# Molecular Thermodynamic Properties of Protein Solutions from Partial Specific Volumes

**P. E. Pjura, M. E. Paulaitis, and A. M. Lenhoff**
Center for Molecular and Engineering Thermodynamics, Dept. of Chemical Engineering, University of Delaware, Newark, DE 19716

*Partial specific volumes at 25°C are reported for α-chymotrypsinogen in aqueous solutions containing NaCl, citrate, and/or polyethylene glycol (PEG) over a range of protein concentrations. The concentration dependence of the partial specific volume can be either positive or negative, depending on the solvent. For example, the partial specific volume increases with increasing protein concentration in NaCl/citrate solutions at high salt concentrations, and decreases with increasing protein concentration in solutions containing PEG. Kirkwood-Buff solution theory has been applied to interpret these results, and it was found that the concentration dependence is determined by two factors: (1) the effective or solvent-averaged interactions between protein molecules in solution, and (2) three-body protein – protein – solvent and protein – solvent – solvent interactions. An approach is proposed for the experimental determination of both contributions that involves measuring osmotic pressures and volumetric properties of dilute to concentrated protein solutions.*

## Introduction

The partial molar volume plays a central role in protein solution thermodynamics. It is required for the experimental determination of many other thermodynamic properties, such as adiabatic compressibilities of proteins in solution from sound velocity measurements (Sarvazyan, 1991) or protein molecular weights from sedimentation measurements (Cantor and Schimmel, 1980). The protein partial molar volume at infinite dilution is also related directly to the protein–solvent pair correlation function (Kirkwood and Buff, 1951), and as such, reflects protein–solvent molecular interactions. This quantity is required in calculations of protein solubilities if McMillan-Mayer fugacities must be converted to Lewis-Randall fugacities (Cabezas and O'Connell, 1993), since it accounts for protein–solvent interactions that are not contained in solvent-averaged protein–protein interactions defined within the context of McMillan-Mayer theory. The concentration dependence of the partial molar volume can also be related to the potential of mean force between protein molecules in solution (Kirkwood and Buff, 1951; Buff and Brout, 1955). Thus, the measurement of this concentration dependence provides an experimental means of obtaining a potential of mean force from volumetric data that would complement conventional measurements of osmotic pressures (Vilker et al., 1981; Haynes et al., 1992) in developing a potential of mean force expression for concentrated protein solutions.

In experimental studies that have addressed the issue of the concentration dependence of the partial molar volume, or equivalently the concentration dependence of the partial specific volume, it has been found that, in some cases, no significant dependence is observed for protein concentrations as high as 0.5 g/g (Dayhoff et al., 1952; Hunter, 1966, 1967; Bull and Breese, 1968; Pilz and Czerwenka, 1973; Ueda and Mashino, 1982; Nolting and Sligar, 1993). However, others have reported partial specific volumes that do vary with protein concentration, depending on pH, salt concentration, and temperature (Kraemer, 1941; McMeekin et al., 1949; Millero et al., 1976; Bernhardt and Pauly, 1980; Kim and Kauzmann, 1980). Kim and Kauzmann (1980), for example, measured partial specific volumes for bovine serum albumin, oxyhemoglobin, and ovalbumin in deionized water, and found larger values at high protein concentrations compared to those obtained at infinite dilution. They concluded that their results were consistent with protein–protein interactions involving the contact of hydrophobic regions or the formation of ion

Correspondence concerning this article should be addressed to M. E. Paulaitis.

pairs of opposite charge on the surfaces of the two proteins, since such interactions would lead to an increase in the specific volume of the solution, presumably as a result of the diminished number of protein–water interactions. Bernhardt and Pauly (1980) reported that the protein concentration dependence of the partial specific volume of hemoglobin in aqueous solutions depended on the salt concentration. Specifically, they observed no concentration dependence for hemoglobin in deionized water, but found that partial specific volumes increased with protein concentration in aqueous solutions containing either sodium or potassium chloride.

In this article, we report measurements of partial specific volumes for $\alpha$-chymotrypsinogen in aqueous solutions containing NaCl, citrate, and/or polyethylene glycol (PEG) over a range of protein concentrations. The measured concentration dependence of the partial specific volume is interpreted within the context of Kirkwood-Buff solution theory to evaluate the relative importance of protein–protein and protein–solvent interactions in describing volumetric properties of concentrated protein solutions. The effect of added salt on the protein concentration dependence of the partial specific volume is also evaluated.

## Materials and Methods

Density measurements were made using the vibrating tube method of Kratky et al. (1969). In this method, the period of oscillation of a vibrating tube containing a fixed volume of sample is measured. The density of this sample is determined by applying Hooke's law, according to which the mass of the vibrating tube and the sample is proportional to the square of the period of oscillation. Therefore, the density of the solution can be calculated from the period using the relation,

$$\rho = (\tau^2 - B)/A \tag{1}$$

where $\rho$ is density, $\tau$ is the measured period of oscillation, and $A$ and $B$ are constants determined from calibrations with air and water. The apparatus used for these measurements was an Anton Paar Model DMA 60 Density Meter coupled to either a DMA 512 or DMA 602 density cell. These cells differ in their range of operating conditions, sample size, and accuracy. The DMA 512 cell was used in only a few of the early measurements.

The protein $\alpha$-chymotrypsinogen A type II (from bovine pancreas) was purchased from Sigma (Cat. No. C4879) as a 6X crystallized, essentially salt-free, lyophilized powder and used without further purification. The actual amount of protein in a given sample of this powder was determined by an independent assay. NaCl was purchased from Aldrich (Cat. No. 22,351-4; 99 + %; A. C. S. reagent), citric acid from Fisher (Cat. No. A-104; $\geq$ 99% by assay), and polyethylene glycol, 10,000 mol wt., from Sigma (Cat. No. P-6667).

Protein solutions were prepared on a mass basis by combining known weights of the protein and solvent. The solvents, consisting of aqueous solutions of NaCl, citrate, and/or polyethylene glycol (Table 1), were filtered through 0.22-$\mu$m Millipore Millex-GV filters before use. The solutions were prepared by gently mixing protein and solvent, taking care to avoid excessive shear or introducing dissolved gas into the solution. Samples at different concentrations were prepared as individual aliquots or by diluting a concentrated stock solution. A range of protein mass fractions up to a maximum of about 0.08 were studied.

The following protocol was used for each measurement. Before a sample was injected into the density cell, the cell was washed five times with deionized water, once with acetone, and blown dry for three minutes with compressed air filtered through cotton and calcium chloride. Samples were injected slowly by hand with a syringe, taking care to avoid introducing bubbles into the cell or the sample. Measurements with the DMA 602 cell were made on three different samples of a given concentration. Five measurements were required when the DMA 512 cell was used to achieve a comparable level of precision in the measured densities. Sample sizes were approximately 2.7 mL for the DMA 512 cell and 1.0 mL for the DMA 602 cell. Calibrations with air and water were also performed prior to each set of experiments. For accurate density measurements, the temperature of the cell must be precisely controlled. This was achieved with either a Neslab model RTE-110 or a Hart model 7011 circulating water bath. All measurements were made at 25°C.

**Table 1. Partial Specific Volumes for $\alpha$-Chymotrypsinogen in Aqueous Solutions at 25°C**

| | Solvent | $\hat{V}_p^\infty$ | $b_0$ | $b_1$ | $b_2$ | $\sigma(b_2)$ | $p(b_2)$ |
|---|---|---|---|---|---|---|---|
| I. | 0.1-M NaCl/ 0.01-M citrate pH 6.5 | 0.7199 | 0.997115 | −0.277109 | −0.000822 | 0.006792 | 0.904787 |
| II. | 0.1-M NaCl/ 0.01-M citrate pH 3 | 0.7124 | 0.997870 | −0.284770 | −0.008216 | 0.005054 | 0.130005 |
| III. | 0.3-M NaCl/ 0.01-M citrate pH 3 | 0.7120 | 0.989749 | −0.276854 | 0.013888 | 0.007454 | 0.087090 |
| IV. | 3% PEG 10 K pH 2 | 0.7197 | 0.996928 | −0.276852 | −0.014312 | 0.011316 | 0.228150 |
| V. | 0.1-M NaCl/ 1% PEG 10 K pH 2 | 0.7165 | 0.996966 | −0.280115 | −0.014533 | 0.005415 | 0.017807 |

The units for $\hat{V}_p^\infty$ are cm$^3$/g. See Eq. 2 for definitions of the $b_i$ coefficients.

For each measurement, a sample was injected into the cell and the period of oscillation, which is updated by the instrument at regular intervals ( ~ 35 s), was monitored. The final value of this quantity was taken to be the arithmetic mean of the last 8 to 12 readings displayed by the instrument after the sample had reached thermal equilibrium and the readings had thus converged to a roughly constant value. In our experiments, this constant value was achieved after the first four or five readings. The standard deviation of the average value was generally less than or equal to $10^{-6}$, and any measurement with a standard deviation greater than this value was rejected. The individual readings were then converted to densities using Eq. 1. The reciprocals of these densities over the entire range of protein concentrations were then fit to the following second-order polynomial for the specific volume as a function of the mass fraction of protein in solution,

$$\hat{V} \equiv 1/\rho = b_0 + b_1\omega + b_2\omega^2 \tag{2}$$

using the IMSL statistical subroutine DRPOLY (Visual Numerics, Inc.). The individual measurements of specific volumes at each mass fraction were the input for this least-squares data regression, rather than an average value at each concentration. The standard deviation of the specific volume was taken to be $1\times10^{-6}$, the nominal accuracy of the instrument.

The partial molar volume is obtained from Eq. 2 by differentiating the total volume with respect to the number of moles of protein at constant temperature and pressure, while holding the number of moles of all other mixture constituents constant (Sandler, 1989). The partial specific volume is equal to the partial molar volume divided by the molecular weight of the protein. The partial specific volume of the protein at infinite dilution is then given by

$$\hat{V}_p^{\infty} = b_0 + b_1 \tag{3}$$

and at finite concentrations by

$$\hat{V}_p = \hat{V}_p^{\infty} + b_2\omega(2-\omega). \tag{4}$$

Three concerns were addressed in applying the vibrating-tube method to measure densities of protein solutions. One was the loss of protein during filtration in the sample preparation. To quantify this loss, a series of measurements were made with the DMA 512 cell on aliquots of a 1% (w/w) protein solution, half of which were filtered through the 0.22-$\mu$m Millex-GV filter and half of which were not. Within the accuracy of the measurements, no significant differences in densities were observed between the filtered and unfiltered solutions. A second concern was the presence of dissolved gas in the samples. Degassing the protein solutions proved to be extremely difficult because the procedure results in water evaporation from the sample, as well as foaming of the solution and subsequent loss of sample, both of which would seriously affect the density. Measurements made with the DMA 512 cell on a degassed sample of deionized water and a second sample that was not degassed showed no significant differences in density. In addition, the protein solutions were filtered before use, which helps to remove dissolved gas. A final concern was the possibility of introducing systematic errors in the density measurements due to high solution viscosities. Bernhardt and Pauly (1980) reported that the vibrating-tube method overestimates the density for highly viscous protein solutions and that the error increases with increasing viscosity. They reported this effect to be measurable when the protein mass fraction exceeded 0.1 for salt-free solutions of bovine hemoglobin and bovine serum albumin, while the presence of even small amounts of salt was found to reduce considerably the viscosity of the bovine serum albumin solutions. Since our measurements on solutions of $\alpha$-chymotrypsinogen were made at protein mass fractions of 0.08 or less, and all solutions contained at least 10-mM salt, the effect of solution viscosity on the measured density was assumed to be negligible.

## Results

Densities of $\alpha$-chymotrypsinogen solutions were measured as a function of protein concentration in five different solvents at 25°C, and the infinite-dilution partial specific volume and the concentration dependence of the partial specific volume (the $b_2$ coefficient) were calculated as described earlier. The results are given in Table 1. The standard deviation and the $p$-value for the $b_2$ coefficients are also tabulated. The $p$-value is the probability that the regressed $b_2$ coefficient is zero. The $p$-values for all regressed $b_0$ and $b_1$ coefficients were found to be negligible.

The literature value for $\hat{V}_p^{\infty}$ of $\alpha$-chymotrypsinogen in 0.001-M HCl solution at 30.4°C is 0.721 cm$^3$/g (Schwert, 1951), which is in excellent agreement with our value of 0.7199 cm$^3$/g in 0.1-M NaCl/0.01-M citrate solution at pH 6.5 (designated solvent I in Table 1). The effect of reducing pH from 6.5 to 3 (solvent I → II in Table 1) is to increase the net positive charge on the protein and to decrease its dipole moment (Haynes et al., 1992; Antosiewicz and Porschke, 1989). The value of $\hat{V}_p^{\infty}$ in this case decreases to 0.7124 cm$^3$/g, which suggests greater electrostriction of waters of hydration at pH 3. The value of $b_2$ is not statistically different from zero for solvent I and is negative for solvent II.

Increasing salt concentration (solvent II → III in Table 1) results in a negligible change in $\hat{V}_p^{\infty}$, but the $b_2$ value at the higher salt concentration is now positive and larger in magnitude by nearly a factor of 2. For $b_2 > 0$, the partial specific volume increases with increasing protein concentration. Adding salt should affect protein–protein interactions by reducing the Debye screening length, thereby decreasing the effective excluded volume (Israelachvili, 1992). Since this would lead to lower values of $b_2$, in contradiction to our observations, it follows that an additional effect of added salt must be invoked to explain this concentration dependence.

For the two PEG solutions (solvents IV and V in Table 1), the measured values of $\hat{V}_p^{\infty}$ fall within the range of values obtained for the other three solutions, and $b_2 < 0$: the partial specific volumes decrease with increasing protein concentration. Adding PEG to these solutions increases the net osmotic attraction between protein molecules because polymer is excluded from the region between them (Mahadevan and Hall, 1990). This net attraction would lead to a decrease in the partial specific volume at high protein concentrations,

which is in agreement with the negative $b_2$ values reported in Table 1. In addition, the enhanced osmotic attraction would result in fewer protein–solvent interactions, which may be offset by a greater number of polymer–solvent interactions. Since PEG at this molecular weight has approximately the same infinite-dilution partial specific volume in water as the protein (Haynes et al., 1993), the interactions of the solvent with the polymer and the protein will be similar. This would also lead to negative $b_2$ values.

## Discussion

The Kirkwood-Buff expression for the partial molar volume of the solute (component 2) in a two-component system is given by

$$\bar{v}_2 = \frac{1 + \rho_1(G_{11} - G_{12})}{\rho_1 + \rho_2 + \rho_1\rho_2(G_{11} + G_{22} - 2G_{12})} \tag{5}$$

where $\rho_i$ is the molar density of component $i$, and the $G_{ij}$ cluster integrals are defined by

$$G_{ij} \equiv \int [g_{ij}(r) - 1]\, d\underline{r} \tag{6}$$

where $g_{ij}(r)$ is the $ij$ pair correlation function (Kirkwood and Buff, 1951). The partial molar volume of the solute at infinite dilution is obtained from Eq. 5 by taking the limit $\rho_2 \to 0$. Thus,

$$\bar{v}_2^{\infty} = 1/\rho_1^0 + G_{11}^0 - G_{12}^0 \tag{7}$$

where $\rho_1^0$ is the molar density of the pure solvent and the superscripts on the cluster integrals denote that these quantities are evaluated for an infinitely dilute solution. In addition,

$$G_{11}^0 = kT\,\kappa_T^0 - 1/\rho_1^0 \tag{8}$$

where $\kappa_T^0$ is the isothermal compressibility of the pure solvent. Hence,

$$\bar{v}_2^{\infty} = kT\,\kappa_T^0 - G_{12}^0, \tag{9}$$

which is the well-known Kirkwood-Buff expression for the infinite-dilution partial molar volume of the solute. To obtain the concentration dependence of the partial molar volume, Eq. 5 can be expanded in powers of $\rho_2$. Neglecting terms higher than first order gives, after rearranging,

$$\bar{v}_2 = \bar{v}_2^{\infty} + \rho_2\left[(G'_{11} - G'_{12})^0 + \bar{v}_2^{\infty}(2G_{12}^0 - G_{11}^0 - G_{22}^0)\right] \tag{10}$$

where

$$(G'_{11} - G'_{12})^0 \equiv \lim_{\rho_2 \to 0} \frac{\partial}{\partial \rho_2}(G_{11} - G_{12})|_{T,P}. \tag{11}$$

A similar expression for the partial molar volume in terms of direct correlation function integrals is given elsewhere (O'Connell, 1971).

Equation 10 allows a qualitative interpretation of the concentration dependence of the partial specific volume, as given by the two terms in brackets. The $(G'_{11} - G'_{12})^0$ term is related to cluster integrals involving three-body correlations (Buff and Brout, 1955)—specifically, protein–protein–solvent and protein–solvent–solvent triplet correlations—and as such, reflects the influence of perturbations in protein–solvent pair correlations due to the presence of a second protein molecule relative to perturbations in solvent–solvent pair correlations due to the presence of the protein. The second term in brackets is related to the Lewis-Randall osmotic second virial coefficient (Cabezas and O'Connell, 1993), while the $G_{22}^0$ cluster integral is related to the McMillan-Mayer osmotic second virial coefficient. The $G_{22}^0$ cluster integral reflects solvent-averaged protein–protein interactions, and can be calculated from a potential of mean force expressed as the sum of various molecular interactions (Vilker et al., 1981; Haynes et al., 1992). The observed concentration dependence of the partial specific volume of $\alpha$-chymotrypsinogen is determined by the extent to which these two terms offset one another. From Eqs. 4 and 10,

$$(G'_{11} - G'_{12})^0 + \bar{v}_2^{\infty}(2G_{12}^0 - G_{11}^0 - G_{22}^0) = 2b_0b_2(M_2)^2 \tag{12}$$

where $M_2$ is the molecular weight of $\alpha$-chymotrypsinogen ($\sim 2.5\times10^4$ g/mol). Taking the $b_0$ and $b_2$ coefficients from Table 1 for solvent III, the righthand side of Eq. 12 is calculated to be $+3.4\times10^7$ (cm$^3$/mol)$^2$. Moreover, $G_{22}^0$ can be estimated from osmotic pressure data for $\alpha$-chymotrypsin in 0.1-M potassium sulfate solutions at pH 3.0 and 25°C (Haynes et al., 1992). Using a value of $-4.2\times10^5$ cm$^3$/mol for this cluster integral and the volumetric data in Table 1 for solvent III, the second term in brackets on the lefthand side of Eq. 12 is found to be $-1.5\times10^{10}$ (cm$^3$/mol)$^2$, which is three orders of magnitude greater than the sum of the two terms. It follows therefore that $(G'_{11} - G'_{12})^0$ must be slightly greater than $+1.5\times10^{10}$ (cm$^3$/mol)$^2$, and the two terms on the lefthand side of Eq. 12 must be nearly equal and opposite in sign.

## Conclusions

Measured partial specific volumes of $\alpha$-chymotrypsinogen in concentrated protein solutions can be either greater than or less than the corresponding value at infinite dilution. This concentration dependence emerges as the small difference between two much larger contributions that reflect (1) solvent-averaged protein–protein interactions in solution, and (2) protein–protein–solvent and protein–solvent–solvent triplet correlations. Little is known about the molecular nature of this second contribution. The approach we propose is to measure osmotic pressures and volumetric properties of dilute to concentrated protein solutions, from which both contributions can be determined. The use of volumetric data in addition to osmotic pressures would undoubtedly lead to better expressions for the potential of mean force than those derived from osmotic second virial coefficients alone. Moreover, an experimental probe of three-body interactions in

concentrated protein solutions is desirable, and would improve our primitive understanding of the molecular origins of these interactions.

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## Notation

$b_i$ = constants of least-squares regression defined by Eq. 2
$G_{ij}^0$ = cluster integral evaluated at infinite dilution of the solute
$k$ = Boltzmann's constant
$T$ = temperature
$\bar{v}_2$ = partial molar volume of the solute
$\bar{v}_2^*$ = partial molar volume of the solute at infinite dilution

### *Greek letters*

$\omega$ = protein mass fraction
$\rho_2$ = molar density of the solute

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