## Introduction

Measurement of volumetric properties of biopolymers in dilute solution has always been a difficult procedure, involving tedious experiments using densitometric or dilatometric methods to determine partial molar volumes. Measuring volume changes (ΔV_{unf}) for biopolymer unfolding is also problematic and most often carried out using large, reinforced optical cells up to pressures of 10,000 atm. These limitations have led to a dearth of volumetric data in the literature, and a recognition that a meaningful understanding of the relationship between structure and volume is lacking.[1]
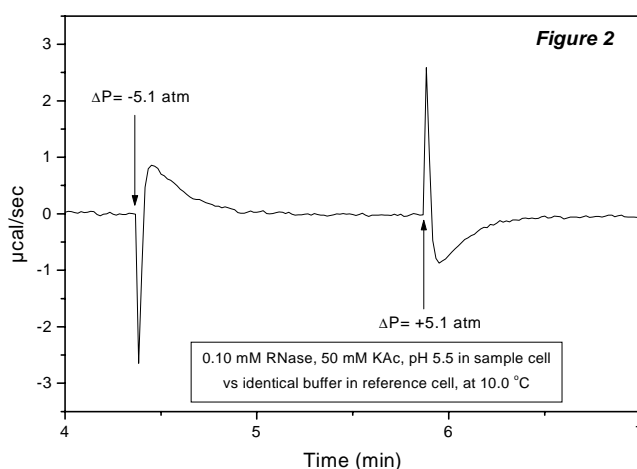
MicroCal has recently developed (patent pending) a calorimetric technique, Pressure Perturbation Calorimetry (PPC), which not only eases the experimental burden but also leads to considerably greater accuracy for measuring critical volumetric parameters of biopolymers in solution. Equally significant, it provides information on biopolymer solvation currently unavailable from any other method.

Method

PPC measures heat change (ΔQ) resulting from a pressure change ΔP above a solution containing a dissolved biopolymer. Measurements are made using a standard VP-DSC instrument equipped with a PPC accessory shown in Figure 1. The total-fill Sample and Reference cells have identical volumes of 0.5 ml. Filling tubes

Figure 1
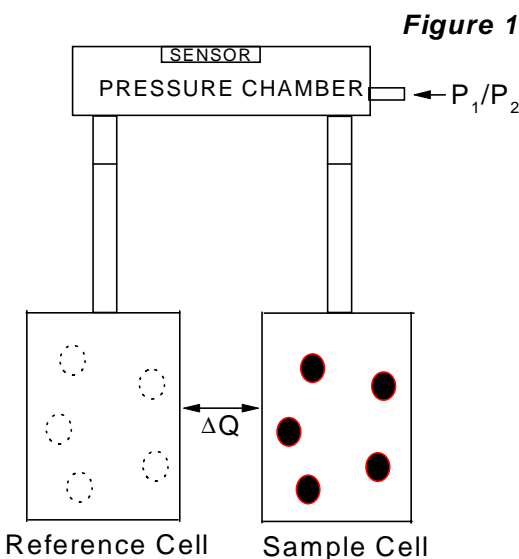
for each cell open into a common Pressure Chamber, containing a Sensor, which transmits data to a a computer for storage. Pressure in the chamber is alternated between P_1 and P_2, using a controlled source operating at selected excess pressures from zero to 5 atm. The sample solution is placed in the Sample Cell and the identical buffer solution in the Reference Cell. Data are shown in Figure 2 for a solution of ribonuclease A (RNase). The calorimetric baseline is first allowed to equilibrate at constant temperature and at pressure P_1 in the chamber. The excess pressure is then changed to P_2, caus-

Figure 2

$\Delta P = -5.1$ atm

$\Delta P = +5.1$ atm

0.10 mM RNase, 50 mM KAc, pH 5.5 in sample cell vs identical buffer in reference cell, at 10.0 °C

ing heat to be absorbed in both cells. Since the solutions in the sample and reference cell are identical except for the small amount of dissolved solute in the sample cell (solid ellipses in Figure 1) counterbalanced by the corresponding volume of buffer in the reference cell (dashed ellipses), differential heats are quite small. The compression and decompression peaks are of identical size and opposite sign. ΔQ values are obtained by integrating each peak.

Measurement of ΔQ at a single temperature requires only a few minutes. Experiments can be set up to automatically to obtain data at numerous temperatures with no operator involvement.

Results on Proteins

Precise measurement of the coefficient of thermal expansion (α) for protein partial specific volumes can be made at concentrations down to 0.25%. Data shown in Figure 3 were obtained

on chymotrypsinogen and RNase from 5 to 90 ^{o}C. The positive peak for chymotrypsinogen at its T_m of 48 ^{o}C shows that ΔV_{unf} is positive, and the area defined by the peak and the progress baseline (dashed) yields ΔV_{unf} of +.18%, or 34 ml/mole. For RNase, ΔV_{unf} is –0.31%, or –30 ml/mole, at its T_m of 60 ^{o}C. Both proteins display a larger α for the unfolded form than for the native form, with $\Delta\alpha_{unf}$ of ca. 1.3×10^{-4} for each at their respective T_m. A summary of parameter values obtained on these two proteins as well as on HEW lysozyme and T4 lysozyme is given in Table 1. When comparison is possible (RNase, chymotrypsinogen, and HEW lysozyme), the values of ΔV_{unf} and $\Delta\alpha_{unf}$ from

Figure 3
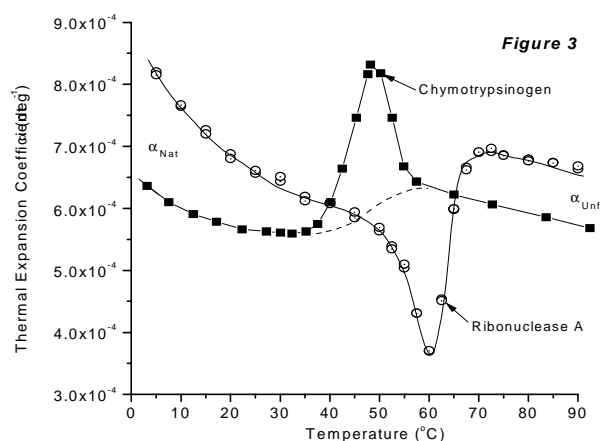
PPC agree well with independent estimates in the literature using high-pressure optical cells.[2-4] Estimates for $\alpha_{25\ C}$ are consistent with results from other methods[5,6] on a number of native proteins. These other methods however, have large experimental uncertainties.

Table 1. PPC Results for four proteins.

| Protein	ΔV_{unf}	$\Delta\alpha_{unf}$ deg^{-1}	$\alpha_{25 C}$ deg^{-1}
Chtg	+.18%	1.3×10^{-4}	5.6×10^{-4}
Rnase A	-.31%	1.4×10^{-4}	6.7×10^{-4}
Lysozyme	-.30%	1.2×10^{-4}	6.1×10^{-4}
T4 Lysozyme	-.22%	1.0×10^{-4}	7.3×10^{-4}

The data in Figure 3 show that α for both native proteins has a strong negative temperature coefficient as well as large positive curvature. This is more pronounced for RNase than for chymotrypsinogen. This same behavior was also

found for HEW lysozyme and T4 lysozyme. Data presented below show that these thermal effects arise directly from the solvation of groups which are exposed at the protein/solvent interface.

Solvation Effects

The partial specific volume of a protein in solution is equal to the sum of the *intrinsic volume of the protein* plus the *change in the volume of solvent* resulting from its interactions with accessible protein groups.

To ascertain the source of this solvation contribution, PPC has been used to measure α values for certain amino acid sidechains and for the peptide group in a polypeptide chain. Results are shown in Figure 4. The three aliphatic sidechains alanine (ala), valine (val), and isoleucine (ileu) are similar in that α values are negative at low temperature, with a strong positive slope and negative curvature as temperature increases. Other aliphatic sidechains show similar behavior, whereas aromatic sidechains

Figure 4
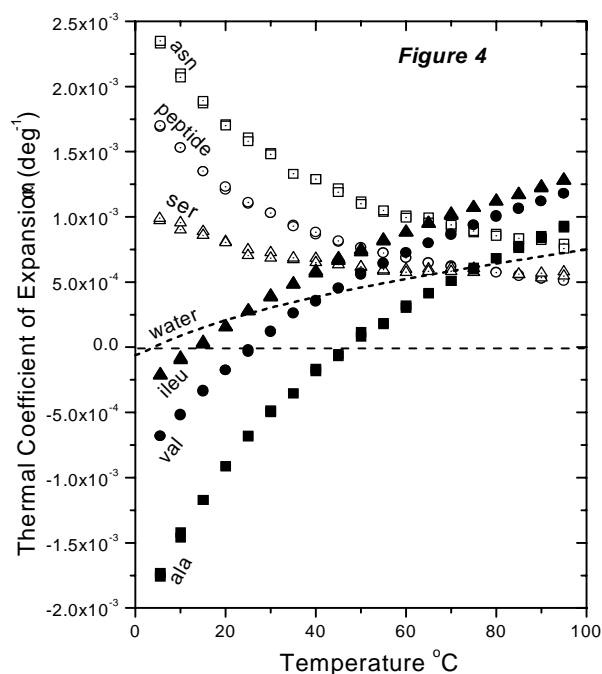
tryptophan and phenylalanine are positive and nearly independent of temperature. The polar sidechains asparagine (asn) and serine (ser), as well as the highly polar peptide group, show a very contrasting behavior with highly positive α values at low temperature, but with a negative

slope and positive curvature with increasing temperature. Other hydrophillic sidehains and various electrolytes (not shown) were found to behave qualitatively similar.

Also shown as the dashed line in Figure 4 is α for pure water, long known to be anomalous compared to other liquids. For water, α is negative below 4 ^{o}C, and exhibits a strong positive slope and negative curvature with increasing temperature. This peculiar behavior has been attributed[7] to the melting of ordered hydrogen-bonded structures existing in liquid water at low temperatures. These ordered structures are of lower density than disordered water so their melting provides a large negative contribution to α particularly at low temperatures. The aliphatic sidechains in Figure 4 tend to mimic the behavior of water but to an even greater degree. These are known to be "structure-makers", promoting a higher degree of order in their water of solvation than exists in pure water, which in turn accentuates the thermal order-disorder transition. The peptide group and polar sidechains behave oppositely, suggesting they are "structure-breakers" which tend to disrupt ordered structures which exist in pure water.

Globular proteins contain a very large preponderance of hydrophilic sidechains and peptide groups relative to apolar aliphatic sidechains, so it is no surprise that both native RNase and chymotrypsinogen exhibit α vs temperature behavior (Figure 3) characteristic of the structure-breakers shown in Figure 4. The three smaller proteins examined (RNase, HEW lysozyme, and T4 lysozyme) have higher α values and larger negative slopes than chymotrypsinogen, due at least in part to the higher surface/volume ratio of the smaller proteins.

When native proteins unfold at high temperature, the solvent accessible surface area (ASA) will increase and α will increase (Figure 3). Disulfide-reduced RNase remains unfolded at all temperatures and PPC results on fully reduced RNase are shown in Figure 5. Relative to the native form at low temperatures, α for the reduced protein is seen to be considerably larger with a stronger temperature dependence. PPC

results such as these can be used to estimate ASA for native RNase in its folded state[12].

Figure 5
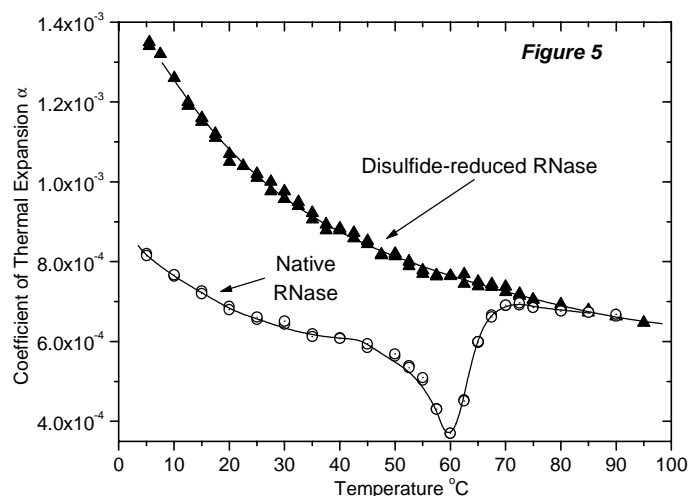
If the thermal behavior of α for proteins is indeed controlled by protein-solvent interactions, then large changes should occur when the protein is observed in solvents known to contain more or less ordered structure than pure water. In the upper frame of Figure 6 are shown α vs temperature plots for pure H_2O, pure D_2O, and 2.8 M guanidinium sulfate. It is known[7] that D_2O is a better hydrogen bonder than H_2O and thereby more highly ordered at low temperature. This is evident in the behavior of α, which is

Figure 6
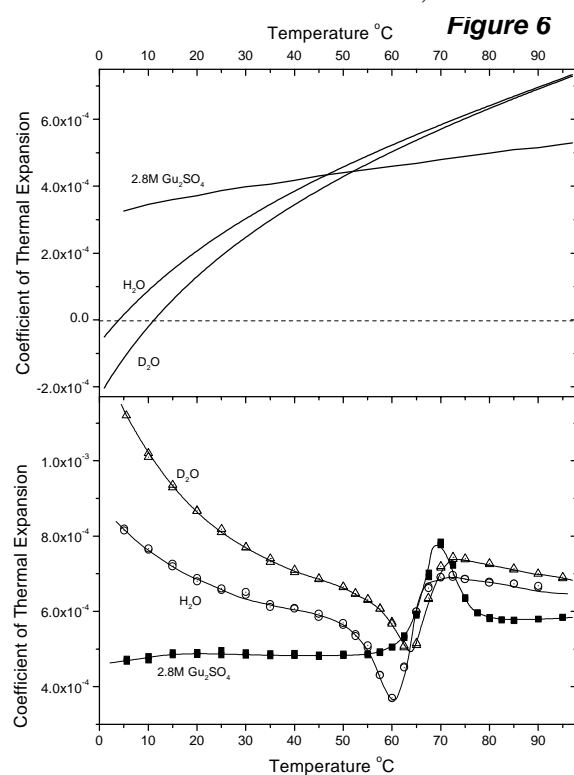
more negative than that of water at 0 $^{\circ}$C and which does not turn positive until a temperature of 11 $^{\circ}$C is reached. That is, D_2O exhibits a more prominent contribution from thermal melting of ordered structure than does H_2O. On the other hand, presence of high electrolyte concentrations tend to accentuate ion-dipole interactions at the expense of hydrogen bond interactions between water molecules so in 2.8 M Gu_2SO_4 there is virtually no indication of the α vs temperature behavior which is attributable to the thermal order-disorder transition so prominent in both H_2O and D_2O.

In the lower frame of Figure 6, PPC results on RNase in these same three solvents are shown. While native RNase has virtually the same 3-D structure in each of these solvents, the behavior of α at temperatures below T_m is drastically different. Since, as mentioned earlier, the ASA of native proteins will be dominated by hydrophilic groups of the structure-breaking type, these results show that the ability of exposed groups to disrupt structure is controlled, as expected, by the amount of structure which exists in the solvent in the absence of protein.

It is also seen in Figure 6 that native RNase is somewhat more stable in both D_2O (T_m of 64 $^{\circ}$C) and 2.8M Gu_2SO_4 (70 $^{\circ}$C) than in H_2O (60 $^{\circ}$C). While the guanidinium ion is strongly destabilizing to native proteins, the sulfate ion is strongly stabilizing, and it was previously shown[8] that the salt is marginally stabilizing on balance. The ΔV_{unf} is nearly the same in D_2O as in H_2O, but is of opposite sign in Gu_2SO_4.

Conclusions

PPC facilitates the measurement of important volumetric parameters for biopolymers in solution. Accuracy is such that the first, second, and third temperature derivative of partial volume can be measured for the first time. These higher order derivatives provide important information on biopolymer solvation, ASA, and solvent structure.

PPC studies (not shown here) on Rnase/2'CMP and transferrin/Fe^{+3} suggest that measurable decrease in ASA occurs when certain ligands bind to proteins[12]. Changes in solvation can be critical in determining binding affinities[9,10], so that extensive PPC data might further a deeper understanding of biomolecular interactions.

Finally, although not yet confirmed, it has been suggested from molecular dynamics studies[11] that thermal expansion of closely related proteins may correlate with stability such that highly stable folded proteins have a smaller α value than do those of lower stability.

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Supplement to Pressure Perturbation Calorimetry (PPC) Application Note

Data Analysis

The equation for the heat Q released by any solution of volume V in response to a pressure change ΔP is easily derived from the second law of thermodynamics[12]. This is expressed as Eq 1, where α is the coefficient of thermal expansion defined in Eq 2.

$$Q = -T\left(\frac{\partial V}{\partial T}\right)_P \Delta P = -TV\alpha\,\Delta P \qquad (1)$$

$$\alpha = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_P \qquad (2)$$

Solutions that contain a solute, protein for example, dissolved in solvent may be treated as two component systems where the total solution volume V is expressed as the sum of volumes from each component as shown

$$V = g_o v_o + g_s \bar{v}_s \qquad (3)$$

in Eq 3. Here g_o and g_s are mass of solvent and solute, respectively, v_o is the specific volume of pure solvent, and v_s is the partial specific volume of the solute. When defined in this manner, v_s includes not just the intrinsic volume of the solute but also includes any volume changes induced in the solvent as the result of its interactions with the solute (ref).

As shown diagrammatically in Figure 1, a certain volume of solvent ($g_s v_s$) is displaced from the sample cell due to the presence of dissolved solute molecules so the net heat ΔQ released when both cells are pressurized simultaneously is equal to the heat produced by the solute particles in the sample cell less the heat produced by the equivalent volume of solvent in the reference cell. This is expressed mathematically[12] in Eq 4, where α_s is the coefficient of thermal expansion of the solute partial volume and α_o is that for pure solvent.

$$\alpha_s = \alpha_o - \frac{\Delta Q}{T\,\Delta P\,g_s \bar{v}_s} \qquad (4)$$

If the solvent is nearly pure water, then α_o can be approximated from density data in the literature. If the solvent, or buffer, has high concentrations of added electrolytes, then α_o may be obtained in a separate PPC experiment with buffer in the sample cell and pure water in the reference cell.