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Molecular Determinants of Expansivity of Native Globular Proteins: A Pressure Perturbation Calorimetry Study

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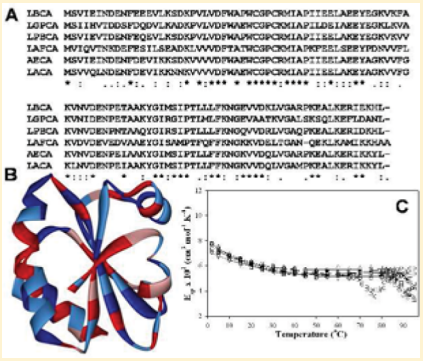
Supporting Information

ABSTRACT: There is a growing interest in understanding how hydrostatic pressure (P) impacts the thermodynamic stability (ΔG) of globular proteins. The pressure dependence of stability is defined by the change in volume upon denaturation, $\Delta V = (\partial \Delta G/\partial P)_T$. The temperature dependence of change in volume upon denaturation itself is defined by the changes in thermal expansivity (ΔE), $\Delta E = (\partial \Delta V/\partial T)_P$. The pressure perturbation calorimetry (PPC) allows direct experimental measurement of the thermal expansion coefficient, $\alpha = E/V$, of a protein in the native, $\alpha_N(T)$, and unfolded, $\alpha_U(T)$, states as a function of temperature. We have shown previously that $\alpha_U(T)$ is a nonlinear function of temperature but can be predicted well from the amino acid sequence using $\alpha(T)$ values for individual amino acids (*J. Phys. Chem. B* **2010**, *114*, 16166−16170). In this work, we report PPC results on a diverse set of nine proteins and discuss molecular factors that can potentially influence the thermal expansion coefficient, $\alpha_N(T)$, and the thermal expansivity, $E_N(T)$, of proteins in the native state. Direct experimental measurements by PPC show that $\alpha_N(T)$ and $E_N(T)$ functions vary significantly for different proteins. Using comparative analysis and site-directed mutagenesis, we have eliminated the role of various structural or thermodynamic properties of these proteins such as the number of amino acid residues, secondary structure content, packing density, electrostriction, dynamics, or thermostability. We have also shown that $\alpha_N(T)$ and $E_{N,sp}(T)$ functions for a given protein are rather insensitive to the small changes in the amino acid sequence, suggesting that $\alpha_N(T)$ and $E_N(T)$ functions might be defined by a topology of a given protein fold. This conclusion is supported by the similarity of $\alpha_N(T)$ and $E_N(T)$ functions for six resurrected ancestral thioredoxins that vary in sequence but have very similar tertiary structure.



INTRODUCTION

Hydrostatic pressure has a profound effect on protein structural stability.[1−8] The dependence of protein stability, ΔG, on hydrostatic pressure, P, is defined by the volume change upon unfolding at constant temperature, $\Delta V = (\partial \Delta G/\partial P)_T$.[9] The volume changes upon unfolding in turn show a strong dependence on temperature. This temperature dependence of the volume changes is defined by changes in thermal expansivity, $\Delta E = (\partial \Delta V/\partial T)_P$. It has been established that changes in expansivity upon unfolding are positive, which means that the expansivity of the unfolded state, E_U, is higher than the expansivity of the native state, E_N, so that $\Delta E = E_U - E_N > 0$.[9−11] This leads to an increase in volume change upon protein unfolding with an increase in temperature. As a first step in deciphering the molecular details of volume changes upon protein unfolding, it is important to understand what defines the changes in ΔE.

The development of the pressure perturbation calorimetry method allowed direct experimental measurements of the thermal expansion coefficient, α, of the proteins.[12−20] This expansion coefficient is directly related to expansivity as $E = \alpha \cdot V$. For the unfolded proteins, it has been established that the expansivity, $E_U(T)$, is a nonlinear function of temperature.[17] Furthermore, it was found that it can be well approximated by the sum of expansivities of individual amino acid residues.[17] The expansivity of the native state of proteins, $E_N(T)$, however, remains largely unexplored.[19] Here we experimentally measured the expansion coefficient and expansivity of the native state of nine different typical globular proteins in a broad temperature range from 2 °C up to 90 °C and then performed comparative analysis with the basic structural and thermodynamic properties of these proteins. In addition, we have analyzed the effect of amino acid substitutions both on the surface and buried positions of three of these proteins on the expansivity of the native state. The results suggest that the three-dimensional topology might be a defining factor of the expansivity function of the native state for globular proteins.

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MATERIALS AND METHODS

Proteins. Hen egg white lysozyme (HEWL), bovine ribonuclease A (Rns), bovine pancreatic trypsin inhibitor (BPTI), horse heart myoglobin (Mb), and bovine ubiquitin (Ubq) were purchased from Sigma-Aldrich and used without purification. Wild type and variants of eglin C (Egl), human acylphosphatase (ACP), and ancestral thioredoxins (Trx) were expressed in *E. coli* and purified as described previously.[21−25] All the recombinant proteins contained 6xHis-tags to aid purification. Purified hyperstable protein G domain (desG)[26] was provided by Dr. Tobin Sosnick. Protein concentrations were measured spectrophotometrically using the following extinction coefficients:[27] ε_{280} = 10 000 M^{-1} cm^{-1} for Rns, ε_{280} = 10 810 M^{-1} cm^{-1} for desG, ε_{280} = 38 460 M^{-1} cm^{-1} for HEWL, ε_{280} = 1280 M^{-1} cm^{-1} for Ubq, and ε_{280} = 14 440 M^{-1} cm^{-1} for the Egl variants, ε_{280} = 5480 M^{-1} cm^{-1} for BPTI, ε_{280} = 13 940 M^{-1} cm^{-1} for ACP variants, ε_{280} = 14 060 M^{-1} cm^{-1} for Trx LBCA, LGPCA, and LPBCA, ε_{280} = 15 340 M^{-1} cm^{-1} for Trx AECA and LACA, and ε_{280} = 8370 M^{-1} cm^{-1} for Trx LAFCA.

Pressure Perturbation Calorimetry (PPC). PPC experiments were performed on a VP-DSC instrument equipped with a PPC attachment (Microcal/GE-Healthcare) as described previously.[15−17] Proteins were extensively dialyzed against corresponding buffers. Buffers were chosen to minimize both enthalpy and volume changes upon ionization:[13,28,29] 30 mM glycine−HCl for pH 2−3.5 or 30 mM sodium−cacodylate for pH 5.5 and pH 7.0. Raw data from PPC experiments was processed using the Origin-PPC (OriginLab, Northampton, MA) software package to obtain the thermal expansion coefficient of a protein in solution as a function of temperature (see refs 15−17 for more details). Briefly, the thermal expansion coefficient of a protein, $\alpha(T)$, 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, $\Delta Q_{H_2O/buf}(T)$, and a buffer/protein scan, $\Delta Q_{buf/pr}(T)$, due to a change in the pressure, ΔP, of the system as

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

where v_{cell} is the volume of the calorimetric cell, c_{pr} is the concentration of protein in the calorimetric cell, and $\bar{V}_{pr}$ is the partial specific volume of protein. The partial molar volumes were calculated from the amino acid composition as described previously[30] and were found to be 0.721 cm^3/g for Rns, 0.729 cm^3/g for HEWL, 0.734 cm^3/g for Mb, 0.747 cm^3/g for Ubq, 0.741 cm^3/g for desG, 0.734 cm^3/g for the Egl variants, 0.743 cm^3/g for Trx LGPCA and LBCA, 0.741 cm^3/g for Trx LAFCA, 0.745 cm^3/g for Trx LPBCA, 0.747 cm^3/g for Trx AECA, 0.751 cm^3/g for Trx LACA, and 0.736 cm^3/g for the ACP variants.

RESULTS AND DISCUSSION

The goal of this work was to establish general features for the temperature dependence of the thermal expansion coefficient of proteins in the native state, $\alpha_N(T)$.

$$\alpha_N(T) = \frac{1}{V_N}\left(\frac{\partial V_N}{\partial T}\right)_p \quad (2)$$

where V_N is the partial molar volume of a protein in solution. The $\alpha_N(T)$ parameter can be measured experimentally using pressure perturbation calorimetry (PPC).[15] Nine different proteins have been used: bovine pancreatic trypsin inhibitor BPTI, hyperstable protein G, ubiquitin, eglin c, acylphosphatase, ancestral thioredoxins, ribonuclease A, hen egg white lysozyme, and horse skeletal myoglobin. These proteins have been well studied by various biophysical methods, and their three-dimensional structures are well-known. This protein set represents an extensive variation in different biophysical properties.[28,31,32] Proteins vary in size from 58 amino acid residues in BPTI to 153 amino acid residues in Mb and in the extent of disulfide bonds (1 in Trx, 3 in BPTI, 4 in Rns and HEWL, and none in Mb, Ubq, Acp, Egl, and desG). The secondary structure content also varies from all α-helical Mb to mixed α/β Ubq, desG, Acp, and Trx, to all-β Rns. Proteins used in this study also differ in their thermodynamic stability, as measured by the Gibbs energy changes upon unfolding (ΔG) and in thermostability, as measured by the transition temperature (T_m).

The $\alpha_N(T)$ and $E_{N,sp}(T)$ Functions for Different Typical Globular Proteins. The thermal expansion coefficients of proteins in the native state as a function of temperature, $\alpha_N(T)$, have been measured experimentally for these nine different proteins using pressure perturbation calorimetry (see the Materials and Methods section). For each protein, at least five independent experiments in the pH range from 2.0 to 7.0 were performed and averaged values for $\alpha_N(T)$ were calculated. The proteins we studied differ in their thermostability. As a result, for some proteins, we were only able to obtain $\alpha_N(T)$ in a relatively narrow temperature range from 2 °C up to 45−50 °C. However, for very thermostable proteins such as BPTI[33] or Trx,[24] that have unfolding temperatures at above 95 °C, a much broader temperature range, up to ∼90 °C, was experimentally accessible. Comparison of the results of $\alpha_N(T)$ measurements for nine different proteins is presented in Figure 1A. However, considering that these proteins have different molecular masses, it is more appropriate to compare the specific expansivity calculated per amino acid residue, $E_{N,sp}(T)$, that is calculated from $\alpha_N(T)$ as

$$E_{N,sp}(T) = \frac{E_N(T)}{N_{aar}} = \frac{\alpha_N(T)\cdot V_N}{N_{aar}} \quad (3)$$

where $E_N(T)$ is the molar expansivity and N_{aar} is the number of amino acid residues in a protein. Specific expansivity $E_{N,sp}(T)$ as defined by eq 3 is independent of protein size, and that allows us to make a direct comparison even though the size of proteins is very different (the smallest of which, BPTI, has 58 amino acid residues while HEWL is twice as large, and Mb is almost 3 times larger than BPTI). A comparison of $E_{N,sp}(T)$ values is presented in Figure 1B. Several general observations can be made. First, both the $\alpha_N(T)$ and $E_{N,sp}(T)$ values are different for these different proteins reflecting the intrinsic variation in these parameters. It seems that there is no meaningful correlation of $\alpha_N(T)$ and $E_{N,sp}(T)$ with protein size. BPTI (58 amino acid residues) and Mb (153 amino acid residues) are the smallest and largest proteins in the studied set, respectively, yet the $E_{N,sp}$(2 °C) for these two proteins are identical, while HEWL (129 amino acid residues) and ACP (99 amino acid residues) have the lowest and highest $E_{N,sp}$(2 °C) values, respectively. The values of $E_{N,sp}$(2 °C) do not appear to depend on the type of secondary structure either. All-α Mb has very

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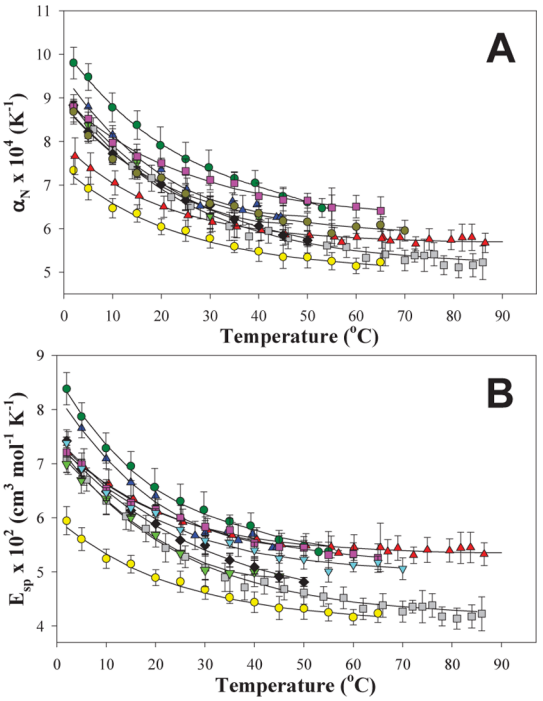

Figure 1. Temperature dependence of the thermal expansion coefficient $\alpha_N(T)$ (A) and specific expansivity $E_{N,sp}(T)$ (B) for the native states of HEWL (yellow ●), BPTI (gray ■), Rns (green ▼), Ubq (black ◆), Egl (blue ▲), ACP (green ●), Trx (red ▲), Mb (light blue ▼), desG (pink ■). The symbols are experimentally measured values, and the lines are fits to an exponential decay function $\alpha_N(T) = A + B \exp(-C \cdot T)$. See the Materials and Methods section for details.

similar $E_{N,sp}(2\ ^\circ C)$ values as largely all-β Rns or mixed α/β Trx. Expansivity could also depend on the density of the solvent. In the case of proteins, the relevant parameter is the packing density that is defined as the ratio of van der Waals volume to the total volume of a protein in the native state.[34−36] This parameter was calculated for studied proteins using their known three-dimensional structure. We found no correlation between packing densities and values of α_N and $E_{N,sp}$ at 2 or 50 °C temperatures (see Figure S1 in the Supporting Information). Finally, there is no correlation between values of $E_{N,sp}$ and stability or thermostability of the proteins. Hyperthermostable proteins such as desG, BPTI ,and Trx that have transition temperatures above 90 °C do not stand out in their $E_{N,sp}(2\ ^\circ C)$ or $E_{N,sp}(50\ ^\circ C)$ from the less thermostable proteins (Figure 1B). We noted, however, that the proteins that have disulfide bridges, and in particular HEWL (4 disulfide bonds), Rns (4 disulfide bonds), and BPTI (3 disulfide bonds), have on average lower values of $E_{N,sp}(T)$ at all temperatures.

Second, the dependences of $\alpha_N(T)$ and $E_{N,sp}(T)$ on temperature are remarkably nonlinear (Figure 1). It appears that there is a steep nonlinear decrease in $\alpha_N(T)$ and $E_{N,sp}(T)$ until ∼50 °C followed by a gradual leveling off above 60 °C. The nonlinear dependence of expansion coefficient and leveling off was also observed for unfolded proteins.[17] In this case, however, the absolute values of the $\alpha_U(T)$ function for unfolded proteins are much higher than $\alpha_N(T)$, consistent with the experimental observation of the positive values for the changes in expansivity upon unfolding.[10,16] Interestingly, such leveling off for the $\alpha(T)$ function was observed for the $\alpha(T)$ of various polar amino acid side chains.[11,15] All of this suggests that hydration plays a significant role in this nonlinear behavior.

Overall, the experimental data establishes the expected range of values and the temperature dependence that the $\alpha_N(T)$ and $E_{N,sp}(T)$ functions can have for typical globular proteins. Next we asked the question of what are the effects of amino acid substitutions on the $\alpha_N(T)$ and $E_{N,sp}(T)$?

Effects of Surface Substitutions on the $\alpha_N(T)$ and $E_{N,sp}(T)$. Figure 2 compares the PPC profiles of the wild-type

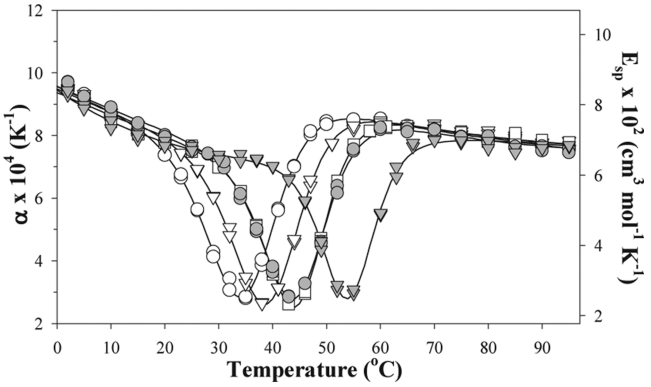

Figure 2. Comparison of $\alpha(T)$ and $E_{sp}(T)$ for the wild type ACPwt (open circles, pH 3.0; open squares, pH 3.25; open triangles, pH 3.5) and stabilized variant ACP-GA2 (filled circles, pH 3.25; filled squares, pH 3.5) containing five surface amino acid substitutions. See text for details.

acylphosphatase and the variant that has five amino acid substitutions on the surface of the protein. These substitutions either reverse the charge of a residue (H60E, E63K, and K72E) or introduce new charges (Q50K and N81K). The variant is ∼10 °C more stable than the wild type.[22] However, more important is that the substitutions significantly change the distribution of charges on the surface of this protein, as well as make the net charge of the variant protein more positive (see PDB 2K7K and 2K7J). The comparison of the $\alpha_N(T)$ and $E_{N,sp}(T)$ between wild type and the charge optimized variant of ACP becomes particularly important considering that one of the proposed factors contributing to the volumetric properties of proteins is electrostriction.[37−41] The electrostriction should be highly enhanced in the case of the designed protein because substitutions not only change the overall electrostatics of the surface but also add net positive charge. However, the $\alpha_N(T)$ and $E_{N,sp}(T)$ for the wild type and a variant of ACP are identical within experimental error (Figure 2). Moreover, the changes in pH, that should also provide additional modulation of the net charge of these two proteins, and thus modulate the degree of electrostriction, do not have a noticeable effect on the $\alpha_N(T)$ and $E_{N,sp}(T)$ for these two proteins. These observations suggest that the substitutions in charged amino acid residues on the surface of a protein (and thus modulate electrostriction) have minimal effect on the $\alpha_N(T)$ and $E_{N,sp}(T)$.

Effects of Substitutions at Buried Position on the $\alpha_N(T)$ and $E_{N,sp}(T)$. If substitutions on the protein surface minimally perturb the $\alpha_N(T)$ and $E_{N,sp}(T)$ of a protein, what are the effects of substitutions at buried positions? In particular, are the differences in $\alpha_N(T)$ and $E_{N,sp}(T)$ for different proteins related to the differences in the relative packing densities in the native state? As we discussed above, no correlation was observed between relative packing densities and $E_{N,sp}(T)$

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among different proteins. To test whether a decrease in the packing density in the same protein will have an effect on the $E_{N,sp}(T)$ function, we compared the wild type of eglin c with the variants that have substitutions of large-to-small amino acid residues at fully buried positions. The large-to-small substitutions will create additional internal voids in the variant proteins. Here internal voids are defined as the volume inside the native structure that is not occupied by the protein's van der Waals volume. If internal voids are indeed important in defining $\alpha_N(T)$ and $E_{N,sp}(T)$ functions, then such perturbations will change the packing density of the native state of the variants relative to the wild type protein, which in turn will have an effect on the $\alpha_N(T)$ and $E_{N,sp}(T)$. Figure 3 compares the PPC profiles of the wild type eglin C and two of its variants that have large-to-small substitutions (V14A and V54A) in fully buried nonpolar sites. Computational analyses suggest that these substitutions introduce internal voids into the eglin c molecule on the order of 60 Å^3.[11] Experimental studies using PPC show that the volume changes upon unfolding, ΔV, of V14A and V54A eglin variants are indeed more negative than the wild type eglin c.[11] Interestingly, the $\alpha_N(T)$ and $E_{N,sp}(T)$ functions for these three proteins are very similar and are independent of pH (Figure 3). These observations suggest that single-site substitutions, even though creating sizable internal voids inside the protein, appear to only minimally perturb $\alpha_N(T)$ and $E_{N,sp}(T)$ functions. The V14A and V54A variants of eglin C have also been studied previously by NMR spectroscopy.[42−44] It has been found that substitutions significantly affect the dynamics of this protein. In particular, the V54A substitution was observed to lead to significant rigidification of the side chain motions, while the V14A substitution had a mixed response leading to both an increase and a decrease of side chain dynamics of various parts of the structure.[42] The results shown in Figure 3 suggest that the changes in the protein native state dynamics do not have a major effect on the $\alpha_N(T)$ and $E_{N,sp}(T)$ functions.

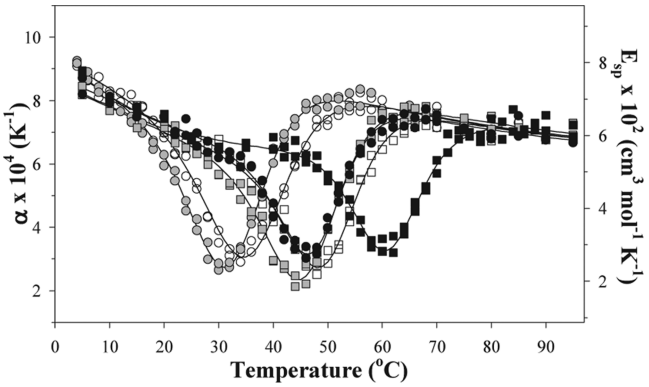

Figure 3. Effect of substitutions at buried positions on the temperature dependence of $\alpha(T)$ and $E_{sp}(T)$ for the wild type Egl (black symbols) and two variants with Val-to-Ala substitutions, V14A (open symbols) and V54 A (gray symbols), at pH 2.75 (circles) and pH 3.25 (squares).

Effects of a Large Number of Surface and Buried Substitutions on the $\alpha_N(T)$ and $E_{N,sp}(T)$. It is possible that single-site substitutions at the buried positions, such as in eglin c or multiple substitutions on the surface such as in ACP, have effects on $\alpha_N(T)$ that are too small to be detected outside the precision of PPC measurements ($\pm 3 \times 10^{-5}$ deg^{-1}). To address this question, we measured $\alpha_N(T)$ for several resurrected ancestral thioredoxin proteins.[23−25] Only 30% of residues (34 out of 106) are identical in all six proteins (Figure 4A). In terms

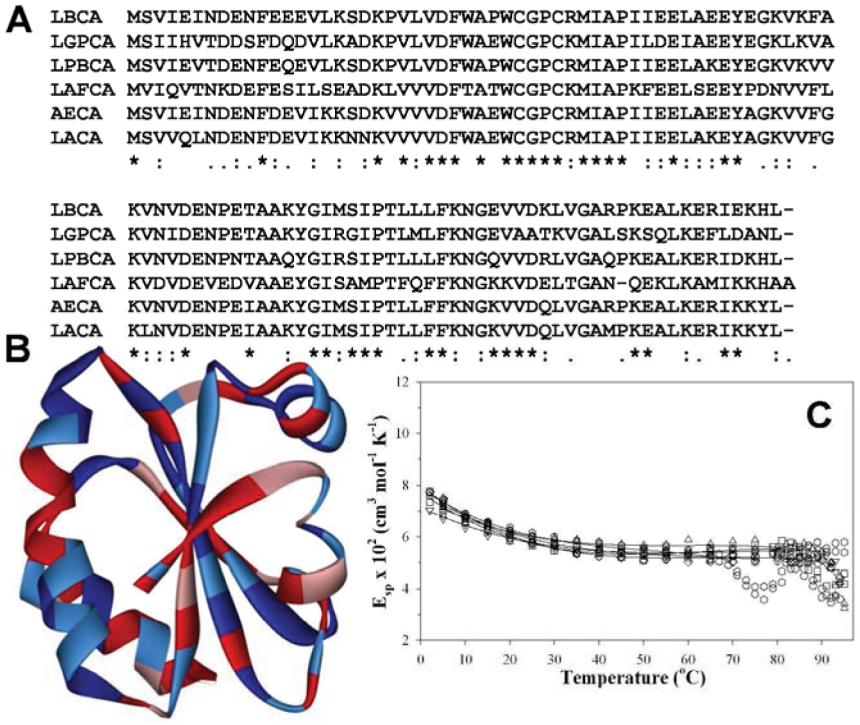

Figure 4. Effect of multiple substitutions in ancestral thioredoxins. (A) Sequence alignment of ancestral Trx.[23−25] (B) Cartoon structure of a thioredoxin (PDB: 2YNX[25]) with color coding of sequence variations: dark blue (identical positions in all sequences), light blue (conserved positions), light red (semiconserved positions), dark red (highly variable positions). (C) Comparison of $E_{sp}(T)$ for the resurrected ancestral Trx at pH 3.0.

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of pairwise identity, the average value is 66% (70 out of 106 residues are identical) with maximal identity of 92% (LACA and AECA, 98 out of 106) and minimal identity of 42% (LBCA and LAFCA, 45 out of 106). More importantly however is the fact that these differences in amino acid sequence are both at the buried and exposed positions (Figure 4B).

Figure 4C compares the PPC profiles of six ancestral thioredoxins. These proteins are very thermostable with transition temperatures well above 80 °C even at low pH. Such high thermostability allows the experimental determination of the $\alpha_N(T)$ and $E_{N,sp}(T)$ functions over a very broad temperature range from 2 to ∼90 °C. Two important observations can be made from the data presented in Figure 4C. First, in agreement with the results obtained for other proteins (see Figure 1), $\alpha_N(T)$ and $E_{N,sp}(T)$ functions depend nonlinearly on temperature. The $\alpha_N(T)$ function shows a significant decrease between 2 and ∼45 °C, dropping from $\alpha_N(2\ °C) = (7.7 \pm 0.4) \times 10^{-4}\ deg^{-1}$ to $\alpha_N(45\ °C) = (5.9 \pm 0.3) \times 10^{-4}\ deg^{-1}$ followed by rather gradual leveling off to $\alpha_N(87\ °C) = (5.7 \pm 0.3) \times 10^{-4}\ deg^{-1}$, at the maximum temperature at which α_N values can be measured experimentally. Second, all six ancestral thioredoxins have the same, within experimental error, $\alpha_N(T)$ and $E_{N,sp}(T)$ functions. This is rather remarkable considering the significant sequence differences between these proteins and the fact that these differences are at multiple positions at both buried and surface sites in the structure (see Figure 4A,B). Taken together, the PPC results for ancestral thioredoxins suggest that $\alpha_N(T)$ and $E_{N,sp}(T)$ functions are not very sensitive to the substitutions either on the surface or interior positions of a protein. This conclusion also agrees with the results of the analysis of $\alpha_N(T)$ and $E_{N,sp}(T)$ for surface variants of ACP and single-site substitutions at buried positions in Egl variants discussed above. Moreover, the high degree of similarity of $E_{N,sp}(T)$ for the six ancestral Trx proteins (Figure 4C) suggests that overall protein topology might be a major factor that defines the differences in $E_{N,sp}(T)$ between different proteins (Figure 1).

CONCLUDING REMARKS

We experimentally determined $\alpha_N(T)$ and $E_{N,sp}(T)$ functions for the native state of nine typical globular proteins. These functions are remarkably different for different proteins. We have eliminated the role of obvious differences in the structural or thermodynamic properties of these proteins such as the number of amino acid residues, secondary structure content, packing density, dynamics, electrostriction, or thermostability. We have shown that $\alpha_N(T)$ and $E_{N,sp}(T)$ functions for a given protein are rather insensitive to the small changes in the amino acid sequence. Substitutions at either surface or buried positions of proteins do not produce significant changes in $\alpha_N(T)$ and $E_{N,sp}(T)$ functions. One possible explanation is that a given protein tertiary fold has intrinsic characteristics that define the $E_{N,sp}(T)$ function. Experiments with six ancestral thioredoxin proteins strongly support this hypothesis but await further experimental validation.

ASSOCIATED CONTENT

Supporting Information

Correlation plots between E_{sp} values of studied proteins and various parameters (packing densities, sphericity, and residue-normalized polar ASA) (Figure S1) and full citation of references 23 and 24. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

D.V. and P.P.P. contributed equally to this work and thus are equal co-first authors.

Notes

The authors declare no competing financial interest.

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