were lyophilized and stored frozen until use. The protein identities were confirmed by mass spectroscopy. Protein concentrations were calculated by recording absorbance at 280 nm and using 5.96 mM^{-1}·cm as the molar extinction coefficient for all variants. The SNase WT; reference Δ+PHS variant; and the cavity containing variants L125A, V66A, I92A, and L103A; and the partially or fully unfolded T62P variants of these were expressed and purified as previously described.[7]

CD Measurements. Spectra were recorded using a Chirascan CD spectrometer in sodium citrate phosphate, pH 6.5 or 3.6 for BTPI proteins and sodium phosphate, pH 7.0 for SNase proteins at 20 °C. All samples were at a concentration of 10 μM. Spectra molar normalization and subtraction were carried out using Pro Fit 6.2.9 (QuantumSoft) software.

PPC and DSC Measurements. All experiments were performed as described previously[19] in a VP-DSC-PPC MicroCal, Northampton, MA, USA, calorimeter. Absolute compression and decompression heats for the buffer−buffer, water−water, and buffer−water were integrated, averaged, and subtracted to the buffer−sample heats as described previously.[11] All samples were scanned twice in the DSC to check for reversibility, which was generally between 70 and 90%, and losses are assumed to be due only to aggregation that occurred at temperatures well beyond the unfolding transition and to a rapid decrease in the temperature in the reverse scans. Protein samples were dialyzed extensively with at least two changes of more than 200 times the sample volume and then degassed. Concentrations for all proteins were in the range of 2−8 mg/mL to avoid protein−protein interactions while ensuring a reasonable signal. For all cases, the partial specific volume of the solute (V) used was 0.73 mL·g^{-1} for the BPTI variants[24] and 0.754 mL·g^{-1} for the SNase.[12] Between measurements, the calorimeter was cleaned with detergent at 60 °C and washed with at least 500 mL of pure water, as described by MicroCal. Data analysis was performed using the Origin data analysis package provided by MicroCal and plotted with pro Fit 6.2.9 (QuantumSoft). The buffer solution was 50 mM sodium citrate phosphate at pH 3.6 or pH 6.5. DSC measurements at a scan rate of 1 °C/min were carried out under the same buffer conditions and protein treatment as for PPC, but at 1 mg/mL of protein concentration; refer to ref 19 for more details. The thermal expansion coefficients of the protein (α) were calculated from measurement of the heat released (ΔQ) upon a small pressure perturbation (Δp) as described by Lin and co-workers:[11]

$$\alpha = \alpha_{solv} - \left(\frac{\Delta Q}{TV_{cell}\Delta p}\right) \tag{1A}$$

where V_{cell} is the active volume of the cell; (α_{solv}), the thermal expansion coefficient of the solvent; and T, the temperature in Kelvin. Molar expansivities were back-calculated by multiplying the expansion coefficient by the molar volume.

Modeling the α-vs-T Profiles of Protein Unfolded States. Here, it is assumed as previously published by the Makhatadze group[14] that the partial molar volume of an unfolded polypeptide chain can be empirically approximated by the sum of the partial molar volumes of its constitutive amino acids. Hence, the temperature profile of the thermal expansion coefficient can be approximated as a volume-weighted sum of the expansivities ($E = V\alpha$) of the individual side chains and peptide bonds, normalized to the total volume as follows:

$$\alpha(T) = \frac{\sum_{i=1}^{N} v_i(T)\,\alpha_i(T)}{\sum_{i=1}^{N} v_i} \tag{2}$$

where the α_i values for the side chains are taken from Lin et al.[11] and N-1 glycine residues are added to account for the contribution from the peptide backbone. The temperature dependence of the volumes of each of the individual side chains and peptide backbone contributions are calculated from the partial molar volume of each side chain at 25 °C, as given in Lin et al.[11] as follows:

$$v_i(T) = v_i(T_0)\exp\left(C_i + a_iT + \frac{b_iT^2}{2} + \frac{c_iT^3}{3} + \frac{d_iT^4}{4}\right) \tag{3}$$

$$C_i = -\left(a_iT + \frac{b_iT^2}{2} + \frac{c_iT^3}{3} + \frac{d_iT^4}{4}\right) \tag{4}$$

Molar volumes for each of the side chains as well as the temperature-dependent expansion coefficients are also taken from Lin et al.,[11] as described by Schweiker et al.[17] Further details of the calculation for unfolded proteins can also be found in ref 11.

RESULTS

Hydration Contributions to Thermal Expansivity Probed with Surface Alanine Variants of BPTI. To probe the contribution of a protein's surface polarity and hydration to its thermal expansivity and to the magnitude of the difference in thermal expansion (ΔE) between the folded and unfolded states of proteins, we used a model protein system, a variant of bovine pancreatic trypsin inhibitor with a single disulfide bond (BPTI-[5,55]A14GA38 V),[22,25] which we will call [5,55] in which eleven surface or partially buried residues were replaced with alanine (BPTI-19, referred to here as 19A) (Figure 1).[21] We note that the 19A variant has an overall global stability similar to that of the reference variant, [5,55].[21] The X-ray crystal structures of both the [5,55] and 19A variants indicated that their backbone structures are nearly identical, with RMSD around 0.5A, and that changes in the side chain

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conformation were limited to a restricted number of residues.[26] Similar structure−stability studies of multiple alanine replacement have been reported in the context of T4 lysozyme.[27] The effect of the eleven alanine substitutions on the temperature-dependent molar expansivity of the unfolded states of the [5,55] and 19A proteins was examined using variants of each bearing one additional mutation in the hydrophobic core (F33A), which leads to full or partial unfolding of the protein under native conditions.

CD spectra (Figure 2A) of the BPTI variants bearing the F33A destabilizing mutation reveal that the 19A variant is fully

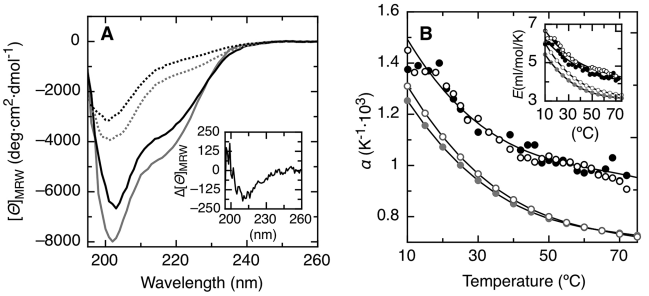

Figure 2. Comparison between experimental and calculated expansivity of the unfolded variant of BPTI protein F33A. (A) CD spectra of BPTI-[5,55] (black solid line), BPTI-[5,55]-F33A (black dotted line), BPTI-19A (gray solid line), and BPTI-19A-F33A (gray dotted line), carried out at pH 6.5 and 20 °C. Inset: CD spectral difference of [5,55]-F33A carried out at pH 6.5 minus pH 3.6. (B) Experimental PPC coefficient of partial molar expansivity values are in black and calculated values are in gray for BPTI-[5,55]-F36A (open circles) and BPTI-19A-F36A (solid circles) proteins. Inset: corresponding experimental and calculated PPC molar expansivity values. Measurements were carried out in 50 mM sodium citrate phosphate pH 3.6. Lines are third-order polynomial data fits.

unfolded at both pH 3.6 and 6.9, whereas the more stable [5,55] variant is unfolded only at pH 3.6. In the model amino acid studies previously reported in ref 11, the α values at low temperature for the alanine side chain were large and negative. However, the magnitude of the effect of the alanine replacements is expected to be attenuated by the small volume of the alanine side chain, since it is the molar expansivities ($E = \alpha \times V$), not the thermal expansion coefficients that are additive. We report both the α values (the norm in the PPC field) and the E values, since these are extensive parameters and, hence, are additive. The magnitude of the effect of each alanine substitution should depend as well on the thermal expansion coefficient and the molar volume of the side chain that is replaced. Of the 11 alanine substitutions, 8 replaced a charged or polar residue (Figure 1), 7 of which are rather large (threonine, lysine arginine, and glutamate), and two replaced a glycine. Only one, L29A, replaced a hydrophobic residue by alanine. Figure 2B shows that a small decrease in the α and E values at low temperatures is expected from modeling the 11 alanine substitutions in the unfolded state of the [5,55] and 19A proteins using the approach of the Makhatadze group,[14] (see Material and Methods) (Figure 2). Although the experimental α-vs-T profiles for the unfolded 19A and [5,55] variants at this low pH are found to be identical (Figure 2B), correction for the difference in molar volume in the E-vs-T profiles brings to light a small decrease in expansivity for the alanine-bearing variant. The observed decrease is slightly smaller than the change predicted by the empirical algorithm of Makhatadze.[14]

There is no empirical model for the expected effect of the alanine substitutions on folded proteins. PPC experiments on the folded forms of [5,55] and 19A (Figure 3) showed that the

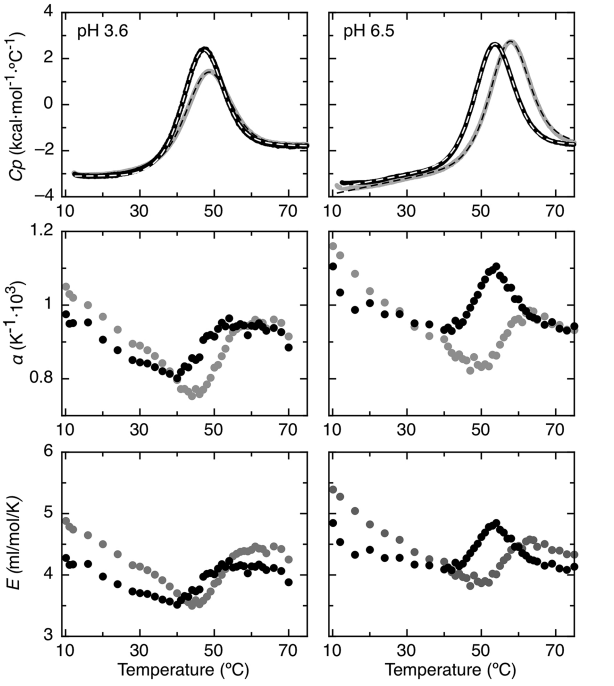

Figure 3. Effect of multiple alanine substitutions on the expansivity profile of BPTI. DSC (top row), PPC coefficient of thermal expansion (middle row) and PPC molar expansivity (bottom row) profiles of [5,55] (gray lines and symbols), and 19A (black lines and symbols) at pH 3.6 (left) and 6.5 (right). The dashed lines represent fits to a two-state unfolding model. DSC and PPC measurements were carried out in 50 mM sodium citrate phosphate buffer using 1 or 6 mg/mL protein concentrations, respectively.

external alanine substitutions led to a small (∼10%) decrease in the E values in the temperature range over which the proteins are folded at pH 3.6 and 6.5. The observed decrease was on the order of that expected for the unfolded forms of the proteins. No difference in α value and a small difference in E between the two variants were observed at temperatures above the unfolding transition. Integration of the plots of α-vs-temperature over the range of the thermal unfolding transition (defined by the DSC profiles) yields the values of $\Delta V_u(T_m)$, the volume change of unfolding (Table 1). Note the very small values of $\Delta V_u(T_m)$ for these proteins and also that near neutral pH, the value of $\Delta V_u(T_m)$ for the 19A variant is positive.

Comparison of the folded and unfolded forms of the [5,55] and 19A variants revealed higher E values (Figure 4 A,C) and α values (Figure 1) for the unfolded proteins of both variants compared with the folded ones at pH 3.6 over the entire range of temperatures under which the [5,55] and the 19A BPTI variants are stably folded. Above the T_m for the folded [5,55] and 19A proteins, their E values and α values are equivalent to those of the unfolded variants bearing the F33A substitution. We note that the relative differences between the E values and α values between folded and unfolded forms of the proteins are the same because the difference in molar volume is negligible for the F33A substitution, which leads to unfolding. Similar

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Table 1. Thermodynamic Parameters Obtained from the PPC and DSC Experiments on the BPTI Variants, [5,55] and 19A[a]

		DSC			PPC	
protein	pH	T_m (°C)	$\Delta H(T_m)$ (kcal·mol^{-1})	ΔC_p (kcal·°C^{-1}·mol^{-1})	$\alpha_{10\ °C}$ (K^{-1}·10^3)	$\Delta V_u(T_m)$ (mL·mol^{-1})
[5,55]	3.6	48.5 ± 0.1	62.9 ± 0.1	1.3 ± 0.1	1.05 ± 0.05	−2.6 ± 1.0
19A	3.6	47.2 ± 0.1	68.6 ± 0.1	1.3 ± 0.1	1.02 ± 0.05	+2.5 ± 1.0
[5,55]	6.5	57.8 ± 0.1	61.4 ± 0.1	1.0 ± 0.1	1.17 ± 0.05	−3.4 ± 0.4
19A	6.5	53.6 ± 0.1	67.1 ± 0.1	1.0 ± 0.1	1.12 ± 0.05	+8.9 ± 0.9

[a]DSC data was fitted to a two states unfolding model. ΔV_u was calculated by integrating PPC data on the unfolding transition window revealed by DSC experiments. Buffer solution was 50 mM sodium citrate phosphate.

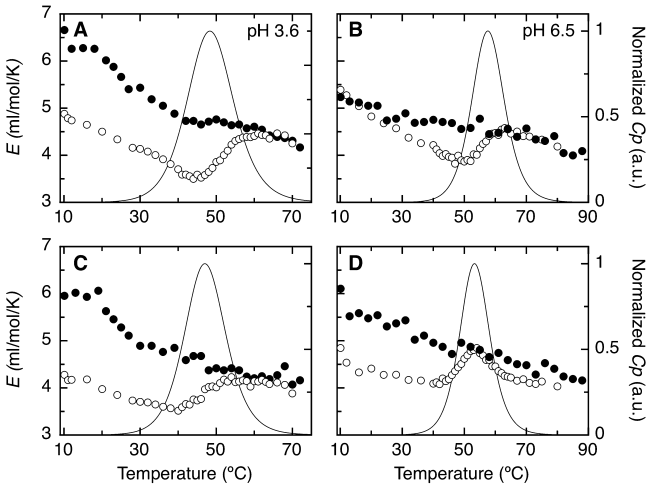

Figure 4. Effect of partial unfolding on the expansivity profile of BPTI protein. Comparison of the PPC molar expansivity temperature profiles of native BPTI-[5,55] (first row) and BPTI-19A (second row) proteins (open circles) with their F33A counterparts (solid circles). First column, PPC measured at pH 3.6; second column, PPC measured at pH 6.5. Also shown in each graph are the corresponding normalized DSC profiles (full line) for BPTI-[5,55] or BPTI-19A proteins.

results were obtained at pH 6.5 (Figure 4 B, D), except that at low-temperature, E and α are equivalent for the stable [5,55] and its F33A variant. This can be explained by the fact that under these conditions, the F33A variant of [5,55] retains a nonnegligible degree of residual structure (Figure 2A). Overall, the PPC experiments on the BPTI variants reveal that, as expected, unfolding by mutation leads to an increase in thermal expansion in the temperature range where the parent proteins are folded. In contrast, probably because of volumetric compensations, large changes to the surface polarity of proteins can be made without significant effects on their thermal expansivity in either the folded or unfolded states.

Internal Void Volume Contributions to Thermal Expansivity Probed with Cavity Variants of SNase. We next sought to probe the contribution of the expansion of the internal solvent-excluded void volume to the thermal expansion of a folded protein. We chose a series of cavity-containing variants of the highly stable Δ+PHS variant of staphylococcal nuclease (SNase) (Figure 5). Crystal structures of these variants confirmed the presence of a cavity at the positions for which leucine, valine, or isoleucine was substituted by alanine, and their volume changes for unfolding were found to be much larger in magnitude than for the reference Δ+PHS.[7] The E-vs-T profiles (Figure 6; see Figure S1 of the Supporting

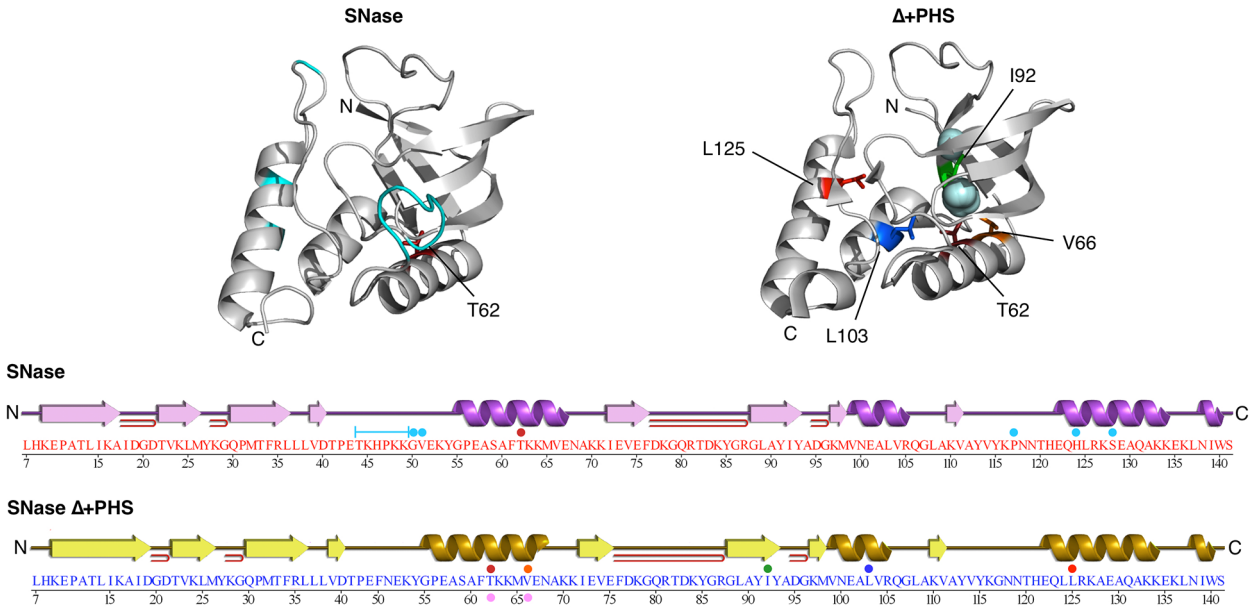

Figure 5. Representation of the structures of SNase and Δ+PHS. Models were made using coordinates from (ID:) 1SNC and (ID:) 3BDC, respectively, for the wild type SNase and the Δ+PHS variant. Residues mutated and deleted in SNase protein leading to Δ+PHS are colored cyan. The positions of single mutants studied in this work are colored burgundy for T62P, orange for V66A, green for I92A, blue for L103A, and red for L125A. The molecular surface of the internal void volumes is colored sky blue on the Δ+PHS protein.[32] Mutated residues are highlighted with colored dots on the protein sequence of SNase and Δ+PHS.[31].

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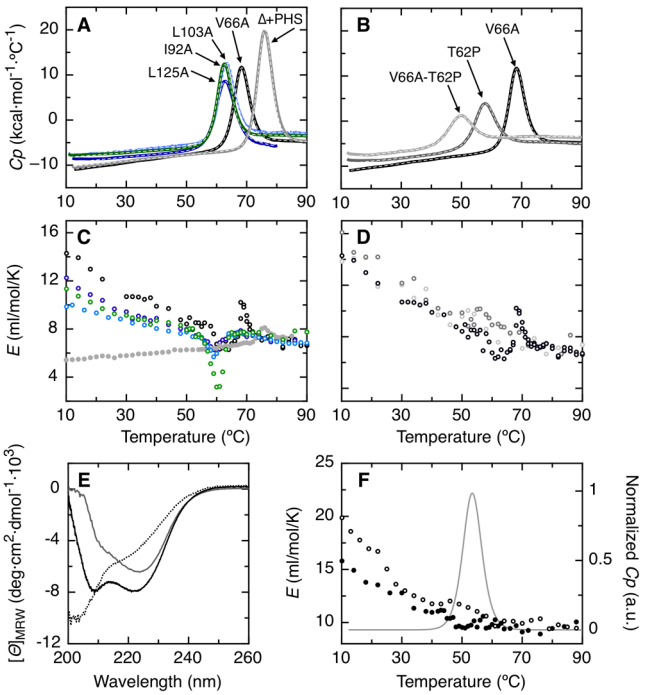

Figure 6. Effect of cavity and disorder creating mutants on the molar expansivity-vs-temperature profile of SNase. Panels A and C show the DSC (A) and PPC (C) molar expansivity vs temperature of Δ+PHS protein (gray) and the corresponding mutants V66A (black), I92A (green), L103A (sky blue), and L125A (blue). Panels B and D show the DSC (B) and PPC (D) molar expansivity-vs-temperature profiles of Δ+PHS protein mutants V66A (black), T62P (gray), and T62P-V66A (light gray). White dashed lines are fits to a two-state unfolding model of DSC data. (E) CD spectra of SNase (black), SNase protein mutant T62P (black dots) and Δ+PHS protein mutant T62P-V66A (gray). (F) PPC molar expansivity vs temperature of SNase (solid dots) and the corresponding mutant T62P (open dots). The normalized DSC profile of SNase protein is shown as a light gray line. Experiments were carried out in 50 mM sodium phosphate pH 7.0 using 0.1, 1, or 6 mg/mL protein concentrations for CD, DSC, and PPC, respectively.

Information for α values) demonstrate that the thermal expansivity of the highly stable Δ+PHS variant is very low and increases slightly with temperature prior to the unfolding transition temperature (~72 °C). This low and nearly temperature-independent thermal expansion for Δ+PHS has been previously observed and reported.[19] Hence, even a very highly charged protein such as SNase can exhibit a very small and constant thermal expansivity, very unlike the large and positive thermal expansivities of polar and charged amino acid side chains. In contrast to the Δ+PHS variant, all of the cavity-containing variants exhibited much larger (~2−3-fold) values of E in the temperature range over which they remain folded and which decrease with increasing temperature. We note that although the $\Delta V_u(T_m)$ for Δ+PHS and its V66A variant are positive at this pH,[19] those for the other cavity-containing variants are negative (Table 2).

Finally, we investigated the thermal expansion of the T62P variant of WT SNase, which is unfolded at pH 7 and 25 °C[28,29] (Figure 6E), in contrast to the same mutation in the Δ+PHS protein (Figure 6B, E). The E values of WT SNase were identical to those published previously,[12,16,19] and as expected, those of the destabilized, unfolded T62P variant in the WT background were higher in the range of 10−40 °C (Figure 6F). The E values in the temperature profiles observed for the Δ+PHS/T62P and Δ+PHS/T62P-V66A variants were significantly larger than those found for Δ+PHS (Figure 6D); however, they were essentially identical to that of the Δ+PHS/V66A variant and significantly lower than that observed for the unfolded T62P variant in the wild type background. The lack of effect of the T62P substitution in the Δ+PHS/V66A background likely arises from residual structure afforded by the stabilizing mutations in the Δ+PHS reference protein.

DISCUSSION

A fundamental understanding of the determinants for the magnitude and sign of the volume change upon protein unfolding requires knowledge of the determinants for the thermal expansivity of the different states of proteins. This is because unfolded states of proteins exhibit much higher expansivities than their folded states, making the magnitude and even the sign of the volume change for unfolding a very strong function of temperature. We found here for both the BPTI and SNase model systems and in agreement with the prior work cited above, larger E values at low temperature for unfolded and even partially unfolded states, as compared with the folded proteins. In the case of the alanine substitutions in BPTI, we observed only very modest effects in the thermal expansivity. This is not surprising, given the small molar volume of alanine, but underscores the fact that the surface polarity of a protein can be significantly perturbed without significantly changing its molar expansivity. In stark contrast with the modest effect of substituting 11 surface residues with alanine on the molar expansivity of BPTI [5,55], single alanine substitutions in the SNase protein interior led to very large

Table 2. Thermodynamic Parameters Obtained from the PPC and DSC Experiments on the SNase Variants[a]

	DSC			PPC	
protein	T_m (°C)	$\Delta H(T_m)$ (kcal·mol^{-1})	ΔC_p (kcal·°C^{-1}·mol^{-1})	$\alpha_{10\ °C}$ ($K^{-1}\cdot10^3$)	$\Delta V_u(T_m)$ (ml·mol^{-1})
SNase WT	53.5 ± 0.1	82.2 ± 0.2	2.70 ± 0.1	1.21 ± 0.05	−20.0 ± 1.0[b]
Δ+PHS	73.8 ± 2[b]	134.3 ± 0.3	2.79 ± 0.1	0.449 ± 0.05	4.0 ± 2.0
Δ+PHS T62P	57.9 ± 0.1	81.7 ± 0.3	1.76 ± 0.1	1.350 ± 0.05	N.A.
Δ+PHS V66A	68.4 ± 0.1	115.7 ± 0.1	1.75 ± 0.1	1.200 ± 0.05	2.7 ± 0.5
Δ+PHS T62PV66A	49.7 ± 0.1	59.6 ± 0.3	1.82 ± 0.1	1.163 ± 0.05	N.A.
Δ+PHS I92A	62.8 ± 0.1	109.5 ± 0.1	2.45 ± 0.1	0.953 ± 0.05	−26.8 ± 1.0
Δ+PHS L103A	63.3 ± 0.1	113.0 ± 0.1	2.12 ± 0.1	0.828 ± 0.05	−9.3 ± 1.0
Δ+PHS L125A	62.1 ± 0.1	111.7 ± 0.2	1.81 ± 0.1	1.030 ± 0.05	−7.7 ± 1.0

[a]DSC data were fitted to a two-state unfolding model. ΔV_u was calculated by integrating PPC data on the unfolding transition window revealed by DSC experiments. Buffer solution was 50 mM sodium phosphate at pH 7. [b]Data published previously.[19]

increases in the molar expansivity observed in the native temperature range. We note the 4-fold difference in the range of the expansivity in Figures 2 and 3 compared with Figure 6.

To aid in the interpretation of this finding, we looked for correlations between the molar expansivity at low temperature and a number of thermodynamic parameters. We found no correlation between the expansivity of the folded states of the SNase cavity variants and either the T_m, the ΔV_u, ΔG_u or the m value of denaturant unfolding. In contrast, a reasonable correlation was observed between the expansivity at low temperature (maximal E) and the ΔC_{pu} of these variants (Figure 7). We assume that it is the heat capacity of the folded

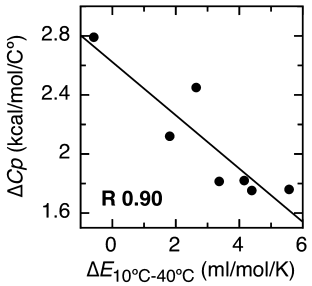

Figure 7. Negative correlation between ΔC_p and the folded state molar thermal expansivity for SNase Δ+PHS variants. Heat capacity changes are taken from Table 2, and $\Delta E = E_{10\ °C} - E_{40\ °C}$ values for Δ+PHS variants are calculated from the data in Figure 5. Lines are linear regression fits with correlation coefficients of −0.90.

states of the proteins, rather than that of their unfolded states, that would be correlated with the expansivity (enthalpy fluctuations) of their folded states. The enthalpy fluctuations of the folded state are linked to the intrinsic network of interactions that stabilize the folded state. These latter must place constraints on its thermal expansion. Unfolded proteins would be expected to have higher expansivities because these constraints have been abolished.

CONCLUSION

The value of the ΔV_u at any given temperature can be thought of as the result of several contributions: (1) some intrinsic structural term at a reference temperature, T_0, defining a theoretical amount of solvent-excluded void volume present in the folded structure (bona fide cavities or interstitial space, particularly between hydrophobic groups);[30] (2) an additional term that takes into account the possible partial occupation of this volume by solvent molecules; (3) any contribution from electrostriction, which may be significant if buried ionizable or charged groups are exposed to solvent upon unfolding; and (4) the degree to which the folded protein can expand with temperature, relative to the expansion of the unfolded state (the ΔE term). On the basis of the present results, we suggest that expansion of the internal void volume is a key contributing factor to the degree to which folded states of proteins can expand with temperature. We suspect that this expansivity is linked to the internal interaction network of the folded state. Although the quantitative and structural relationship between enthalpy fluctuations and expansivity remain to be determined, the present observations reveal that protein volumetric properties are intimately linked to their sequence specific energetic properties and underscore the usefulness of pressure perturbation for understanding protein folding.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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