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Partial specific volumes and interactions with solvent components of α -globulin from *Sesamum indicum* L. in urea and guanidine hydrochloride «

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Abstract. The interaction of α-globulin with urea/guanidine hydrochloride was investigated by determining the apparent partial specific volumes of the protein in these solvents. The apparent partial specific volumes were determined both under isomolal and isopotential conditions. The preferential interaction parameter with solvent components calculated were 0.08 and 0.1 g of urea and guanidine hydrochloride respectively per g protein. In both the cases the interaction was not preferential with water. The total binding of denaturant to α-globulin was calculated both for urea and guanidine hydrochloride and the correlation between experimentally determined number of mol of denaturant bound per mol of protein and the total number of peptide bonds and aromatic amino acids were found to be in excellent agreement with each other. The changes in volume upon transferring α-globulin from a salt solution to 8 M urea and 6 M guanidine hydrochloride were also calculated.

Keywords. Partial specific volume; denaturation; preferential interaction; α-globulin; urea and guanidine hydrochloride.

Introduction

Urea and guanidine hydrochloride (GuHCl) are classical protein denaturants and their use has been extensive. GuHCl has been frequently used as a subunit dissociating agent for multimeric proteins. Urea, although less effective, is still used extensively as a protein denaturant. The mechanism of denaturation of proteins by urea and GuHCl and the thermodynamic aspects of this reaction have been the subject of extensive studies and the present state of knowledge has been summarised by Tanford (1968, 1970) and Pace (1975).

α-Globulin is the major storage protein of sesame seed, *Sesamum indicum* L. and constitutes nearly 65-70% of the total proteins present in the seeds. The protein has been-isolated and characterized and some of its properties studied by various physico chemical techniques (Nath *et al.,* 1957; Sinha and Sen, 1962;

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Abbreviations used: GuHCl, Guanidine hydrochloride; PS buffer, 0.1 M phosphate buffer, pH 6.5 containing 1 M NaCl.

Ventura and Lima, 1963; Prakash, 1976; Prakash and Nandi, 1976, 1977a, b; Prakash, 1978; Prakash, 1980; Prakash *et al.,* 1980; Prakash, 1981 a, b; Prakash, 1982; Lakshmi and Nandi, 1977, 1978). The weight average molecular weight of the protein has been estimated to be 274000±20,000 by sedimentation equilibrium in phosphate buffer, 0.1 M, pH 6.5 containing 1 M NaCl (Prakash, unpublished results). It is shown to consist of at least 12 subunits (Prakash and Nandi, 1978).

The partial specific volume, $\bar{V}$ of a protein in a particular solvent is a parameter which is necessary for the determination of the molecular weight of the protein from sedimentation equilibrium data, as well as for the interpretation of small angle x-ray scattering results. In practice, this quantity, $\bar{V}$ is frequently assumed or calculated from amino acid composition. A small error in the partial specific volume can lead however, to a large error in the calculated molecular weight ultimately leading to erroneous conclusions on the stoichiometry of assembly of macromolecules and also to serious errors in the calculation of the various thermodynamic parameters of associating systems. In particular if the experiment is performed in concentrated denaturant solutions such as 6 M GuHCl or 8 M urea the problem becomes more serious due to a large change in partial specific volume of proteins in these solvents. Although several methods are currently used for the measurement of partial specific volume like pycnometry, density gradient columns (Linderstrom-Lang and Lanz, 1935; Hvidt, *et al*., 1954; Reithel and Sakura, 1963), H_2O-D_2O exchange technique (Edelstein and Schachman, 1967; Schachman, 1957), magnetic float method (Ulrich *et al*., 1964) and precision densimetry (Stabinger *et al*., 1967), the last two methods are used more extensively in the determination of the $\bar{V}$ of proteins due to their high precision and ease of operation. The measurement of the partial specific volume as a thermodynamic parameter can reflect and give an insight into protein denaturation, and its contribution is two fold. First, through changes in the volume of the protein upon denaturation; second, through interactions which the protein may undergo with the solvent component *i.e*. preferential interaction either with water or with the denaturant. It is feasible to measure both the preferential interaction parameter as well as volume change upon denaturation by determining the partial specific volume of the protein under various thermodynamic conditions.

α-Globulin has been shown to undergo association-dissociation in the presence of various additives like electrolytes, detergents, urea, GuHCl, acid and alkali. With some of the reagents, the association-dissociation process is also accompanied by denaturation and conformational changes in the protein (Prakash, 1976; Prakash and Nandi, 1976, 1976a, b, 1978; Prakash *et al*., 1980; Prakash, 1981; Lakshmi and Nandi, 1977,1978). Of particular interest were urea and GuHCl, in the presence of which α-globulm initially undergoes aggregation and dissociation and ultimately denaturation (Prakash and Nandi, 1977b) leading to 100% aperiodic structure (Prakash *et al*., 1980). Hence it was of interest to look into the thermodynamics of the interaction of these denaturants with α-globulin. The present paper describes the measurements of the preferential interaction parameters, volume changes upon denaturation and the calculations of the various thermodynamic parameters in 8 M

urea and 6 M GuHCl solutions to get an insight into the dynamics of the multimeric protein α-globulin, in these solvents.

Experimental procedures

Materials

Extreme purity GuHCl from Heico Inc., USA was used after purification as described under Methods. Ultrapure urea from Schwarz/Mann was used after purification as described under Methods. Reagent grade sodium phosphate, sodium acetate, methylamine hydrochloride, phoshorus pentoxide and acetic acid were from Fischer Scientific Co., USA. All other chemicals were of Reagent grade and were used without further purification.

Methods

Purification of GuHCl: GuHCl dissolved in water was passed through a 40μ pore size millipore filter system and the ultraviolet absorbance of this solution was measured in a Cary 118 spectrophotometer against deionized water from 240-400 nm. Only solutions which had less than 0.05 absorbance at 240 nm and no absorbance between 260-400 nm were used.

Purification of urea: A concentrated solution of urea in water was treated with activated charcoal and stirred for 4 h. The solution was filtered through Whatman filter paper No. 44 to remove coarse char coal particles and further filtered twice through millipore filter and to the filtrate methylamine hydrochloride was added (final concentration-0.012 M) and stirred well. The solution was passed through a column previously gravity packed with Bio-Rad Analytical grade mixed bed resin, AG 501-X8(D) 20-50 mesh, fully regenerated. The eluate concentration of urea was measured both densimetrically and refractometrically with water. The final concentration of urea was adjusted to 8 M with proper buffer salts to have a 8 M urea solution in buffer as described elsewhere (Prakash *et al*., 1981b).

Preparation of α-globulin solution in dilute buffer

α-Globulin was isolated in a homogeneous form by the previously described method (Prakash and Nandi, 1978). A solution in 0.1 M phosphate buffer, pH 6.5 containing 1 M NaCl (PS buffer) was prepared.

Preparation of α-globulin solution in 8 M urea buffer or 6 M GuHCl

The partial specific volume of α-globulin in 8 M urea buffer and 6 M GuHCl was measured (i) at constant molality and (ii) at constant chemical potential as described elsewhere (Prakash *et al*., 198 1b).

For measurements at constant molality, 5-25 mg sample of the protein was weighed into acid-cleaned test tubes and dried again at 40°C over phosphorus pentoxide for at least 56 h. Air was admitted into the vacuum oven by bubbling it through a concentrated sulphuric acid trap. Immediately after taking the protein out of the oven, an aliquot of urea or GuHCl solution pre-eqilibrated to room temperature was added and the test tubes were sealed with parafilmquickly. The protein was allowed to dissolve slowly without vigorous shaking. The tubes were

kept in a humidity-controlled constant temperature room at 20°C for 4 h. Then the tubes were centrifuged at 15,000 g for 20 min at 10°C and any evaporative losses were minimised. These tubes were equilibrated overnight at 20°C before isomolal measurements were done. This time of equilibration permitted elimination of any air bubbles from the solution, since with experience it was found that microbubbles caused serious problems in density measurements. The same solutions were used for concentration measurements by ultraviolet absorbance and for the measurement of V at constant chemical potential, conditions. For routine measurements at constant chemical potential, the protein solutions were dialyzed at 4°C against 250 ml of 8 M urea buffer or 6 M GuHCl for 2-3 days and at 20°C against 250 ml of PS buffer for measurements in buffer alone. The last 24 h of dialysis in the presence of urea or GuHCl was performed at 20° C, which is the temperature at which the density measurements were performed. Thermal equilibrium of the samples were established as described elsewhere (Prakash *et al*., 1981b).

Determination of protein concentration: Protein concentrations were measured routinely by ultraviolet absorption. For the accurate determination of the extinction coefficient the deionized protein (~2 g) was dissolved in water and again dialyzed overnight against deionized water. The protein solution was then centrifuged at 100,000 g for 1 h to remove any undissolved materials and suspended aggregates or particles in the solution. It was then filtered through a millipore filter; from the filtrate an aliquot was then transferred to the 8 M urea in buffer or phosphate buffer containing 6 M GuHCl such that the protein absorbance was well within the range of 1.0 absorbance unit. After 4 to 5 h the ultraviolet absorption spectrum of the solution was obtained in triplicate from 250 to 400 nm in a Cary 118 spectrophotometer. The light scattering corrections were made as described elsewhere (Prakash *et al*., 1981b). The average of the ultraviolet absorbance of the three samples was then determined. A known volume (~300-400 ml) of the above stock protein solution was freeze-dried and then dried under vacuum at 40°C in the presence of phosphorus pentoxide for 72 h. The exact weight of the protein in each sample was obtained from the difference in weight between the predried, tared flask prior to the addition of the protein solution and after exhaustive drying of the protein. The extinction coefficient of the protein was calculated from a knowledge of the corrected ultraviolet absorbance in the buffer or in 8 M urea buffer or in 6 M GuHCl solution. The values of the extinction coefficients for α-globulin at 280 nm in various solvents are: 0.2 M phosphate buffer pH 6.5 containing 1M NaCl, 9.54 dl/g-cm; 8 M urea buffer pH 4, 8.94 dl/g-cm and 6 M GuHCl, 8.93 dl (g-cm).

Handling of protein solutions for density measurement: Extreme care was taken to avoid foaming of the protein solution during transfer to any container or to the densimeter as also to minimize evaporation. The solutions from the test tubes were drawn slowly and carefully with a disposable 1 ml syringe. The needle was then replaced by a female luer adapter to facilitate the transfer of the solution to the densimeter. For measurements at constant chemical potential, the dialysis bag was retrieved from the dialyzing system with stainless steel forceps and the solution was taken out of it with a 1 ml disposable syringe and needle and immediately transferred to the densimeter.

Density measurements: The densities of water, the solvent and the protein solutions of various concentrations were measured with a precision Densitymeter DMA-02 (Anton Paar, Gratz), as described by Lee and Timasheff (1974a).

The density of each solution was measured with reference to a known standard. The difference between the densities of two samples is given by:

$$\rho_1 - \rho_2 = (1/A)\ (T_1^2 - T_2^2) \tag{1}$$

Where A is the instrument constant determined with solutions of known density, ρ_1 and ρ_2 are the densities of the known and unknown solutions and T_1 and T_2 their respective time periods as read directly from the display of the densimeter. All measurements were made at 20°C with the densimeter cell compartment maintained at 20°±0.02°C with a refrigerated and heated form Scientific Company Circulating water bath with a large reservoir, the entire system being kept in a chamber where humidity was controlled and maintained at 20°C.

Analysis of data: The densities of the solvent and the protein solution were converted to an apparent partial specific volume ϕ using the following equation (Schachman, 1957, Kielly and Harrington, 1960, Cassassa and Eisenberg, 1961, 1966).

$$\phi = \left(\frac{1}{\rho_0}\right) \left\{ 1 - [(\rho - \rho_0)/c] \right\} \tag{2}$$

where ϕ is the apparent partial specific volume, ρ and ρ_0 are the densities of the solution and solvent, respectively in g per ml, and C is the concentration of protein in g per ml. The obtained values of ϕ, were then plotted as a function of protein concentration and the extrapolated value at zero protein concentration was taken as the partial specific volume $\bar{V}$ For three component systems, the notation of Scatchard (1946) and Stockmayer (1950) was adopted setting components, 1, 2 and 3 as water, protein and the added diffusible material, *i.e.*, urea or GuHCl.

The preferential interaction parameter

$$\left[\frac{\partial g_3}{\partial g_2}\right] T, \mu_1, \mu_3 = \xi_3 \tag{3}$$

of the solvent components with the macromolecule was then calculated from the proper combination of densities obtained at constant chemical potential and constant composition of solvent components (Cohen and Eisenberg, 1968).

$$\left[\frac{\partial g_3}{\partial g_2}\right] T, \mu_1, \mu_3 = \left\{ \left[\frac{\partial \rho}{\partial g_2}\right] T, \mu_2, \mu_3 - \left[\frac{\partial \rho}{\partial g_2}\right] T, P, m_3 \right\} \Big/ \left[\frac{\partial \rho}{\partial g_3}\right] T, P, m_2 \tag{4}$$

where T is the absolute temperature, P is pressure, μ is the chemical potential of the particular component i, g is the concentration expressed as of i per g of principal solvent, m is the mol of component i per 1000 g of water and ξ_3 is preferential

interaction parameter. From the definition of the partial specific volume, at infinite dilution, the above equation may be re written as

$$(-\phi'_2\rho_0)^o = (1-\phi_2\rho_0)^o + \xi_3(1-\overline{V}_3\rho_0) \tag{5}$$

Where, ϕ'_2 and ϕ_2 are the partial specific volumes measured at constant chemical potential and constant solvent molality respectively. The superscript *o* indictates the values corrsponding to infinite dilution of the macromolecular species. In practice, however, it was possible to use ϕ_2 measured at the given concentration since generally it was found that the apparent partial specific volumes were independent of protein concentration.

The volume change upon transferring a negative protein to a denaturing environment is given by (Eisenberg, 1976)

$$\Delta V = M_2\ (\phi_1 - \overline{V}_2) \tag{6}$$

where M_2 is the molecular weight of the protein, and $\overline{V}_2$ is the partial specific volume of the native protein in dilute buffer and ϕ_1 is the partial specific volume in urea or GuHCl under constant molality.

Strictly speaking, the above value of ΔV, does not correspond just to the volume change of the protein itself upon unfolding, but it is also a function of the contribution from all other volume changes, such as differences between the changes of volume of solvent components when they interact with the protein, difference in electrostriction in the two media and volume changes associated with changes in ionization of protein side chains upon denaturation.

Calculation of the number of mol of denaturant bound per mol of the protein

The apparent partial specific volume of the protein, at infinite dilution of the macromolecular species, is related to the preferential interaction parameter (Lee and Timasheff,1974a)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3} \simeq \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu,\mu_3} = \xi_3 \tag{7}$$

by

$$\phi'_2 = \phi^*_2 - \xi_3\left(\frac{1}{\rho}\phi V_3\right) \tag{8}$$

where $\overline{V}$ is the partial specific volume of the protein in dilute buffer and the other symbols have their usual notation as described before. The above equation is valid since (Cassassa and Eisenberg, 1966; Cohen and Eisenberg, 1968)

$$\xi_3 = \left[\left(\frac{\partial\rho}{\partial g_2}\right)_{\mu_3} - \left(\frac{\partial\rho}{\partial g_2}\right)_{m_3}\right] \Big/ \left(\frac{\partial\rho}{\partial g_3}\right)_{m_2} \tag{9}$$

The preferential interaction parameter is related to the actual amount of solvent components bound to the protein by (Noue and Timasheff, 1972).

$$\xi_3 = A_3 - g_3 A_1 \tag{10}$$

where all interactions are expressed in units of g of ligand bound per g of protein; A_3 is absolute solvation, *i.e.* the actual amount of denaturant bound to the protein; A_1 is the absolute hydration, and g_3 is the solvent composition, expressed as g of denaturant per g of water. A combination of equation (8) and (10) gives

$$\phi_2' = \phi_2^* - \left(\frac{1}{\rho} - \bar{V}_3\right)(A_3 - g_3 A_1) \tag{11}$$

from which the apparent partial specific volume of the protein chemical equilibrium with solven φ'_2 can be calculated if

$$\phi_2^*, A_3, A_1, g_3, \text{ and } \bar{V}_3 \text{ are known.}$$

Calculation of A_3: The extent of urea or GuHCl binding to protein in 8 M urea of 6 M GuHCl is calculated according to Lee and Timasheff (1974b) and Prakash *et al.* (1981a) for GuHCl and urea respectively.

Results and discussion

The partial specific volume of α-globulin were determined under both isomolal and isopotential conditions in (i) PS buffer (ii) 8 M urea buffer and (iii) 6 M GuHCl solution.

(i) PS buffer

The concentration dependence of apparent partial specific volumes is presented in figure 1 for α-globulin in PS buffer. The isomolal value at zero protein concentration was 0.728±0.002 and the isopotential value at zero protein

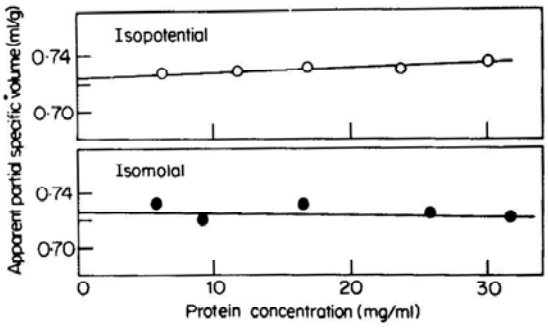

Figure 1. Concentration dependence of the apparent partial specific volume of α-globulin under isomolal and isopotential conditions in 0.1 M phosphate buffer pH 6.5 contaning 1M NaCl.

concentration was 0.725 ± 0.002 indicating almost no preferential interaction of the protein with the solvent components to cause any distinguishable hydrodynamic change in the protein. However, it could very well be a compensatory effect of the hydration as well as that of buffer salts and NaCl. Since in an isopotential experiment, the chemical equilibrium is established, the value is taken as the partial specific volume of the protein in native condition and is listed in table 1.

Table 1. Partial specific volumes and preferential interaction parameters of α-globulin in urea and GuHCl solutions.

	Solvent	
	8 M Urea	6 M GuHCl
$\bar{V}_2°$ (native)	0.725 ± 0.002	0.725 ±0.002
$\phi_{2,\, m_3=\phi_{m_3}}$ C → O[a]	0.708 ± 0.004	0.707 ±0.003
$\phi_{2,\, m_3'=\phi_{m3}}$ C → O[a]	0.692 ± 0.002	0.684 ± 0.002
ξ_3 (from eq. 5)[b]	0.08 ± 0.02	0.10 ± 0.02
$\partial m_3 \partial m_2)_{\bar{\mu}_1, \mu_3}$	133 ± 40	105 ± 20

[a] Values given in ml/g; b Values given in g/g;
[c] Values given in mol/mol.

(ii) 8 M Urea buffer

The concentration dependence of apparent partial specific volumes is presented in figure 2 for α-globulin in 8 M urea buffer. At constant molality of urea a value of 0.708 ± 0.004 is obtained at zero protein concentration and at constant chemical

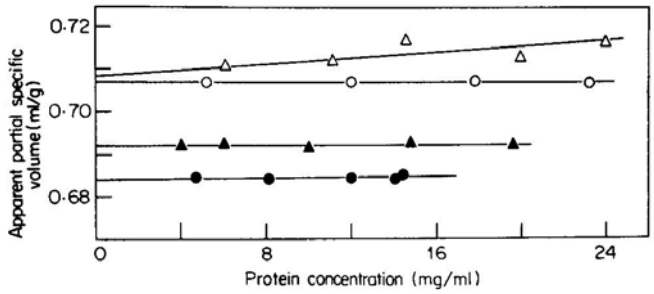

Figure 2. Concentration dependence of the apparent partial specific volume of α-globulin in 8 M urea buffer and 6 M GuHCl solutions under isomolal and isopotential conditions. Urea: Isomolal (△) and isopotential (▲); GuHCl: Isomolal (o) and isopotential (●).

potential a value of 0.692 ± 0.002 is obtained at zero protein concentration as shown in table 1. The results indicate a preferential interaction of solvent component with α-globulin leading to a decrease in the partial specific volume of the protein and the protein concentration has not much effect on the partial specific volume of the protein in isopotential condition and has a significant effect under isomolal conditions.

(iii) 6 M GuHCl

Figure 2 shows the partial specific volume of α-globulin in 6 M GuHCl solution at various protein concentrations. Under isomolal conditions a value of 0.707 ± 0.003 at zero protein concentration is obtained for α-globulin and the value is fairly independent of protein concentration. On the other hand, under constant chemical potential an interpolated value of 0.684 ± 0.002 is obtained at zero protein

concentration and the partial specific volume of the protein is dependent on the protein concentration. The results indicate a preferential interaction of solvent components with α-globulin leading to a decrease in the partial specific volume of the protein.

Also it can be seen from table 1 that the partial specific volume in 6 M GuHCl is less than in 8 M urea solution indicating a much higher preferential interaction of the solvent components in 6 M GuHCl with the protein.

The difference between the partial specific volume of a protein in the native and unfolded states under conditions of constant molality reflects mainly the volume change which accompanies denaturation. On the other hand, the difference between partial specific volume of the native and unfolded protein after dialysis should reflect a combination of two factors: (i) the volume change due to denaturation and (ii) preferential interaction with solvent components. From table 1, it is difficult to distinguish between the contribution from volume change upon denaturation and from preferential interaction with solvent components. In 8 M urea buffer, the volume change observed in the case of proteins such as β-lactoglobulin, papain and α-chymotrypsin is due mainly to denaturation (Lee and Timasheff, 1974a). On the other hand in the case of lysozyme, α-lactalbumin and chymotrypsinogen *A*, the preferential interaction parameter makes a more significant contribution to volume change (Prakash *et al*., 1981b).

Data on partial specific volume of proteins in urea solution is scarce in the literature (Prakash *et al*., 1981b). Most of the reported values for denatured proteins were obtained in 6 M GuHCl solution. Upon comparison of the partial specific volume of α-globulin in urea and GuHCl, it would seem that ϕ_2,m_3 values in 8 M urea are somewhat higher than those in 6 M GuHCl. This may reflect either the known fact that 8 M urea is less effective denaturing agent than 6 M GuHCl (Aune and Tanford, 1967; Salahuddin and Tanford, 1970; Tanford, 1968; 1970; Green and Pace, 1974) or simply a difference in the electrostriction in the presence of ionic (GuHCl) and nonionic (urea) denaturants. Prakash and Nandi, (1977b) have reported the viscosity of α-globulin in both 6 M GuHCl and 8 M urea solution in triethanolamine buffer, 0.05 M, pH 9.0 containing 0.5 M KCl. In 8 M urea solution, they obtain a value of 0.28 dl/gm and under the same conditions they obtain a value of nearly 0.4 dl/gm in 6 M GuHCl solution, indicating that α-globulin is unfolded to a greater extent in 6 M GuHCl than in 8 M urea solution. This probably indicates that just as in many other proteins urea is a much less effective denaturing agent than GuHCl for α-globulin and probably electrostriction difference between the two denaturants is not playing a major role. *Protein solvent interactions:* The preferential interaction parameter for α-globulin in both 8 M urea and 6 M GuH Cl was calculated according to equation 5, from the partial specific values. The volues o ξ_3 and $\partial m_3/\partial m_2$ are listed in table 1. In these calculations, the values of $\bar{V}_3$ and ρ_0 used were: for 8 M urea, $\bar{V}$ = 0.763 ml/g and ρ_o = 1.115 3 g/ml (Prakash *et al*., 198 1b) and for 6 M GuHCl, $\bar{V}_3$ ml/g (Reisler and Eisenberg, 1969) and ρ_o = 1.1427 g/ml (Lee and Timasheff, 1974a). The value of $\bar{V}_3$ was determined by the same methods as those employed for the proteins with measurements done at molarities higher than 8 M urea or 6 M GuHCl followed by

extrapolation back to 8 M urea (Prakash *et al.*, 198 1b) and 6 M GuHCl (Lee and Timasheff, 1974a).

In general, the value ξ_3 varies between zero (ribonuclease A) and 0.14 (pepsinogen) g of urea/g of protein (Prakash *et al*., 1981b); and zero (ribonuclease A) and 0.17 (α-chymotrypsin) g of GuHCl/g of protein (Lee and Timasheff, 1974a). It was observed that in the well-worked systems of urea and GuHCl, the interaction was not preferential with water (Prakash *et al*., 1981b; Lee and Timasheff, 1974a). The values of ξ_3 for α-globulin also indicate that the interaction preferential with solvent components and not with water. From table 1, one can see that the preferential interaction parameter is much higher in GuHCl (0.10 g/g) than in urea (0.08 g/g) solution. It is known that most proteins are devoid of specific structural features in 6 M GuHCl (Tanford, 1968, 1970). This conclusion may be extended to the 8 M urea system although the extent of randomization of structure may be smaller in that solvent and the kinetics of denaturation may be slow in 8 M urea (Aune and Tanford, 1967; Salahuddin and Tanford, 1970; Tanford, 1968 and 1970; Green and Pace, 1974 and Pace, 1975). It is shown by Lee and Timasheff (1974a) and Prakash *et al.* (1981b) that the preferential interaction parameter ξ_3 in either 6 M GuHCl or 8 M urea, is related to the intrinsic properties of the protein molecule, especially the amino acid composition. This is more so since it is known that the different amino acid side chains differ from each other in their affinities of water, urea and GuHCl (Nozaki and Tanford, 1970).

Since preferential interaction parameter varies from one protein to another, it seemed reasonable, just as in the case of 6 M GuHCl (Les and Timasheff, 1974a) or 8 M urea (Prakash *et al*., 1981b) to normalize the data in terms of the actual apparent binding of urea to each protein. This can be calculated from the preferential interaction parameter if the total degree of hydration is known from equation 10. A close examination of this equation reveals that a proper choice of the parameter A_1 is critical, since a range of values for A_1 of proteins can be found in the literature (Kuntz, 1971; Bull and Breese, 1968; Squire and Himmel, 1979) and also A_1 depends on the technique used (Kutz, 1971; Bull and Breese, 1968; Tanford, 1961; Timasheff, 1963). In the absence of experimental value of A_1 for α-globulin it was calculated from the hydration of its constituent amino acids according to the method of Kuntz (1971). The above procedure assumes that (i) the majority of the residues are exposed to and are in contact with the bulk solvent in the presence of 8 M urea or 6 M GuHCl and (ii) the hydration values for the proteins investigated are equal to the summation of those of their constituent amino acids. This approach appears to have a greater validity than that of taking A_1 values measured by different experimental techniques and in different solvents.

However, the values of hydration of the amino acids depends on the pH of the system also. This is exemplified in the data of Kuntz (1971) where aspartate, glutamate and tyrosyl residues show a strong pH dependence of their hydration. Since in the present study the protein α-globulin, just like other major seed proteins is rich in aspartic and glutamic acids proper hydration values have to be taken into account both in the urea system (where pH = 4) and in the GuHCl system. Values of 2 for both aspartic and glutamic acids at pH 4 and of 6 and 7.5 at

pH 7 are used for the hydration of aspartic and glutamic acids (Kuntz, 1971). The value of g_3 used in the calculation of A_3 was 0.752g of urea/g of water and 1.007 g GuHCl/g of water, as determined gravimetrically. Incorporation of these values of A_1, g_3 and $(\partial g_3 / \partial g_2)_{T, P, \mu_3}$ (all in g/g) into equation (7) gives the value of A_3 for α-globulin. The results are summarised in table 2.

Table 2. α-Globulin-solvent interactions in urea and GuHCl solutions.

Solvent	$(\partial g_3/\partial g_2)$ T, p, μ_3 (g/g)	A_1 (g/g)	A_3 (g/g)	A_3 (mol/mol)	A_3 Calcd (g/g)
8 M Urea	0.08	0.252	0.270	449	0.285
6 M GuHCl	0.10	0.401	0.480	507	0.454

The interaction of urea and GuHCl with α-globulin may occur at a variety of sites. The mechanism of denaturation has been generally examined from three points of view: (i) the interaction of urea or GuHCl with a variety of sites on the protein (ii) the effect of urea or GuHCl on the solvent structure itself and (iii) a combination of these two factors. Due to the complex nature of the solvent itself and since no precise information is available on the equilibrium structure of water around various groups of a macromolecule, it is difficult to interpret the results from the point of view of solvent structure. On the other hand the favourable free energy of transfer of hydrophobic side chains and in particular of aromatic residues from aqueous medium to 6 M GuHCl or 8 M urea suggests these are likely sites of interaction (Tanford, 1970; Nozaki and Tanford, 1970). However, the hydrogen bonding ability of the guanidinium group in urea molecule (bifunctional reagent) should favour their interaction with peptide bonds. On this basis Rohinson and Jencks (1965) have postulated that GuHCl/urea molecule could hydrogen bond to two peptide bonds forming a cyclic structure. Based on this Lee and Timasheff (1974a, b) and Prakash *et al*., (1981 a, b) have proposed models for the interaction of GuHCl and urea respectively with the various proteins. From these models, the theoretical values of A_3, the value of absolute solvation, is calculated for α-globulin both in 8 M urea and 6 M GuHCl and is listed in table 2 last column. It can be seen from table 2 that in urea the experimentally determined A_3 is in excellent agreement with the theoretically calculated value of A_3. On the other hand in GuHCl even though there is a good correlation the experimentally determined A_3 has a slightly higher value.

Volume change: Using the data in table 1 and equation 6, the changes in volume upon tansfer of α-globulin from dilute salt solution to 8 M urea or 6 M GuHCl were calculated from a knowledge of the apparent partial specific volumes measured at constant molality of urea/GuHCl and under native conditions. A value of –4700 ml/mol. and –5000 ml/mol were obtained upon transferring α-globulin from dilute salt solution to 8 M urea and 6 M GuHCl solutions respectively. These values are in good agreement with the viscosity data of Prakash and Nandi (1977b)

where it is shown that α-globulin is more extended in GuHCl solution than in urea solution. The magnitude of the values appears to be comparable to many other proteins of similar nature, although not much work has been done in measuring △V of multimeric proteins of very high molecular weight. Also, in α-globulin, all the difference in △V cannot be attributed to unfolding only since Katz and Miller (1971) and Krauz and Kauzmann (1965) have shown that the △V values obtained at different pH's cannot be interpreted directly unless the contribution of protonation to △V is accounted for. From the △V values are also calculated ∂V in ml/mol and are shown in the last column of table 3. From a comparison of the ∂V values of many other proteins in urea and GuHCl (Lee and Timaseff, 1974a;

Table 3 Change in volume for α-globulin upon transferring from native to the denatured state in 8 M urea and 6 M GuHCl.

Solvent	$-\Delta V$ (ml/mol)	$-\partial V = \Delta V$/residue (ml/mol of residue)
8 M Urea	4700 ± 1600	2.35 ± 0.8
6 M GuHCl	5000 ± 1400	2.50 ± 0.7

Prakash *et al*., 198 1b), it appears that the value is close to that of β-lactoglobulin in 6 M GuHCl and papain in 8 M urea solution. However, no quantitative comparison can be made since various denaturants could interact differently with different proteins and such interactions need not unfold the proteins to the same final .thermodynamically stable form. Even assuming it does so unless the equilibrium strucutre of water around the various groups of the macromolecule is known precisely it is difficult to interpret all the observed results of denaturation of proteins from a thermodynamic and mechanistic point of view.

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