Probing the contribution of internal cavities to the volume change of protein unfolding under pressure

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Abstract

The structural origin of the decrease in system volume upon protein denaturation by pressure has remained a puzzle for decades. This negative volume change upon unfolding is assumed to arise globally from more intimate interactions between the polypeptide chain and water, including electrostriction of buried charges that become exposed upon unfolding, hydration of the polypeptide backbone and amino acid side chains and elimination of packing defects and internal void volumes upon unfolding of the chain. However, the relative signs and magnitudes of each of these contributing factors have not been experimentally determined. Our laboratory has probed the fundamental basis for the volume change upon unfolding of staphylococcal nuclease (Snase) using variable solution conditions and point mutants of Snase (Royer CA et al., 1993, *Biochemistry* 32:5222–5232; Frye KJ et al., 1996, *Biochemistry* 35:10234–10239). Our prior results indicate that for Snase, neither electrostriction nor polar or nonpolar hydration contributes significantly to the value of the volume change of unfolding. In the present work, we investigate the pressure induced unfolding of three point mutants of Snase in which internal cavity size is altered. The experimentally determined volume changes of unfolding for the mutants suggest that loss of internal void volume upon unfolding represents the major contributing factor to the value of the volume change of Snase unfolding.

Keywords: high pressure; protein folding; staphylococcal nuclease; volume change

Understanding protein folding remains one of the most challenging problems in modern molecular biophysics. Considerable progress has been made in the last decade in the characterization of the fundamental thermodynamic and kinetic aspects of the process, as well as in the structural characterization of the various states, native, intermediate, and unfolded, implicated in the transitions. From a thermodynamic point of view, the fundamental parameter in protein temperature stability, the heat capacity change at constant pressure, which is responsible for the temperature dependence of the entropy, enthalpy, and free energy of folding, has been parameterized and can now be modeled adequately, in terms of the changes in exposure of hydrophobic and hydrophilic moieties of particular proteins based on their sequences and structures (Privalov & Gill, 1988; Murphy & Freire, 1992).

A thorough understanding of the thermodynamics of protein folding requires, in addition to the temperature effects, a fundamental comprehension of pressure effects as well. However, in terms of the behavior of proteins under pressure, our level of understanding is much less advanced than in the field of temperature denaturation. (For reviews of pressure effects on proteins see Jaenicke, 1981; Her-

emans, 1982; Weber & Drickamer, 1983; Silva & Weber, 1993; Mozhaev et al., 1996.) At a very basic level, we know from experiment that in cases where the application of pressure leads to the unfolding of proteins, it is because there is a decrease in the volume of the protein-solvent system upon unfolding. In early seminal work on the pressure denaturation of proteins, Brandts et al. (1970) found that pressure lead to the unfolding of ribonuclease A, and that the volume changes for unfolding (ΔV_u) deduced from the equilibrium unfolding profiles fell in the range of -45 to -5 mL/mol, depending on the pH and temperature at which the experiment was carried out. A few years later, Zipp and Kauzmann (1973) studied the pressure denaturation of metmyoglobin over a wide range of temperature, pH, and pressure. They found the ΔV_u to be negative for all conditions studied and on the order of -100 mL/mol in good agreement with dilatometric measurements of Katz et al. (1973). Moreover they found that the volume change was independent of pressure. Negative values of ΔV_u have also been determined for lysozyme (Li et al., 1976; Samarasinghe et al., 1992), chymotrypsinogen (Hawley, 1971; Li et al., 1976; Wong & Heremans, 1988), myoglobin (Marden et al., 1986), ovalbumin (Suzuki et al., 1963), ribonuclease A (Takeda et al., 1995), and staphylococcal nuclease and a series of mutants of this protein (Eftink et al., 1991; Royer et al., 1993; Vidugiris et al., 1995; Eftink & Ramsay, 1996; Frye et al., 1996; Frye & Royer, 1997).

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Although the experimentally determined values for ΔV_u are, in general, negative, leading to pressure-induced unfolding, their absolute values are quite small, typically less than 1% of the total protein volume. Zipp and Kauzmann (1973) mused at these unexpectedly small values and their independence on pressure (i.e., the compressibility term is even smaller). The subject has continued to elicit confusion and controversy in the literature ever since (see Dill, 1990). Generally, three factors are thought to contribute to the value of ΔV_u :

- electrostriction of charged and polar groups that become exposed to solvent upon unfolding,
- elimination of packing defects,
- and the volume effects of transfer of hydrophobic groups from the protein interior to water.

There is general agreement that the first two factors should provide a negative contribution to ΔV_u , but their magnitudes are not well defined. The significance of the contribution of electrostriction to the overall volume change is thought to be minimal because most charged side chains in proteins are already exposed, and thus, solvated in the native structure and undergo no change in solvent exposure upon unfolding (Weber & Drickamer, 1983). Nogochi and Yang (1963) estimated a volume decrease of -1 mL/mol peptide group for the helix-coil transition of polyglutamate. Presumably since no change in charge exposure and little contribution of hydrophobic groups is involved in this reaction, the volume change reflects primarily the interaction of water with the exposed polar peptide backbone.

While proteins present highly densely packed interiors (Richards, 1974, 1977), the void volumes should be significantly larger than those of the unfolded protein chain exposed to small solvent molecules such as water. For example, the dilution of pure dimethylacetamide in a continuum solvent results in a 30% decrease in specific volume (Assarsson & Eirich, 1968). Weber and Drickamer (1983) predict these packing defects to provide the major contribution to the value of ΔV_u . Prehoda and Markley (1996) estimate a negative contribution from elimination of the packing voids of about 10% of the total protein volume.

The third factor, the exposure of hydrophobic groups to water, has caused the largest controversy, as both its sign and magnitude remain under debate. Kauzmann predicted that the exposure of buried hydrophobic residues should provide a large negative contribution to ΔV_u (near -20 mL/mol aliphatic side chain) based on the relatively large and negative transfer volumes of model hydrophobic compounds from pure liquid or hydrophobic solvents to water (Masterton, 1954; Kauzmann, 1959). Brandts et al. (1970) pointed out that if the model compound data were applicable to proteins then the experimentally observed volume changes should be much larger in absolute value because all of the contributing factors are negative. They conclude that "The volume and compressibility changes during denaturation are not completely dominated by the hydrophobic effects which are described by the usual model compound transfer reactions." Through the years others have concluded that the volume changes following interactions between aliphatic groups and water are actually positive (Masterton & Seiler, 1968; Bøje & Hvdt, 1972). If such is the case, then the small, negative values observed for ΔV_u would result from the combination of rather large contributions of opposite sign. Chalikian and Breslauer (1996) have proposed a similar compensating situation involving negative contributions from the elimination of packing defects and solvent interactions with the polar moieties of

the protein chain, offset by a large positive contribution from the thermal volume which results from thermally induced mutual molecular vibrations of the solute and the solvent.

To try to define the factors contributing to the value of ΔV_u , we have chosen an approach in which we study the pressure denaturation of a particular protein with point mutations that should perturb the protein system in such a manner as to lead to predictable differences in the experimentally observed volume change of unfolding. The protein model that we have employed is staphylococcal nuclease (Snase). Snase is a small, single domain monomeric protein with no disulfide bridges that folds in a two-state manner in equilibrium unfolding assays (Shortle & Meeker, 1986; Carra & Privalov, 1995). In fact, although the kinetics of Snase folding are complex at atmospheric pressure (Chen et al., 1991, 1992; Su et al., 1996), this complexity disappears with pressure, presumably because the intermediate species occupy larger system volumes than either the folded or unfolded states and thus are destabilized by pressure (Vidugiris et al., 1995, 1996). Particularly useful to our studies is the fact that Snase is relatively unstable, such that with a ΔV_u of -77 ± 8 mL/mol, at pH 5.5, the midpoint of the folding transition is 1.5 kbar, well within the pressure range available in our pressure apparatus (1 atm, 2.5 kbar). We have previously shown that while ΔG_u changes considerably, the value of ΔV_u is independent of pH (Royer et al., 1993), eliminating electrostriction as an important contributor to ΔV_u of this protein. Moreover we have shown the value of ΔV_u of Snase to be independent of both the concentration guanidinium hydrochloride (GuHCl) (Vidugiris et al., 1996) and the concentration of xylose, a stabilizing colligative agent (Frye & Royer, 1997). Since the midpoint for the pressure-induced unfolding of Snase under these varying conditions occurs at pressures ranging from 500 to 2,500 bar, we can conclude, that in the case of Snase, the value of ΔV_u is apparently pressure independent as well (i.e., the compressibility term is negligible, in agreement with the work of Eftink & Ramsay, 1996).

The contribution of the solvation of exposed surface area upon unfolding was previously investigated by our group (Frye et al., 1996) by determining the change in volume upon unfolding for two mutants of Snase with altered denaturant m -values of unfolding (Shortle et al., 1989) such that the amount of exposed surface area upon unfolding was either significantly larger (+28%) or significantly smaller (-28%) than that of the wild-type (WT) Snase. The results of these previous studies indicated that there was no significant difference in the experimentally determined ΔV_u between these mutants and the WT Snase. We concluded from those studies that either the hydration of exposed surface area does not contribute significantly to the decrease in system volume upon unfolding or that the negative volume change upon exposure of polar surface area is nearly perfectly compensated by a positive contribution from the exposure of nonpolar surface area (Frye et al., 1996).

With the contributions of electrostriction and solvation of exposed surface area eliminated as significant contributing factors to the value of ΔV_u for Snase, the contribution of the elimination of packing defects upon unfolding remained to be examined. If the protein in the native state contains solvent inaccessible cavities or packing voids, as the protein unfolds, those voids become exposed to the solvent which, being a small molecule, can interact with the protein surface with fewer and smaller voids, resulting in a decreased system volume. It is unclear, however, how large a contribution the elimination of the packing defects makes to the value of ΔV_u . For Snase, the change in partial molar volume of the

protein upon unfolding at 21 °C is about 0.5% of the total hydrated volume of the folded protein (Royer et al., 1993). The volume sizes of internal cavities of various proteins, reported by Rashin et al. (1986), ranged from 0 to 2% of the total volume, and are, therefore, large enough to contribute significantly to the total volume change of unfolding. Moreover, small packing voids exist in the folded structure, analogous to those obtained upon stacking spherical objects in a container.

In the present study, three cavity mutants of Snase were investigated in order to determine the differences in ΔV_u relative to WT Snase. All three mutations were made at position 66, which is a valine residue in the WT Snase (Fig. 1). Two of the mutations were to smaller amino acid side chains (glycine, V66G, and alanine, V66A) while the third was mutated to a larger amino acid side chain (leucine, V66L). The crystal structure has been solved for the V66G mutant, bearing the largest cavity, and no crystallographically determined water molecules were found in the cavity (Ed Lattman, pers. comm.). Moreover, the main-chain atoms in the WT Snase and V66G structures superimpose with a mean-square deviation of only 0.776 Å (Fig. 2), and only minor readjustments of the side chains around residue 66 are observed, indicating that the chain does not collapse into the cavity formed by substitution of V66 by G. We expected, therefore, that the V66G and V66A mutations, which introduce a cavity in the core of Snase, should result in a larger negative value for ΔV_u , with respect to WT Snase, while the V66L should decrease the absolute value of ΔV_u .

Results

Since the overall stabilities of the mutant proteins were quite different from that of WT Snase (V66G and V66A less stable and V66L more stable), the pressure-induced unfolding experiments were carried out under different conditions. In the case of V66G, tuning the stability to a value similar to that of WT Snase to obtain a complete unfolding profile and, thus, accurate values of ΔV_u ,

Fig. 1. The three-dimensional structure of staphylococcal nuclease determined from X-ray crystallographic data (Hynes & Fox, 1991). The figure was drawn using the program Molscript (Kraulis, 1991). Valine 66 is shown in ball-and-stick presentation.

Fig. 2. The crystal structure of V66G (gray) superimposed on top of the WT crystal structure (black). The atomic coordinates of V66G were the gracious gift prior to their publication from E. Lattman and coworkers.

required the addition of 19.6 mM fraction xylose at pH 7.0. The cavity formed in V66A is less destabilizing and the experiments were simply carried out at pH 7.0, rather than at pH 5.5 as in the case of WT Snase. Due to the increased stability of the V66L mutant, the experiments were carried out in the presence of 0.5 M guanidine at pH 5.5. We remind the reader that we have previously demonstrated that the value of ΔV_u for Snase unfolding is independent of pH (Royer et al., 1993), xylose (Frye & Royer, 1997), and GuHCl (Vidugiris et al., 1996). While we could not carry out studies on the GuHCl or xylose concentration dependence of these mutants due to their relative stability and the limits of our instrumentation, we point out that the lack of effect of pH and GuHCl on the value of the volume changes upon unfolding has been observed for the pressure-induced unfolding of the *trp* repressor dimer and that of a site specific mutant of *trp* repressor. Thus, this lack of effect of solution conditions may be a general phenomenon and can be understood if solvation of exposed surface area contributes little to the overall value of ΔV_u , as we have concluded from the study of the cooperativity mutants. In all cases the unfolding was observed by monitoring the decrease in fluorescence intensity as a function of pressure. We have also demonstrated previously that this loss in fluorescence correlates both at equilibrium and kinetically with the loss in discrete histidine proton NMR signals (Royer et al., 1993; J. Markley, pers. comm.), the increase in the radius of gyration of the protein, and the loss in α -helix and β -sheet secondary structure (Panick et al., 1998).

Figure 3 shows representative results for V66G in the presence of 19.6 mM fraction xylose at pH 7.0, V66A at pH 7.0, and V66L in the presence of 0.5 M guanidine at pH 5.5, and WT Snase at pH 5.5, respectively. The data were analyzed for the volume change upon unfolding ΔV_u and free energy of unfolding ΔG_u as described in Materials and methods and the values for the average of four to five experiments along with the values from the global analysis of all of the experiments for each mutant are shown in Table 1. The analysis was always carried out directly on the raw data, and then,

Fig. 3. Normalized representative experiments for the unfolding of (●) WT at pH 4.5, (■) V66G at pH 7.0 and 21 °C with 19.1 mM xylose, (▲) V66A at pH 7.0 and 21 °C, and (◆) V66L at pH 5.5 and 21 °C with 0.5 M guanidine. The solid lines represent fits to the data.

for purposes of comparison, the data and fits were normalized to the percent folded as described in Materials and methods. The free energies of unfolding cannot be compared directly since the denaturation conditions for all three mutants were different. The value of ΔV_u for WT has been found previously (Vidugiris et al., 1995; Frye et al., 1996; Frye & Royer, 1997) to be -77 ± 8 mL/mol. The values of ΔV_u for V66G (-112 ± 8 mL/mol) and V66A (-104 ± 6 mL/mol) are both significantly larger in absolute magnitude than WT, as would be expected from creating larger cavities within the interior of the protein, but are within error of one another. Unexpectedly, the V66L mutant also has a volume change larger in absolute magnitude than WT (-94 ± 12 mL/mol). This value within error of the WT value (77 ± 8 mL/mol), but nonetheless, we would have expected it to be smaller.

Discussion

Although proteins have been shown to be as tightly packed as small organic crystal molecules (Richards, 1974, 1977), Honig and

Table 1. Experimental volume change and free energy change upon unfolding of the cavity mutants of Snase

Protein	ΔG_u (kcal/mol) ^a		ΔV_u (mL/mol)	
	Average	Global	Average	Global
V66G	2.0 ± 0.2	2.0 ± 0.2	-112.6 ± 8.2	-111.9 ± 7.7
V66A	2.2 ± 0.2	2.1 ± 0.2	-105.7 ± 7.6	-103.8 ± 6.1
V66 (WT) ^b	3.2 ± 0.2	—	-77 ± 8.0	—
V66L	2.4 ± 0.3	2.3 ± 0.4	-95.7 ± 4.1	-94.2 ± 11.7

^aThe mutants and WT SNase were pressure denatured under different solution conditions, which are given in Materials and methods and also in the text. Thus, the ΔG_u values should not be compared.

^bThe values for WT SNase are taken from Vidugiris et al. (1995).

Table 2. Side-chain volumes and predicted volume change upon unfolding

Amino Acid	van der Waals volume (mL/mol) ^a	$\Delta V_{\text{mutation}}$ (mL/mol)	Predicted $\Delta V_{\text{unfolding}}$ (mL/mol)
Valine	63.2	0	-77
Glycine	28.9	34.3	-111
Alanine	40.3	22.9	-100
Leucine	74.7	-11.4	-66

^aCalculated based on the values given by Richards (1974).

coworkers determined that out of 12 proteins, all but one contained internal cavities (Rashin et al., 1986). In addition, these investigators found that the total volume of the cavities was between 0 to 600 Å³ (0–361 mL/mol) and increased with molecular weight such that the cavities make up less than 2% of the total protein volume. Since the change in volume upon unfolding for nuclease is 0.5% of the total protein volume, in principle, cavities could account for the entire observed volume change upon unfolding (Royer et al., 1993). The mutants involved in this study modify the size of the internal cavity near amino acid 66, which is located in the hydrophobic core of Snase. As noted above, it can be seen (Fig. 2) from the superposition of the WT and V66G structures around the position of the mutation that the cavity does still exist and that there has only been a minimum amount of local readjustment of the backbone.

Using the van der Waals volumes of the amino acid side chains determined by Richards (1974), one can calculate crudely the change in volume expected for the substitution of the valine at position 66 by glycine, alanine, and leucine. Table 2 shows the values for such a calculation. The predicted value of ΔV_u for the three mutants based on side-chain van der Waals volumes would be -111, -100, and -66 mL/mol for V66G, V66A, and V66L, respectively. The actual experimental values for V66G and V66A are -112 ± 8 and -104 ± 6 mL/mol, respectively, while that for V66L was -94 ± 12 mL/mol. The first two are in good agreement with the predicted values, while that of the V66L mutant is larger in absolute value than expected.

Using the program Voidoo (Kleywegt & Jones, 1994) and a probe radius of 1.2 Å, we have analyzed the pdb coordinates of WT Snase (Hynes & Fox, 1991) and the V66G mutant (a gift from Lattman & co-workers) for the existence of internal cavities in the structure. This program detected cavities only in the V66G structure with a total cavity volume of 71 Å³, which corresponds to 42 mL/mol. Thus, the value of ΔV_u for the V66G mutant would be expected to be near -119 mL/mol (77 (WT) + 42 mL/mol), also in relatively good agreement with the experimentally determined value (-112 ± 8 mL/mol). While Voidoo did not detect any cavities in WT Snase, using the program GRASP (Nicholls et al., 1993) and a probe radius of 1.4 Å, we detected a cavity of 19 Å³ (12 mL/mol) in WT Snase adjacent to residue 66. This cavity in WT Snase may contribute to the value of ΔV_u of -75 mL/mol observed for the WT protein. Moreover, internal void volumes smaller than the volume required for a probe radius of 1.4 Å will also contribute to ΔV_u . Wynn et al. (1996) also with GRASP (Nicholls et al., 1993) and a probe radius of 1.4 Å detected cavities of 31 and 17 Å³ in the Ca²⁺/pdTp bound form of the WT Snase

also adjacent to residue 66. GRASP also detected a large cavity of $118 \text{ \AA}^3$ (71 mL/mol) in the V66G mutant. Relatively small differences in the coordinates can lead to substantially different results using cavity calculating programs, and different programs also yield substantially different results. Nonetheless, it is clear that (barring the obstruction of the cavity around position 66 by noncrystallographically determined water molecules) a small cavity exists in the WT Snase, which is considerably increased in volume upon mutation of V66 to G.

The results from the V66G and V66A mutants of Snase suggest a significant contribution from solvent inaccessible cavities to the value of ΔV_u . However, the experimental value of ΔV_u for V66L was found to be $-94.2 \pm 12 \text{ mL/mol}$, compared to the predicted value of -66 mL/mol . A slightly larger negative ΔV_u for the V66L mutant as compared to the WT Snase might arise from local rearrangements in the structure, which may actually create a new small cavity or small packing defects. New cavities were created in a packing mutant of lysozyme (Eriksson et al., 1992). And interestingly, Wynn et al. (1997) made unnatural amino acid substitutions at the cysteine in the V23C variant of Snase, exactly opposite V66, the position of the cavity mutants in the present study. These authors found that the displacement of helix 1 away from the β -barrel in the pentyl-disulfide adduct at C23 results in the creation of a new cavity of $29 \text{ \AA}^3$ (18 mL/mol) between the two elements of secondary structure. The future refinement of the V66L structure may reveal new cavities in this mutant, although we note that the small observed increase in the absolute value of ΔV_u for the V66L mutant could also arise from small packing defects, that would not necessarily be apparent in cavity calculations on atomic coordinates. We rule out any undetected non-two-state behavior of the mutants as providing the cause for the larger absolute values of ΔV_u observed for these mutants. The existence of equilibrium intermediate states will decrease, not increase, the value of the apparent volume change obtained from analysis of a pressure denaturation profile assuming two-state behavior.

It could be serendipity, rather than the creation of cavities, that leads mutations at position 66 in Snase to cause a change the value of ΔV_u ; however, we point out that the introduction of cavities within Snase has been the only change made to this protein or the solution conditions of pressure denaturation that resulted in a value of ΔV_u significantly different than that of the WT. Based on the present results, we suggest that packing defects may indeed provide a significant contribution to the value of the volume change of unfolding of Snase.

Materials and methods

Protein expression and purification

The Snase mutants were produced using the λ expression system in the *Escherichia coli* strain Ar λ 9 as described by Shortle and Lin (1985). The cells were grown up and protein was purified as described previously (Frye et al., 1996).

High pressure experiments

High pressure experiments were carried out in a Vascomax high pressure cell using high pressure generating system similar to that described by Paladini and Weber (1981). The pressure generator

(High Pressure Equipment Co., Erie, Pennsylvania) is connected to a high pressure gauge and the pressure cell through a series of metal tubing and high pressure valves. The steady-state fluorescence unfolding profiles were measured using an ISS spectrofluorometer (ISS, Urbana, Illinois). Exciting light from a xenon arc lamp at 295 nm was coupled to the pressure cell from the monochromator via a UV multifiber optic bundle (Oriel Corp., Stratford, Connecticut). The tryptophan emission was monitored at a 90° angle through a Corion 340 nm high pass filter to minimize scattering contributions that can be significant in the high pressure cell if emission is monitored at lower wavelengths. Pressure jumps of 100 bar were made, and the fluorescence intensity was measured at a 5 s integration time. Equilibrium was reached after 20 min for all pressures, and those intensity values were used for the equilibrium unfolding profiles. The experiments were carried out at a concentration less than $10 \text{ }\mu\text{M}$ (as determined by an extinction coefficient at 280 nm of $18,050 \text{ cm}^{-1} \text{ M}^{-1}$) to avoid any aggregation that can occur in unfolding experiments at high concentrations. The buffer was 10 mM bis-Tris for all proteins with 19 mM fraction xylose at $\text{pH } 7$ for V66G, with 0.5 M guanidine at $\text{pH } 5.5$ for V66L, and at $\text{pH } 7$ without any additional chemicals for V66A. The different buffer conditions were used to poise the unfolding transition so that the entire unfolding profile could be observed under 0 to $2,500 \text{ bar}$. The experiments were repeated four to five times.

Data analysis

Analysis of the curves of fluorescence intensity vs. pressure p was performed using the analysis program BIOEQS (Royer et al., 1991; Royer & Beechem, 1992; Royer, 1993) fitting the intensity data in terms of a two-state transition from the native to the fully unfolded state ($F \rightarrow U$) to yield a free energy ΔG_u and volume change ΔV_u for the transitions, as previously described (Vidugiris et al., 1995).

$$\Delta G = -RT \ln K_{eq} = -RT \ln((I(f) - I(p))/(I(p) - I(u))) \quad (1)$$

and

$$d\Delta G_u/dp = \Delta V_u \quad (2)$$

where $I(f)$, $I(u)$, and $I(p)$ correspond to the fluorescence intensities of the native and unfolded states and that at pressure p . The data for each mutant were first analyzed in the unlinked or single curve mode, and the volume change for each curve was averaged and the standard deviation calculated. No baseline slope was apparent in the data. Then the profiles for each mutant were globally analyzed linking the value for the free energy and volume change across all data sets for each mutant. For purposes of visual comparison, the raw data and fits from the unfolding profiles were normalized to a scale from 0 to 100% native protein by calculation the percentage decrease in intensity at each pressure relative to the asymptotic intensity values recovered from the fits (Fig. 3).

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