International Journal of

Biological Chemistry

ISSN 1819-155X

Academic
Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 5 (6): 327-341, 2011
ISSN 1819-155X / DOI: 10.3923/ijbc.2011.327.341

Polyols Stabilize the Denatured States of Multidomain Protein Ovomucoid

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ABSTRACT
The present study represents the first attempt to gain a quantitative estimate of the protective influence of polyols on a multidomain protein ovomucoid in its native and chemically-induced denatured states. The polyols selected were such that they enabled the comparisons of the stabilizing effects of 6-C epimers (sorbitol and mannitol), 5-C epimers (adonitol and xylitol) and a 3-C sugar (glycerol) against isothermal denaturation induced by guanidinium chloride and urea. The stabilizing effect of these polyol osmolytes on the multidomain protein ovomucoid against guanidinium chloride (GdmCl) and urea denaturation was studied using different optical probes. Circular Dichroism (CD) measurements were done by monitoring changes at 222 nm. Absorption measurements were followed by observing changes at 287 and 288 nm for guanidinium chloride (GdmCl) and urea-induced denaturation of ovomucoid, respectively. Both of the observations at 287 nm and 288 nm showed denaturation as a two steps process involving at least one stable intermediate state and it was seen that polyols stabilize both the transitions with less or no effect on intermediate state of ovomucoid. We have also examined by fluorescence studies that the intermediate state obtained with guanidinium chloride and urea is possibly a molten globule. This was further proved by DLS studies where it showed an overall increase in 32.36% of the hydrodynamic radius of the protein from native to denatured state, with an increase of 15.52 and 19.94% from native to intermediate and from intermediate to denatured sate, respectively.

Key words: Protein stability, protein denaturation, polyol osmolytes, ovomucoid, molten globule

INTRODUCTION
Protein stability plays an extremely important role not only in biological function but also in medical sciences. Certain tissues and cells are often subjected to stress as from sharp change in solute concentration, high temperature, high salinity and metabolic substances which can cause changes in the cellular proteins structures. A strategy which nature uses to stabilize the inventory of the cell especially proteins under these extreme conditions is by accumulating the low molecular weight compounds known as osmolytes. These molecules are also known as chemical chaperones as they have been shown to stabilize protein and protect them against aggregation. They fall into the following categories: Polyhydric alcohols (polyols) such as glycerol, xylitol, etc., amino acids and its derivatives like tuarine and β-alanine, proline and methylamines like TMAO (trimethylamine

N-oxide). They exert a dramatic effect on protein folding reaction, without making or breaking covalent bonds and thus are the best additive for the protection of proteins against harsh stresses. Osmolytes stabilize proteins, not by interacting with them but altering the solvent properties of the surrounding water and hence protein-solvent interaction (Timasheff, 1993). They stabilize the native state because they are preferentially excluded from the protein surface, for the preferential exclusion increase the chemical potential of the proteins proportionally to the solvent exposed area. Their effect seems to be general for all proteins. Those having enhancing effect on protein function are called counteracting osmolytes and those having no inhibitory or enhancing effect are compatible osmolyte. Compatible osmolytes are amino acids and their derivatives and polyols (Yancey, 2004). Among these, polyols are the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure and freezing (Yancey *et al.*, 1982; Carpenter *et al.*, 1993). They have also been found to be effective stabilizers of proteins when added at high concentrations, for they raise the mid-point of heat and chemical-induced denaturation (Taneja *et al.*, 1994; Xie and Timasheff, 1997). It has been reported that when a protein is present in the polyol solution, two types of interactions are observed, namely the osmophobic effect (i.e., the unfavorable interaction between peptide units and polyols) and solvophobic effect (i.e., the unfavorable interaction between side chains and polyols). Both of these effects will be larger in guanidinium chloride-induced denatured protein than that in the heat denatured protein for the heat denatured state retains residual structure (i.e. some peptides units and side chains are exposed to solvents) (Ahmad *et al.*, 1983). Recently molecular basis for polyol-induced protein stability was revealed by molecular dynamics simulation. It was found that polyols protection is positively correlated with both the molecular volume and the fractional polar surfaces and former contributes more significantly to protein stability (Liu *et al.*, 2010).

The final step in protein biosynthesis is the folding of polypeptide chain into the unique native and functional conformation. Useful information about the mechanism of the overall process of protein folding can be obtained by characterizing the different conformational states involved in the folding↔unfolding process. The intermediate state (s) of most of the protein denaturation studies is (are) either present transiently or is (are) accumulated in amounts too low to be detectable by the conventional analytical techniques. The existence of stable intermediate state on the pathway of protein unfolding has been rarely reported and wherever such intermediate state (s) has been implicated, it has not been characterized systematically. Consequently to date there are not enough evidences to show a relationship between polyols and those intermediate states. Interestingly, at least one such stable intermediate which accumulates in significant amount has been recognized in the reversible denaturation of ovomucoid, a multidomain protein (Baig and Salahuddin, 1978). This was one of the reasons to choose this as a model protein for this study. Other reason was that there is no literature available how the relationship between polyols stabilization and a multidomain protein works.

Ovomucoid is a glycoprotein (trypsin inhibitor) from egg-white (Kato *et al.*, 1976). Its native conformation is resistant to extreme values of pH and temperature and to concentrated chemical denaturants. In contrast, the results presented in this study have demonstrated unambiguously that ovomucoid undergoes conformational change in the presence of increasing concentrations of guanidinium chloride (GdmCl) and urea. The unfolding reaction involved at least three stable conformational states: they are the native (N), intermediate (X) and completely denatured (D) states. These findings are surprising; for ovomucoid with eight disulfide bonds is expected to show minimal conformational fluctuations which would tend to reduce the number of stable intermediate state(s) on the folding pathway (Waheed *et al.*, 1977). Under such situations even if such an intermediate state has to exist it is likely to do so only transiently. This communication describes for the first time systematic study of the GdmCl-induced unfolding of ovomucoid by circular dichroism and absorption measurements, in presence of cosolvents and also showing the intermediate state as molten globule by fluorescence and DLS studies.

We made attempts to see the stabilizing effects of polyols on the three stable conformational states which were involved in the overall unfolding process. Here we report how these guanidinium chloride and urea-induced intermediate states are stabilized by polyols. Theoretical studies and experimental data suggest that the single domain proteins may fold on as smooth free-energy landscape within milliseconds and without detectable kinetic intermediates (Dill and Chan, 1997) whereas the folding of larger, multidomain and multimeric protein is much slower and is characterized by accumulation of kinetic measurements (Radford *et al.*, 1992). This study represents the first attempt to gain quantitative estimate of the protective influence of polyols on chemically denatured states of a multidomain protein.

MATERIALS AND METHODS

Ovomucoid was isolated from chicken egg white by the method described by Waheed and Salahuddin (1975). Concentration of the protein solution was determined experimentally using, the molar absorption coefficient (ϵ) value of 142800 M^{-1} cm^{-1} at 280 nm for ovomucoid (Baig and Salahuddin, 1978). All solutions for optical measurements were prepared in the desired degassed 50 mM sodium phosphate buffer pH 7.0 containing 0.1 M KCl. On the addition of GdmCl or urea pH of each solution was also measured after the denaturation experiment and it was observed that the change in pH was not significant. However, it should be noted that no corrections were made for the possible effect of co-solvents on the pH of protein solutions. The other chemicals used were of analytical grade and were used without further purification.

Preparation of GdmCl stock solution: Concentrated solution of GdmCl containing 0.1 M KCl was prepared. Refractive index measurements were made in an Abbe refractrometer after filtering the solution through whatman filter paper No.1. This was done in order to determine the concentration of the buffered stock solution of GdmCl using tabulated values of the solution refractive indices, (Nozaki, 1972). The stock solution of GdmCl was stored at 4°C.

Preparation of urea stock solution: Concentrated solution of Urea containing 0.1 M KCl was prepared. Refractive index measurements were made in an Abbe refractrometer after filtering the solution through whatman filter paper No.1. The concentration of urea stock solution was also determined by refractive index measurements (Pace, 1986) same as that of GdmCl. To avoid generation of cyanate ions all urea stock were made fresh before use.

Preparation of polyol stock solutions: Maximum solubility of all polyols at 25°C was used to prepare stock solutions of maximum molarity. All solutions were prepared in 50 mM sodium phosphate buffer containing 0.1 M KCl. Final pH of polyol stock solutions was maintained at 7.0 and finally before storing at 4°C, the solutions were filtered through 0.45 μm Millipore filter paper.

Preparation of 8-anilino-1-naphthalenesulfonic acid (ANS) stock solution: Stock solution of ANS was prepared by dissolving ANS in 50 mM sodium phosphate buffer of pH 7.0. The concentration of the stock solution was determined spectrophotometrically using an 'ϵ' value of 5000 $M^{-1}cm^{-1}$ at 350 nm (Muluqueen and Kronmann, 1982).

Preparation of solution for isothermal studies: For isothermal experiments fixed volume of the protein stock solution using lambda pipette (Lang-Lavy type) in 1 mL volumetric flask followed by adding appropriate volumes of buffer and denaturant solution by micropipette, final volume was adjusted by the buffer. The reason for using volumetric flasks was to make sure that the final volume of each solution is constant. The solution were mixed thoroughly and incubated overnight at room temperature, which was more than sufficient time to attain equilibrium, although no difference was noticed between the observations made at 10 min and those made after 12 h.

Analysis of Gdmcl-induced denaturation curves: GdmCl-induced denaturation curves of ovomucoid in the absence and presence of polyols were measured by following changes in $[\theta]_{222}$ at 25°C. Assuming that the GdmCl-induced denaturation process proceeds through a two-state mechanism, the free energy change (ΔG_D) for folding↔unfolding reaction was calculated using the relation:

$$\Delta G_D = -RT \ln\{(y-y_N)/(y_D-y)\} \tag{1}$$

where, y is the measured optical property at given denaturant concentration, while y_N and y_D are, respectively properties of the native and denatured states at the same denaturant concentration at which y has been measured, R is gas constant and T is temperature in Kelvin. Values of y_N and y_D for any point in the transition region are obtained by linear extrapolation of the pre- and post transition baselines. The variation in these baselines with the change in denaturant concentration is known as solvent perturbation or solvent effect. Values of ΔG_D in the range $-1.3 \le \Delta G_D$ (kcal moL^{-1})≤ 1.3 were plotted against [GdmCl], the molar concentration of the denaturant and least-squares analysis was used to fit the (ΔG_D, [GdmCl]) data to the relation:

$$\Delta G_D = \Delta G_D^\circ - m[d] \tag{2}$$

where, ΔG_D° is the value of ΔG_D in the absence of denaturant and m is the slope, $(\partial \Delta G_D / \partial [d])_{T,P}$ and [d] is the molar denaturant concentration. The midpoint of transition curve, C_m was calculated from $C_m = \Delta G_D^\circ / m$.

Circular Dichroism (CD) measurements: Circular dichroism spectra were measured in JASCO spectropolarimeter, model J-810 equipped with peltier type temperature controller (PTC-423S). Acquisition of data was carried out using the software provided by JASCO. The instrument was calibrated with (+)-10-camphorsulfonic acid. Protein concentration used was in the range of 18-20 µmole in a quartz cuvette of 0.1 cm path length. Data were obtained at an interval of 0.1 nm in the far-UV region. Scanning rate of 100 nm min^{-1} at the response time 4 sec were used. At least three accumulations of scanning were carried out to average out the spectrum to improve upon the signal to noise ratio in each case including base line. All results of Circular Dichroism (CD) measurements are expressed as mean residual ellipticity $[\theta]$ in deg $cm^2 dmol^{-1}$.

Absorption measurements: Various states of ovomucoid have been characterized by solvent perturbation of the absorption and fluorescence spectra of ovomucoid as well as by viscosity measurements (Waheed *et al.*, 1977). These measurements were done to observe the effect of polyols at different concentrations on various thermodynamically stable states of ovomucoid. All the spectroscopic measurements were carried out in a Spectroscan 80-DV UV/Vis double beam spectrophotometer equipped with Peltier-type temperature controller (PTC-348 WI) using quartz cells of 1 cm path length. The temperature was maintained at 25°C. Protein concentration used was in the range of 7-10 μmole. The GdmCl-induced and urea-induced denaturation was monitored by measuring absorbance of the protein in the presence and absence of polyols. The absorption spectra of the exposed and unexposed protein by guanidinium chloride (GdmCl) and urea in presence of polyol osmolyte were recorded at 287 and 288 nm, respectively. Appropriate blanks were run simultaneously.

Fluorescence measurements: ANS binding measurements of ovomucoid in the absence and presence of various guanidinium chloride (GdmCl) concentrations were carried out in Perkin Elmer L-50B spectroluminiscencemeter. Samples were prepared in 50 mM sodium phosphate buffer pH 7.0. Protein to ANS ratio used was 1:30. The spectra were measured at a protein concentration of 7-8 μmole and reading was taken in a 5 mm quartz cuvette. Excitation and emission slit width were set at 12 mm. The samples were excited a 360 nm and an emission was recorded between 400 to 600 nm at the interval of 1 nm. All the samples were equilibrated overnight.

Dynamic light scattering: The hydrodynamic radius of native and various guanidinium chloride (GdmCl) induced denatured ovomucoid states were measured by Dynamic Light Scattering (DLS). The protein samples were filtered through 0.22 μm filter and 50 μL of (3 mg mL^{-1} in 50 mM sodium phosphate buffer, pH 7.0) protein sample was used. The cell holder was thermostated at 22°C. Measurements were done at a fixed angle of 90° using an incident laser beam of 633 nm. Twenty measurements were made with an acquisition time of 30 sec for each measurement at the sensitivity of 10%. The data was analyzed using graphical size analysis software PMgr v3.01 P17, provided with (RiNA laser spectroscatter 201). The mean peak value of hydrodynamic radius distribution was taken as an apparent hydrodynamic radius of scattering particle ($R_{h,app}$). For each of the solutions studied, measured values of $R_{h,app}$ nm were converted into apparent molecular masses (Mr,app) using relationship known for globular proteins (Creighton, 1993).

RESULTS

Isothermal denaturation studies: To understand the unfolding behaviour of ovomucoid by guanidinium chloride (GdmCl) and urea in the absence and presence of polyols, we carried out the GdmCl-induced denaturation of the protein at pH 7.0 and 25°C by observing changes in $[\theta]_{222}$. Denaturation curves of near-UV absorption of the protein were monitored by measurements of $\Delta\varepsilon_{287}$ and $\Delta\varepsilon_{288}$, respectively for GdmCl and urea in the absence and presence of polyol.

Far-UV CD measurements

GdmCl-induced denaturation in the absence and presence of various polyols at pH 7.0 and 25°C: GdmCl-induced transition curves of ovomucoid were measured in the absence and presence of 10, 20, 30 and 40% (w/v) sorbitol; 10, 20, 30 and 40% glycerol (v/v) and 0.25, 0.50, 0.75 and 1.0 M xylitol, adonitol and mannitol at pH 7.0 and 25°C. All the transition profiles were found

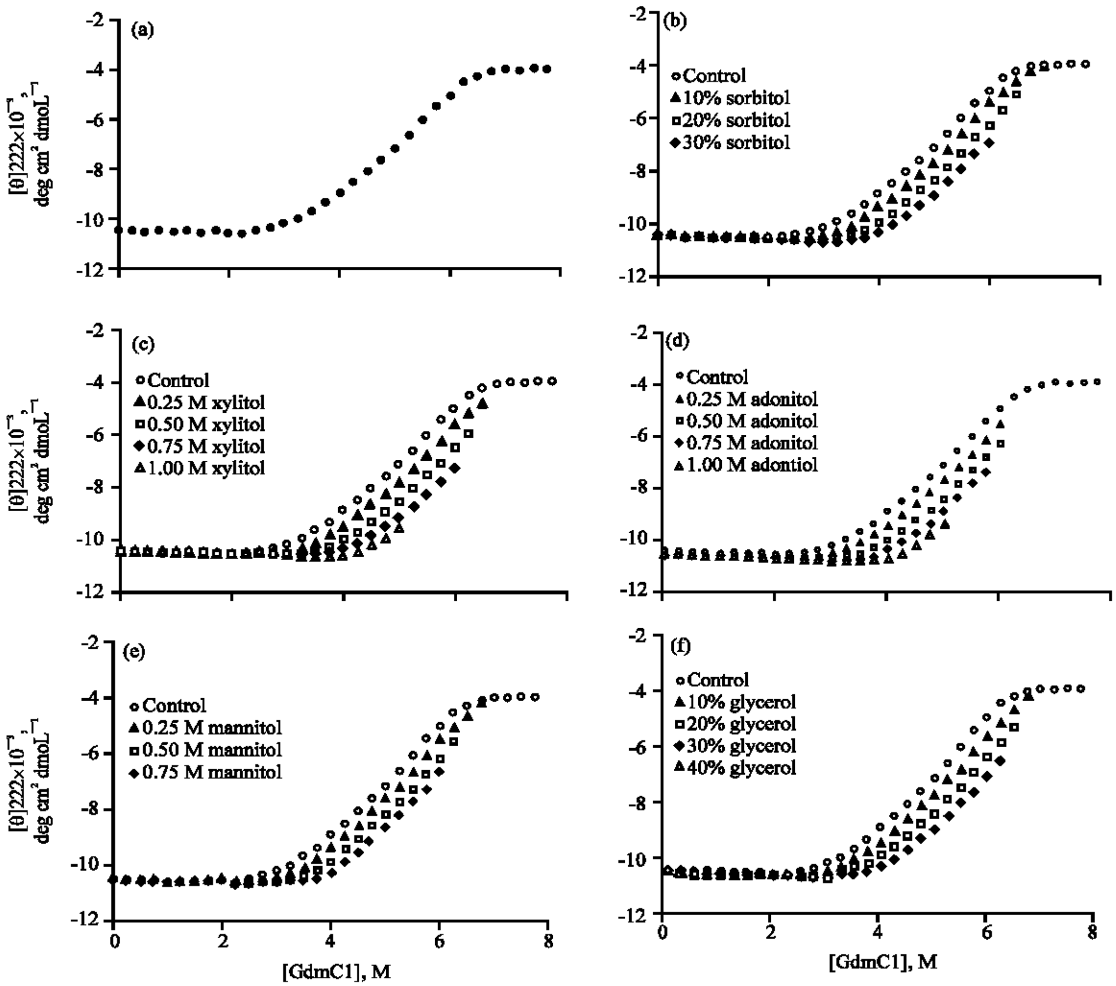

Fig. 1(a-f): GdmCl-induced unfolding profiles of ovomucoid in the presence and absence of different concentrations of polyols at pH 7.0 and 25°C. Panel (a-f) shows denaturation curves in the absence (O) and presence of 10 (▲), 20 (□), 30 (●) and 40% (Δ) sorbitol (w/v) and glycerol (v/v); 0.25 (▲), 0.50 (□), 0.75M, (●) and 1.0 M (Δ) mannitol, adonitol and xylitol. In order to maintain clarity some data points are not shown

to be reversible. Fig. 1(a-f) shows the denaturation profiles of ovomucoid in the absence and presence of different polyols at different concentrations. It has been observed that y_N is independent of polyol concentration Fig. 1(a-f). For comparison, the stability curve in the absence of polyol is included in (b-f) of Fig. 1. It should be noted that due to the experimental constraints we were unable to get complete transition curves in the presence of higher concentrations of polyols. However, in such cases, curves were analysed assuming the same dependence of y_D on [GdmCl]. ΔG_D values of ovomucoid in the presence of different concentrations of polyols were calculated using equation 1. Fig. 2(a-f) shows ΔG_D versus [GdmCl] plots. GdmCl-induced curves were used to estimate the value of ΔG_D in the range-$1.3 \leq \Delta G_D$, kcal moL$^{-1} \leq 1.3$ as a function of [GdmCl] using equation 1. All ΔG_D versus [GdmCl] plots were analysed for ΔG_D° and m according to equation 2. For comparison, the plot in the absence of polyol is included in (b-f) of Fig. 2. Table 1 shows the value of ΔG_D° and C_m, the midpoint of denaturation (= $\Delta G_D/m_d$) in the absence and presence of

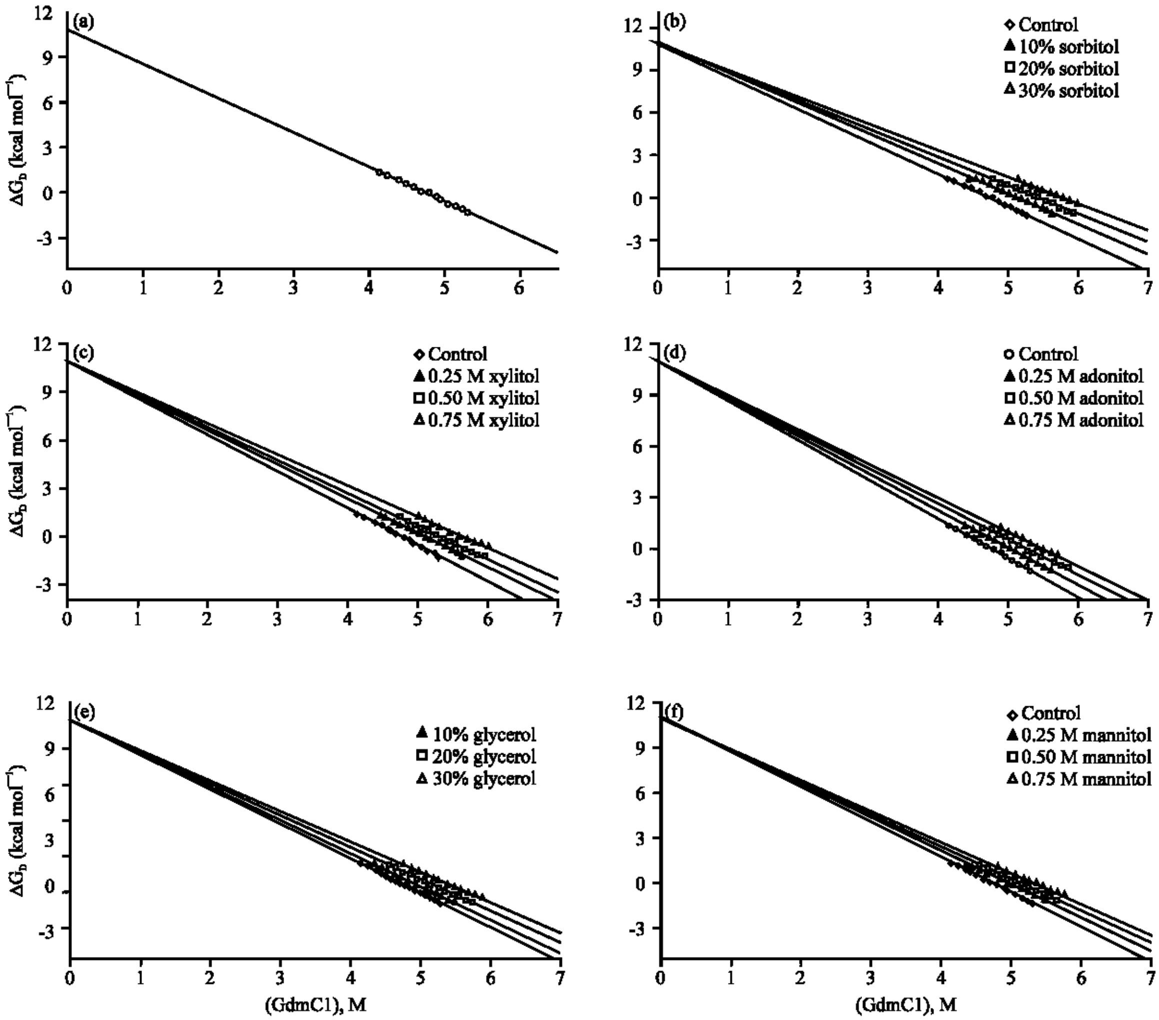

Fig. 2(a-f): Panel (a-f) shows ΔG_D versus [GdmCl] plots of ovomucoid in the absence (O) and presence of 10 (▲), 20 (□), 30 (Δ), 40 (●) sorbitol (w/v) and glycerol (v/v); 0.25 (▲), 0.50 (□), 0.75 M, (Δ) and 1.0 M (●) mannitol, adonitol and xylitol at pH 7.0 and 25°C. In order to maintain clarity some data points are not shown

different concentrations of various polyols. Each parameter given in Table 1 is the mean of three independent measurements. The midpoint concentration of unfolding (C_m) of GdmCl-induced denaturation of ovomucoid increases from 4.75 M to 5.79, 5.62, 5.47,5.32 and 5.34 M at the highest concentration of sorbitol, xylitol, adonitol, mannitol and glycerol, respectively. This means that the ability of polyols to protect ovomucoid against GdmCl-induced denaturation decreased in the order of sorbitol, xylitol, followed by adonitol, mannitol and glycerol.

Absorption measurements

GdmCl-induced denaturation in the absence and presence of sorbitol: The GdmCl-induced unfolding of ovomucoid in the absence and presence of different concentration of sorbitol was followed by absorption measurements at pH 7.0 and 25°C Fig. 3. The GdmCl-induced unfolding showed a two-step transitions involving at least three major conformational states, namely the

Table 1: Parameters characterizing the GdmCl unfolding of ovomucoid in the absence and presence of different concentrations of polyols at pH 7.0 and 25°C

Polyol	Concentration	$\Delta G_D°$ (kcal moL^{-1})	m (kcal mol^{-1} M^{-1})	C_m (M)
Sorbitol	0	10.80±0.64	2.27±0.13	4.75±0.07
	10%	10.87±0.66	2.12±0.12	5.13±0.06
	20%	10.89±0.54	1.98±0.08	5.44±0.04
	30%	10.82±0.75	1.87±0.11	5.79±0.03
Xylitol	0.00 M	10.80±0.64	2.27±0.13	4.75±0.07
	0.25 M	10.78±0.78	2.12±0.10	5.08±0.04
	0.50 M	10.81±0.71	2.03±0.09	5.32±0.03
	0.75 M	10.85±0.62	1.93±0.14	5.62±0.05
Adonitol	0.00 M	10.80±0.64	2.27±0.13	4.75±0.07
	0.25 M	10.80±0.91	2.15±0.15	5.02±0.05
	0.50 M	10.75±0.86	2.04±0.11	5.26±0.03
	0.75 M	10.84±0.78	1.98±0.09	5.47±0.06
Mannitol	0.00 M	10.80±0.64	2.27±0.13	4.75±0.07
	0.25 M	10.78±0.54	2.17±0.15	4.96±0.03
	0.50 M	10.80±0.75	2.10±0.08	5.14±0.06
	0.75 M	10.80±0.85	2.03±0.12	5.32±0.05
Glycerol	0%	10.80±0.64	2.27±0.13	4.75±0.07
	10%	10.81±0.71	2.21±0.08	4.91±0.05
	20%	10.78±0.84	2.10±0.16	5.13±0.03
	30%	10.85±0.95	2.032±0.11	5.34±0.04

ΔG_D^o : Gibbs free energy change at 25°C; Cm: midpoint of transition curve

Table 2: Characteristic feature of GdmCl-induced transition of ovomucoid and its stability by sorbitol at pH 7.0 and 25°C

Transition	Near onset of transition	At midpoint of transition	At and above which transition is complete
First transition N↔X			
0% sorbitol	-	1.25	2.50
10% sorbitol	-	1.50	3.00
20% sorbitol	-	1.75	3.25
Second transition X↔D			
0%sorbitol	4.25	5.40	6.50
10%sorbitol	4.50	5.65	6.50
20%sorbitol	4.75	5.90	6.50

native (N), intermediate (X) and fully denatured (D) states. Both the transitions were seen to be reversible. The characteristic features of the transitions N↔X and X↔D in absence and presence of polyol are summarized in Table 2.

Urea-induced denaturation in the absence and presence of sorbitol: The urea-induced unfolding of ovomucoid in the absence and presence of polyol was followed at pH 7.0 and 25°C by absorption measurements (Fig. 4). The concentration of urea used was in the range of 0-9.8 M. The extent of unfolding was determined by following changes in $\Delta\varepsilon_{288}$. In case of urea induced denaturation of ovomucoid the choice of 288 nm as the wavelength for measuring absorbance was dictated by the observation that the direct difference spectra of the urea-denatured ovomucoid recorded against that of the native protein solution of identical protein concentration showed a pronounced trough near 288 nm. It has been observed that urea induced denaturation of ovomucoid is reversible. The unfolding is seen to occur in two steps both in the absence and

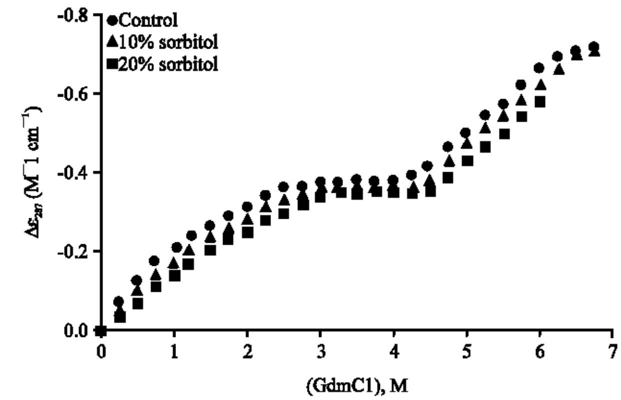

Fig. 3: Effect of sorbitol on different states of ovomucoid obtained by GdmCl-induced denaturation at 287 nm at pH 7.0 and 25°C

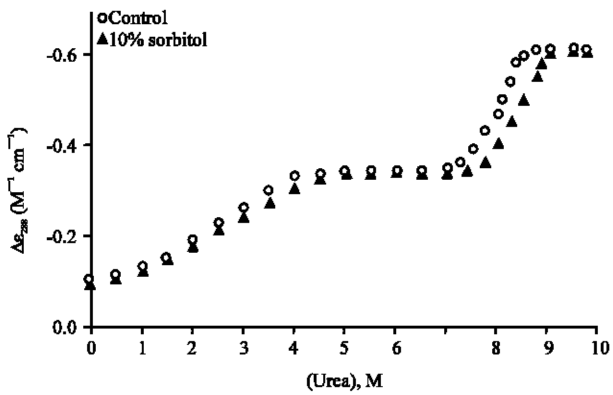

Fig. 4: Effect of sorbitol on different states of ovomucoid obtained by urea-induced denaturation at 288 nm at pH 7.0 and 25°C

Table 3: Characteristic Features of Urea-induced transition of ovomucoid stability by sorbitol at pH 7.0 and 25°C

		[Urea], M	
Transition	Near onest of transition	At midpoint of trasition	At and above which transition is complete
First transition N↔X			
0% sorbitol	1.50	2.10	4.00
10% sorbitol	1.50	2.60	4.50
Second transition X↔D			
0% sorbitol	7.00	8.10	8.90
10% sorbitol	7.40	8.50	8.90

presence of polyol osmolytes, involving at least three major conformational states, namely the native (N), intermediate (X) and fully denatured (D) states (Fig. 4). Small pre-transition zone is observed in this case, which is absent in GdmCl-induced unfolding. The characteristic features of two transitions, i.e., N↔X and X↔D in the absence and presence of polyol are summarized in Table 3.

Fluorescence measurements: 1-Anilinonaphthalene, 8-sulfonate (ANS) is a fluorescent dye that binds hydrophobic regions of proteins. The binding of this polar dye to protein is associated with

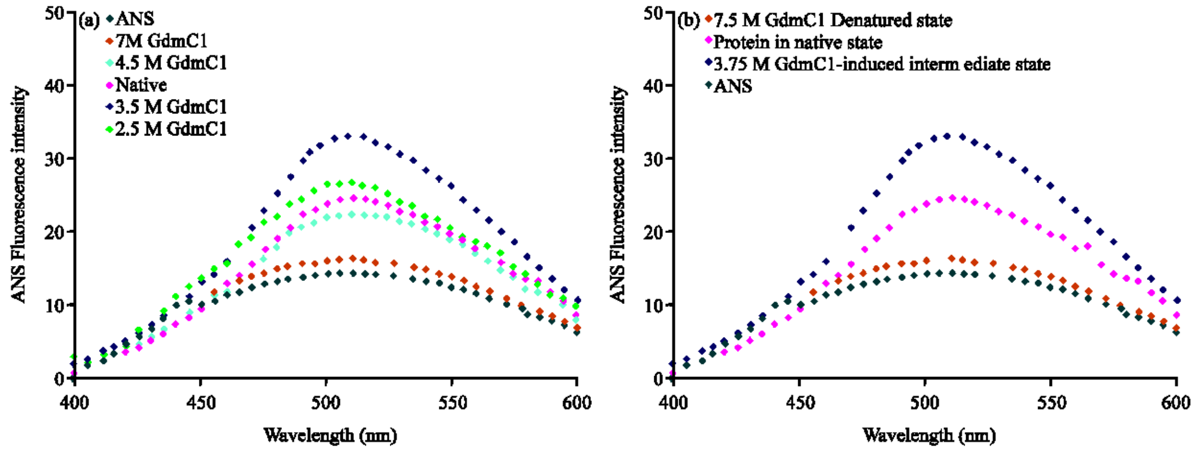

Fig. 5 (a-b): ANS binding measurements of ovomucoid in presence and absence of various concentrations of GdmCl. Enhancement in fluorescence and a blue shift in presence of GdmCl was seen up to the point where ovomucoid behaves as random coil. (Protein to ANS ratio used was 1:30).

an enhanced fluorescence and a blue shift in the wavelength of peak emission (λ_{max}).Use of this fluorescent probe has been particularly helpful in the identification of equilibrium intermediates termed molten globules. The probe binds and fluoresces intensely to the molten globule state presumably because the loose tertiary interactions present in molten globules enable the probe to find exposed hydrophobic regions. Binding and fluorescence of ANS in unfolded proteins has a much lower quantum yield due to quenching by the surrounding solvent. ANS fluorescence spectra of the native and various GdmCl-denatured states of ovomucoid are shown in Fig. 5 (a-b). It is seen in the (Fig, 5 a-b) that the fluorescence of ANS in the presence of the protein existing in 'X' state shows an increase in fluorescence intensity accompanied by a blue shift for GdmCl treated ovomucoid as compared to that obtained in the native state. Increasing GdmCl concentration does not affect the peak position.

Dynamic light scattering: Dynamic Light Scattering (DLS) or "the photon correlation spectroscopy" is a technique used to determine the size of particle. In DLS, intensity fluctuations are analyzed. The decay of the autocorrelation function of the signal gives the hydrodynamic radii. Fluctuations are a result of Brownian motion and can be correlated with particle diffusion coefficient and size and the typical measurement was quite fast with duration of about 1 min. The data can be analyzed to directly give the diffusion coefficients of the particles doing the scattering. When multiple species are present, a distribution of diffusion coefficient is seen.

Traditionally, rather than presenting the data in terms of diffusion coefficient, the data are processed to give the size of the particle (radius or diameter). The relation between diffusion and particle size is based on theoretical relationships for the Brownian motion of spherical particles, originally derived by (Einstein, 1956). DLS measurements on ovomucoid in the absence and presence of GdmCl were done using Laser spectroscatter 201 at 25°C. The hydrodynamic radii of the native and GdmCl-induced intermediate state and denatured state ovomucoid in sodium phosphate buffer pH 7.0 was found to be 2.34, 2.77 and 3.46 nm, respectively. The results of DLS measurements are summarized in Table 4 which shows that the intermediate state obtained in a

Table 4: Dynamic light scattering measurements

Protein states	Hydrodynamic-radii (nm)	Polydispersity (%)	Molecular mass (kD)
Native	2.34±0.24	7.50	24.8
Intermediate	2.77±0.13	4.60	36.7
Denatured	3.46±0.60	17.40	62.0

wide range of GdmCl denaturation may possibly be a molten globule with an increase of 19.94% in hydrodynamic radii higher from native state. The generation of two species is evident from the profile of hydrodynamic radii.

DISCUSSION

In the present study GdmCl -induced denaturation of ovomucoid in the presence and absence of various polyols at different concentrations were carried out at pH 7.0 and 25°C. These denaturation curves were analyzed for $\Delta G_D°$ and C_m (midpoint of denaturation) to measure the shift of the equilibrium, N state↔D state. Since the analysis involved three assumptions, a few comments are necessary. First, it has been assumed that the GdmCl-induced denaturation of ovomucoid in the absence of polyols follows a two-state mechanism, which is indeed true for most proteins in the absence of osmolyte (Griko and Privalov, 1994). We have assumed that a two-state assumption is also valid in the presence of polyols. The second assumption is that the dependence of ΔG_D of the protein on the denaturant concentration is linear in entire [GdmCl] range at a given concentration of each polyol. It was seen that at each fixed concentration of polyol, the plot of ΔG versus [GdmCl] is linear at least in the narrow denaturation concentration range. Since there is neither direct interaction between polyols and proteins (Xie and Timasheff, 1997) nor there is any interaction between denaturant and polyol (Kim *et al.*, 2003), it seems reasonable to assume a linear dependence of ΔG on [GdmCl] range upto 0 M. In fact, earlier reports have shown that ΔG_D of several proteins in the absence of polyol varies linearly with [GdmCl] throughout the denaturant concentration range (Ahmad *et al.*, 1994; Gupta and Ahmad, 1999). C_m values obtained from the analysis of the GdmCl-induced transition curves in the absence and presence of polyols given in Table 1 which shows Increase in C_m values of the protein was also observed, which suggest polyols increase in stability of GdmCl denatured ovomucoid. C_m of ovomucoid at pH 7.0 increases with an increase in the osmolyte concentration and stability decreases in the order of sorbitol>xylitol> adonitol>mannitol>glycerol. This can be explained by the fact that sorbitol, adonitol, mannitol and xylitol have osmophobic effect on protein while glycerol is solvophobic (Becktel and Schellman, 1987; Kaushik and Bhat, 1998; Timasheff, 2002). Further, that the stabilizing effect of glycerol is significantly less than that of other sugars, is also an expected result, for the more is the number of hydroxyl groups, the more is the stabilization of the protein (Gerlsma, 1968; Gerlsma and Stuur, 1972). This provides that polyols provide the protective environment for ovomucoid against GdmCl and urea-induced denaturation in a concentration dependent manner and also suggest that protecting ability decreases with the decrease in chain length.

We have also observed that $\Delta G_D°$ of a protein at physiological pH remains unchanged in the presence of polyols. $\Delta G_D°$ values calculated from the analysis of transition curves were in the range of 10.78-10.85 kcal moL^{-1} (average, 10.81 kcal moL^{-1}). If we consider our $\Delta G_D°$ measurements in physiological context, the important physiological role played by the osmolytes in governing protein stability-function relationship may be appreciated. If osmolytes were to increase $\Delta G_D°$, it would mean that the protein turnover rate would be decreased, for there exist an inverse relation between

ΔG_D° and *in vivo* rate of degradative (Pace *et al.*, 1981; Mcledon, 1977). We have also used various other optical probes to see the effects of polyols on the chemically denatured ovomucoid. Literature studies have shown that GdmCl and urea-induced unfolding of ovomucoid involves two steps when monitored by $\Delta\varepsilon$ measurements. The first step N↔X and second step X↔D (Baig and Salahuddin, 1978). Present results also confirm the three observable stable states N, X and D states during denaturation of ovomucoid in the presence of GdmCl and urea.

In order to see whether the isothermal denaturation of ovomucoid involves three states even in the presence of polyols and how polyols stabilize these different states, we have measured denaturation transitions of ovomucoid in the absence and presence of sorbitol using absorption spectroscopy as optical probes. Sorbitol was chosen because it has the most stabilizing effect. Measurements of $\Delta\varepsilon_{287}$ were done for GdmCl (Fig. 3). We observed that sorbitol stabilized both the GdmCl-induced transitions N↔X and X↔D. In case of GdmCl the notable feature of the first transition is the absence of pre-transition zone. The first transition was complete at and above 2.5 M GdmCl. The second transition started from 4.25 M GdmCl and was complete at 6.5 M GdmCl. Thus, the intermediate conformation state exists between 2.5 to 4.25 M. However, in presence of 10 and 20% sorbitol, first transition was complete at and above 3 and 3.25 M GdmCl, respectively. Second transition in presence of 10 and 20% sorbitol started from 4.50-4.75 M, respectively, thus the intermediate state in presence of 10 and 20% sorbitol existed from 3 to 4.50 M and from 3.25 to 4.75 M, respectively (Table 2). An obvious shift of the intermediate state from 2.5-3.25 to 4.25-4.75 in the absence and presence of polyols, respectively, indicated stabilising effect.

Also the mid points of both transitions shifted in presence of sorbitol. The increase was from 1.25-1.75 M and 5.40-5.90 M for first and second transition, respectively.

In order to see whether this GdmCl-induced denaturation was due to the ionic nature of the denaturant, nonionic denaturant urea was taken. It was found to induce unfolding of ovomucoid (Waheed *et al.*, 1977).which was same as that of the GdmCl, i.e., involving three observable stable states N, X and D (Fig. 4). The notable feature of the first transition was the presence of small pre-transition zone, which was absent in the GdmCl-induced unfolding. Possible reason for the absence of pre-transition zone in the presence of GdmCl is because of it being strong denaturant than urea. The first transition in urea denaturation of ovomucoid was is complete at and above 4 M urea. The second transition started from 7.0 M and was complete at 8.9 M urea. However in presence of 10% sorbitol, the first transition was complete at and above 4.5 M and the second transition started from 7.4 M urea. Thus, the intermediate state existed in the [denaturant] range from 4-7 and 4.5-7.4 M urea in absence and presence of sorbitol, respectively (Table 3), which leads to the conclusion that sorbitol stabilizes both the transitions.

In both GdmCl and urea denaturations it is seen from the Figure 3 and 4 that the polyols have less or no effect on the intermediate state which supports the thermal denaturation studies (data not published). The long transition regions suggest that the intermediate state of the protein is highly stable and is not fairly affected on addition of polyols even at high concentrations.

In order to ascertain whether X state has molten globule like character we have carried out fluorescence measurements of ANS in the presence of ovomucoid at various concentrations of GdmCl. ANS fluorescence shows the exposure of hydrophobic groups and suggested that this protein passes through an intermediate state as ANS shows increase in fluorescence intensity with a blue shift in the emission maximum on binding with exposed hydrophobic clusters which are known to be present in the molten globule (Semisotnov *et al.*, 1991). Present observations suggest that the fluorescence intensity and wavelength maximum of ANS emission spectrum are unperturbed in the presence of the native protein, whereas there is a significant change in the intensity and a blue shift in the emission maximum of ANS in the presence of protein in 'X' state obtained by a range of GdmCl (2.5, 3.5, 4.5 and 7 M) concentrations. These results suggest that 'X' state has a molten globule characteristics namely loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent. On increasing the concentration of GdmCl, fluorescence intensity increases gradually with a blue shift upto the concentration at which ovomucoid is fully denatured and behaves as a random coil. No increase in the intensity was seen after that due to the fact that no hydrophobic patches were available for the ANS to bind. As circular dichroism and fluorescence studies provided information about the species present in the solution as a whole and constitute an average measurement of their physical properties in an ensemble to deal with individual property Dynamic Light Scattering (DLS) measurements were done. The homogeneity of the solution was reflected in the parameter percent of polydisersity (Pd). Polydispersity is the measurement of standard deviation of the size of the particle. Native ovomucoid shows polydispersity around 7.5% and it increased to 17.4% with an increase in concentration of GdmCl. This increase in polydispersity means that there is decrease in homogeneity of the solution. The results obtained from DLS experiments also showed an increase in the hydrodynamic radius of the protein on GdmCl-induced denaturation, which might be probably due to the formation of the molten globule state. The change in R_h (hydrodynamic radius) during chemical denaturation is a good measure of protein radius during unfolding. Since the radius of ovomucoid increases gradually from 2.34 to 3.46 nm on increasing the GdmCl concentration, it provides a direct evidence for the generation of an intermediate during the unfolding process of ovomucoid as exemplied here may be a molten globule. Also the long transition region suggests that the molten globule of this protein is highly stable. DLS has been used to monitor change in dimension of the protein during denaturation and renaturation. This has been extended here for studying chemical denaturation of ovomucoid. Ovomucoid, the multidomain protein, exhibits scattering behavior, which is consistent with a globular protein as supported from the DLS studies. These results supported the ANS measurements.

CONCLUSION

The overall conclusion of the study is that effect of polyols on different states of ovomucoid obtained by GdmCl and urea-induced denaturation was found to be stabilizing and the stabilizing effect was observed for both the transitions ($N \leftrightarrow X$ and $X \leftrightarrow D$) with less or no effect on the intermediate state. They stabilizes ovomucoid in terms of C_m but ΔG_D° of the protein remains significantly unchanged. Flourescence and DLS measurements showed that the intermediate state obtained may possibly be a molten globule.

ACKNOWLEDGMENTS

GM acknowledges CSIR for fellowship. Department of Biochemistry, JH acknowledges UGC for support.

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