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VOLUMETRIC CHARACTERIZATION OF α-CHYMOTRYPSINOGEN A IN AQUEOUS UREA AND ALKYLUREA SOLUTIONS AT 25 °C†

Nataša Poklar, Romana Cerc-Korošec, Branko Èeh and Gorazd Vesnaver

Department of Chemistry, University of Ljubljana, Aškerèeva 5, 1000 Ljubljana, Slovenia

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Abstract: The apparent specific volume, $\varphi_{2,}$ of α-chymotrypsinogen A (α-ctg A) in a aqueous solutions containing different amount of urea, methylurea, N,N'-dimethylurea or ethylurea was determined from density measurements. Since no dependence of φ_2 on protein concentration was observed its values were equated with the corresponding specific volumes at the infinite dilution, $\bar{v}_2^0$. The small positive changes in $\bar{v}_2^0$ observed in urea and methylurea solutions at the highest denaturant concentration were ascribed to the complete unfolding of α-ctg A while significantly larger positive changes observed in N,N'-dimethylurea and ethylurea solutions were explained in terms of the incomplete unfolding of α-ctg A into a compact intermediate state.

INTRODUCTION

The partial molar volume, $\bar{V}_2^o$ of a protein is a macroscopic observable which is sensitive to the hydration/solvation properties of the protein atomic groups exposed to the solvent, as well as to the structure, dynamics, and conformational properties of the protein interior inaccessible to the solvent [1, 2].

† Dedicated to the memory of Professor Dr. Anton Šebenik

According to scaled particle theory [3] the partial molar volume at the infinite dilution, $\overline{V}_2^o$, of a solute can be considered as a sum of four terms [4-6]:

$$\overline{V}_2^o = V_M + V_T + V_I + ß_{TO}RT \quad (1)$$

where V_M, is the intrinsic molar volume of the solute, which corresponds to the solute domain into which solvent cannot penetrate; V_T is the "thermal volume" of the solute, which corresponds to an "empty" domain around the solute molecule that results from the mutual thermal motions of the solute and solvent molecules; V_I is the "interaction volume" which represents the change in the solvent volume due to the hydration/solvation; and $ß_{TO}RT$ is the ideal term, where $ß_{TO}$ is the coefficient of the solvent isothermal compressibility and R is the universal gas constant [7].

The ideal term ($ß_{TO}RT$) in equation (1) is small (about 1 cm^3 mol^{-1} for aqueous solutions) and therefore can be neglected when considering macromolecules such as proteins, which usually have large partial molar volumes, $\overline{V}_2^o$, (order of magnitude ~10^4 cm^3 mol^{-1}) determined primarily by contributions V_M, V_T and V_I [8, 9]. Consequently, the partial specific volume at the infinite dilution, $\overline{v}_2^0$, of a protein of a molecular mass M can be expressed as:

$$\overline{v}_2^0 = v_M + v_T + v_I \quad (2)$$

where $v_M = V_M/M$; $v_T = V_T/M$; $v_I = V_I/M$.

For globular proteins, the intrinsic volume, V_M, is equal to the sum of the van der Waals volumes of the constituent atoms plus the total volume of the voids inside the protein molecule, which result from its imperfect packing. The thermal volume, V_T, can be considered to correspond to a layer of "empty" space around the solute molecule, which results from the mutual thermal motions of the solute and solvent molecules. For low-molecular-mass compounds, it has been shown that, on average, the thickness, Δ, of such a layer equals ~ 0.50 Å at 25°C and does not significantly depend of the shape and the chemical nature of the solute molecule [6]. The interaction volume, V_I, results from the interactions of each atomic group on the protein surface

with surrounding water molecules (electrostriction, hydrophobic interactions, hydrogen bonding).

EXPERIMENTAL PROCEDURES

Materials

α-Chymotrypsinogen A (α-ctg A) from bovine pancreas was purchased from Sigma as a six-time crystallized powder. Before use it was dialyzed against three times distilled water for 24 hours and then lyophilized for 48 hours and kept in exicator.

Ultra pure urea was a product of Kemika (Zagreb, Croatia). Methylurea, N,N'-dimethylurea and ethylurea were supplied by Fluka (Buch, Switzerland). Before use, all ureas were recrystalized from hot ethanol and dried for 48 hours under a vacuum at 40 ^{o}C in the presence of phosphorus pentoxide.

Solutions for density measurements were prepared by weighing dried protein into 10 ml Erlenmeyer flask and by adding a three times distilled water and dry urea or alkylureas into the flask on a precision analytical balance (Sartorius Research RC 210S, Goettingen, Germany). Series of ten α-ctg A solutions in urea or alkylurea solutions of different concentrations were prepared in the concentration range from 0 to 20 mg per g of solution. Solutions of urea and alkylureas used as a solvent for densitometry were prepared in the same way.

Density measurements

All densities were measured at 25°C with a precision of ± 1.5 x 10^{-6} g cm^{-3} using a vibrating tube densimeter (DMA-60, Anton Paar, Austria). In solutions containing m_2 grams of solute and m_1 grams of solvent the apparent specific volume of α-ctg A in triple distilled water and different urea, methylurea, N,N'-dimethylurea and ethylurea solutions, φ_2, defined as:

$$j_2 = \frac{V_{solution} - V_{solvent}}{m_2} \qquad (3)$$

was calculated from the well-known relation [10]:

$$j_2 = \frac{1}{w_2}\left(\frac{1}{r} - \frac{1-w_2}{r_o}\right) \qquad (4)$$

in which w_2 is the weight fraction of solute in the measured solution and ρ and ρ_o are the densities of the solution and the solvent, respectively. The corresponding partial specific volumes, $\bar{v}_2$, can be obtained as:

$$\bar{v}_2 = m_2\left(\frac{\partial j_2}{\partial m_2}\right)_{m1} + j_2, \quad P,T = \text{const.} \qquad (5)$$

and since at all measured concentrations of α-ctg A (between 1 and 20 $mg/g_{solution}$) no concentration dependence of φ_2 was observed the measured φ_2 values were equated with the corresponding partial specific volumes at the infinite dilution, $\bar{v}_2^0$.

RESULTS AND DISCUSSION

The $\bar{v}_2^0$ values of α-ctg A in pure water and in the presence of different concentrations of urea and alkylureas are listed in Table 1 and plotted in Figure 1.

The value of partial specific volume of α-ctg A at infinite dilution of $\bar{v}_2^0$ = 0.731 cm^3g^{-1} obtained in triple distilled water at 25 °C is comparable with the literature values of 0.733 cm^3g^{-1} [11] and 0.730 cm^3g^{-1} [12]. In the literature we can find that the partial specific volumes of the majority globular proteins in their native states fall in a surprisingly narrow range between 0.70 and 0.75 $cm^3\ g^{-1}$ [9, 13]. This similarity in $\bar{v}_2^0$ values suggests that the globular proteins are essentially similar with respect to their "average" intrinsic packing and hydration properties when normalized per gram of a protein (or per amino acid residue).

The dependence of $\bar{v}_2^0$ of α-ctg A on denaturant concentration in aqueous solutions of urea and alkylureas is presented in Fig. 1. In urea solutions, similarly as observed before [11], $\bar{v}_2^0$ increases with concentration, reaches a maximum at concentration of about 4 M and after that it starts decreasing. In 8 M urea the measured $\bar{v}_2^0$ value of 0.731 cm^3g^{-1} is exactly the same as the value of $\bar{v}_2^0$ observed in

triple distilled water. In methylurea solutions increasing of denaturant concentration to 8 M results only in a slightly increase of $\bar{v}_2^0$ from 0.731 to 0.734 cm^3g^{-1}.

Table 1: *The partial specific volumes at infinite dilution of a-chymotrypsinogen A in water and aqueous urea, methylurea, N,N'-dimethylurea and ethylurea solutions at 25°.*

Solvent	$\bar{v}_2^0$ (cm^3g^{-1})*	Solvent	$\bar{v}_2^0$ (cm^3g^{-1})*
Distilled Water	0.731	N,N'-Dimethylurea	
Urea		2 M	0.730
2 M	0.733	4 M	0.736
4 M	0.736	6 M	0.745
6 M	0.735	7 M	0.750
8 M	0.731	8 M	0.752
Methylurea		Ethylurea	
2 M	0.731	2 M	0.733
4 M	0.732	4 M	0.741
6 M	0.733	6 M	0.744
8 M	0.734	7 M	0.745

*The estimated error is ± 0.001 cm^3g^{-1}.

In contrast, a pronounced increase in $\bar{v}_2^0$ up to 0.752 cm^3g is observed in N,N'-dimethylurea solution when denaturant concentration is increased to 8 M. In ethylurea solutions $\bar{v}_2^0$ also increases significantly with denaturant concentration and around 6 M it nearly levels off at around 0.745 cm^3g^{-1}.

Comparing the results of our previous dialysis and fluorescence studies of α-ctg A in the presence of urea and alkylureas [14-16] with the results obtained in this study we can notice the correlation between the urea concentration at the maximum urea binding to α-ctg A (5 M), the urea concentration at which half of the protein undergoes a conformational transition (5 M) and the urea concentration at which the $\bar{v}_2^0$ starts to decrease (Fig. 1). In methylurea, a similar correlation between methylurea

binding [15] and $\bar{v}_2^0$ was observed; up to 8 M concentration they both increase continuously. For the other two alkylureas, N,N'-dimethylurea and ethylurea, a similar dependence of $\bar{v}_2^0$ and α-ctg A fluorescence intensity on concentration was observed, they both show the same denaturant concentration interval in which the α-ctg A unfolding occurs [16].

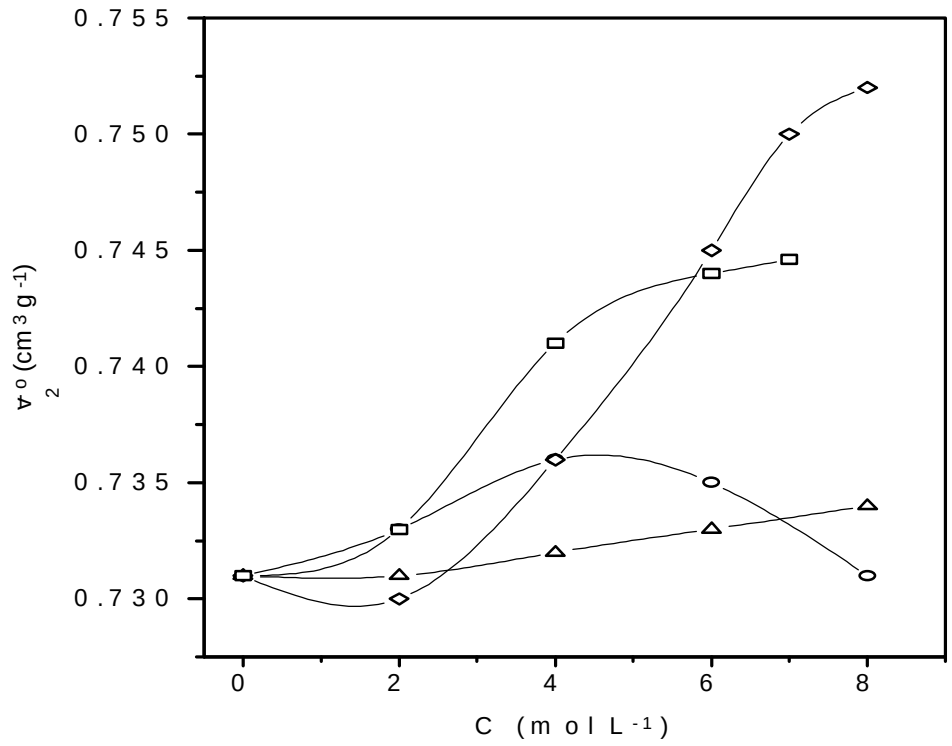

***Figure 1**: The partial specific volume at infinite dilution, $\bar{v}_2^0$, of α-ctg A at 25 °C in the presence of urea (O), methylurea (D), N,N'-dimethylurea (à) and ethylurea (□) as a function of denaturant concentration, C.*

From the standpoint of volume changes, protein denaturation can be considered as a three step process: (i) disruption of the tightly packed interior of the folded protein; (ii) creation of the cavity in the solvent large enough to accommodate the unfolded protein chains, followed by the placement of these chains into the cavity; (iii) "switching on" the interaction between the solvent and the formerly buried polar,

and charged groups of the protein molecules. Disruption of the protein interior brings a negative specific volume change, due to a decrease in the size and number of the intermolecular voids. Switching on the new solute-solvent interactions also brings about negative specific volume changes for charged and polar group, primarily due to the electrostriction and hydrogen bonding. In contrast, cavity formation around the solute molecules caused by mutual thermal motion of solute and solvent molecules will lead to positive changes in the thermal volume due to an increase in the accessible surface area of the protein [7].

The small urea induced increasing of $\bar{v}_2^0$ of α-ctg A observed up to about 4 M (Figure 1) can be interpreted in terms of an electrostriction effect. Namely, due to a partial replacement of electrostricted water molecules in the solvation sheaths of the protein charged groups by urea molecules, the amount of electrostricted water is reduced, and consequently, the $\bar{v}_2^0$ is increased [17]. Another contribution, a negative one, could stem from hydrogen bonding of urea molecules to the peptide group, however, it has been found that such contribution is much smaller than the one due to electrostriction [18]. Then, there is the volume change originating from the hydrophobic hydration/solvation whose sign is negative and the magnitude also small compared with the electrostriction effect [19].

The disruption of the protein interior caused by the increasing of urea concentration brings a negative specific volume change, due to a decrease in the size and number of the intermolecular voids. Switching on new solute-solvent interactions also brings about negative specific volume changes for charged and polar group, primarily due to electrostriction and hydrogen bonding. The observed $\bar{v}_2^0$ of α-ctg A in 8 M urea solution has the same value as in the native state. It means that there have to be some positive contribution to the $\bar{v}_2^0$. Recently, Chalikian and Breslauer [7] suggest a new interpretation of partial volume data of biopolymers, which provides a rationale for the long-standing “protein-volume paradox” that is, the inconsistency between significant negative volume changes one would expect to accompany the protein denaturation and the experimentally observed small negative or even positive changes

in $\bar{v}_2^0$. According to his suggestion the observed near-zero volume changes accompanying the unfolding of small globular proteins reflect fortuitous compensations between the negative contributions due to enhanced hydration (hydrogen bonding, electrostriction) and reduced intramolecular void volume and the positive contribution due to the increased thermal volume that results from increased surface area of the protein accessible to the solvent.

The observed changes in $\bar{v}_2^0$ of α-ctg A in the presence of methylurea can be explained in a similar way with the only difference that methylurea is weaker denaturant than urea. The introduction of N,N'-dimethyl and ethyl groups into urea molecule give rise to stronger hydrophobic interactions with nonpolar groups and lowers the ability of these molecules to form a hydrogen bonds with the protein. Studies on some model dipeptides [20] have shown that the volume changes accompanying their transfer from water into 4 M and 8 M urea and methylurea, to 4 M and 7 M N,N'-dimethylurea and to 4 M and 6 M ethylurea are small and positive. These data also show that in solutions of alkylureas the contributions to the apparent specific volumes due to hydrophobic and other noncoulombic interactions are small and blurred by much stronger electrostriction effect [20]. This means that in case of complete unfolding of α-ctg A the observed large positive contribution to $\bar{v}_2^0$ cannot be explained simply in terms of lower ability of these two denaturants to form hydrogen bonds with the protein. A possible reason for the observed behavior might be that even at the highest concentrations of N,N'-dimethylurea and ethylurea α-ctg A unfoldes only to an intermediate compact state. If this is so, the negative contribution to $\bar{v}_2^0$ due to electrostriction and hydrogen bonding may be more reduced than the positive contribution due to the accessible surface of the protein and the net result would be positive $\bar{v}_2^0$. This suggestions that α-ctg A may unfold to different final states in urea and methylurea than in N,N'-dimethylurea and ethylurea is in line with the results of circular dichroism studies performed in the far-UV CD range on α-ctg A in urea and alkylurea solutions [21]. According to these studies increasing of urea and methylurea concentration induces collapse of the α-ctg A secondary structure into the

random coil form while in N,N'-dimethylurea and ethylurea solutions it gives rise to an increase of the α-helical structure of α-ctg A secondary structure, an increase that is typical for formation of compact intermediate states of proteins. Finally, it has been reported recently, that increasing of temperature or decreasing pH of α-ctg A solutions results in the protein transition into compact intermediate states accompanied by positive changes in $\bar{v}_2^0$ [22].

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POVZETEK

Navidezni specifièni volumni, φ_2, α-kimotripsinogena A (α-ctg A) v vodnih raztopinah razliènih koncentracij seènine, metilseènine, N,N'-dimetilseènine in etilseènine so bili doloèeni iz izmerjenih gostot. Vrednosti φ_2 niso koncentracijsko odvisne zato so kar enake ustreznim parcialnim specifiènim volumnom pri neskonènem razredèenju, $\bar{v}_2^0$. Majhne pozitivne spremembe $\bar{v}_2^0$ α-ctg A opažene pri visokih koncentracijah seènine in metilseènine pripišemo popolnemu razvitju α-ctg A, medtem ko pa znatnejše pozitivne spremembe v $\bar{v}_2^0$ opažene v raztopinah N,N'-dimetilseènine in etilseènine razložimo z nepopolnim razvitjem α-ctg A v kompaktno intermediatno stanje.