Effect of Guanidine Hydrochloride on the Thermal Stability of Hen Egg White Lysozyme

Thesis Submitted

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Submitted by:

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Certificate

This to certified that the thesis entitled ***"Effect of Guanidine Hydrochloride on the Thermal Stability of Hen Egg White Lysozyme"*** *being submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***the School of Chemistry and Biochemistry, Thapar University, Patiala*** *is a bonafide work carried under the supervision of* ***Dr.Rajesh Kumar,*** *Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.*

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CANDIDATE'S DECLARATION

I hereby declared that the work presented in this thesis entitled ***"Effect of Guanidine Hydrochloride on the Thermal Stability of Hen Egg White Lysozyme"*** *submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***the School of Chemistry and Biochemistry, Thapar University, Patiala*** *is an authentic record of my own work carried out under the supervision and guidance of* ***Dr. Rajesh Kumar,*** *Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and refers other researcher's work which are duly listed in the reference section.*

The matter embodied in this thesis has not formed the basis for the award of any other degree of this or any other university.

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Date: 15 July, 2012

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Regards,

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1.0 Introduction

All biological processes depend on proteins being stable and in appropriate folded conformation. It is important to know how proteins fold into their biologically active states, and how these states are stabilized. The complete characterization of any protein requires its stability determination and the forces which lead to stability and correct folding. Several factors such as hydrophobic interactions, electrostatic interactions, hydrogen bonding, and conformational entropy are accountable for correct folding and stability of native proteins [1]. The role of electrostatic [2-6], hydrophobic [7-8] and hydrogen bonding [9-10] interactions in determining the stability of proteins are well known, but the relative contributions of each of these factors vary from protein to protein and with the solution conditions to which the protein is exposed [6-8]. Protein folding study is medically important because misfolding of protein can lead to various diseases like Alzheimer's disease, cystic fibrosis, Parkinson's disease, Huntington's disease, Creutzfeldt–Jakob disease, and some form of cancers are also related to proteins misfolding [11]. So in order to cure these diseases, it is important to understand how protein misfolding and aggregation occurs.

Folding of some translates in the cellular milieu is assisted by cellular helper factors, including proline isomerase, protein-protein disulfide isomerase, and molecular "chaperones". The reaction in most cases can be adequately reproduced in the test tube by using chaotropic denaturants that disrupt protein structures. The exact mechanism of action of chaotropic denaturants such as guanidine hydrochloride (GdnHCl) on proteins is poorly understood. The functional dependence of structural and energetic properties of proteins with solvent composition is not clear either. These issues are quite relevant to the understanding of structure, stability, function, and folding of proteins. Using hen egg white lysozyme (Figure 1), the present study endeavors to show how such properties vary in the reaction space. In the

present work, we particularly determined the GdnHCl dependence of denaturation free energy of lysozyme in the range of 0-6M GdnHCl at pH 5. The denaturation free energy in the subdenaturing limit was determined from the thermodynamic analysis of fluorescence monitored thermal denaturation transitions of lysozyme at different concentration of GdnHCl. The denaturation free energy at higher GdnHCl concentration (*i.e.,* in the transition region) was determined by thermodynamic analysis of fluorescence monitored GdnHCl-induced denaturation curves of lysozyme at pH 5.

Lysozyme is a globular protein of about 129 amino acids folded in two domains, α – domain-characterized by α-helical structure and β-domain-organized in β-sheets. Both the domains are functional for the active site cleft which is formed between them [12]. Lysozyme has six tryptophans, two are located in the β-domain (Trp 62 and 63) and other are in α-domain (Trp 28, 108, 111, 123). Trp 62 and 63 are attributed to the main contribution to the intrinsic tryptophan photoluminescence (>80%). Trp 62 has binding capability with denaturants (GdnHCl) and the other one (108) being buried in the protein near the active cleft [13-14].The remaining four tryptophan (Trp 28, 63, 111, and 123) are involved in electron transfer, or interaction with disulfide bond or other amino acid quenchers and therefore become less important for the global luminescence signal [15]

Figure 1. Ribbon schematic of hen egg white lysozyme (PDB accession ID: 2 lyz, showing α helix and β sheet respectively).

Tyrosine and tryptophan are used experimentally because their quantum yield is high enough to give a good fluorescence signal. In case of intrinsic PL experiment of lysozyme, to minimize the excitation of tyrosine residue, the excitation wavelength was set at 290nm rather than 280nm corresponding to the maximum absorption band of tryptophan [16].

2.0 Materials and Methods

Hen egg white lysozyme was purchased from sigma and was used without further purification. GdnHCl was from USB. Other analytical grade chemicals were from Sigma or Merck. All experiments were done in 50mM glycine and 25mM sodium acetate buffer at pH~5.

2.1 Thermal unfolding of lysozyme in the presence of different concentration of GdnHCl, pH 5

PL measurements were performed on luminescence spectrometer equipped with a temperature controlled cell holder and an optical cuvette of 1cm path length. Samples were prepared in buffer (50mM glycine and 25mM sodium acetate) solution containing 0-4M GdnHCl, and containing ~10μM protein (lysozyme). Thermal unfolding of lysozyme was followed by measuring intrinsic emission fluorescence in the wavelength range of 280 to 480 nm and temperature from 10 to 90 °C. Pelteir controlled heating rate was 1°C/min. The temperature of the cell holder was regulated by an external circulating water bath. Slits were set as 6 nm for both excitation and emission.

2.2 GdnHCl-induced unfolding of lysozyme at pH 5

The Samples were prepared in buffer (50mM glycine and 25mM sodium acetate) solution, containing 0-6 M GdnHCl, and ~10μM protein and were incubated for 30 minutes. Fluorescence emission spectra were recorded in a Perkin Elmer LS-55 fluorescence spectrometer setting the excitation and emission slits to 6 nm each and excitation wavelength of 280 nm. Relative fluorescence intensity values were plotted against molar concentration of

GdnHCl to obtain the optimum denaturant concentration at which the change in fluorescence intensity was at a maximum.

3.0 Results

3.1 Effect of GdnHCl on thermal stability of lysozyme

To determine the effect of GdnHCl on the thermal stability of lysozyme, we recorded a series of intrinsic tryptophan PL-monitored thermal-denaturation curves for lysozyme in the presence of varying concentration of GdnHCl at pH~5. Figure 2a shows the representative thermal denaturation process measured by intrinsic tryptophan PL of lysozyme in the absence of denaturant (GdnHCl) at pH~5. At low temperatures λ_{max} of these thermal denaturation curves remains unchanged. Starting from 60°C, the tryptophan luminiscence band shows a progressive red shift which ends at a temperature depending on the concentration of denaturant used. Accordingly, the signal intensity decreases, probably due to the thermal activation of nonradiative channels, which quench the luminiscence from the excited electronic state [16].

Figure 2b shows the results of λ_{max} analysis on the steady-state PL of lysozyme. The thermodynamic parameters associated with the temperature-induced denaturation of lysozyme were obtained by nonlinear least squares analysis of the temperature dependence of the protein. In the analyses, a two-state denaturation process was assumed when fitting the data to the van't Hoff equation:

$$y(T) = \frac{(y_N + m_N T) + (y_U + m_U T)\, exp\left[\frac{-H_m}{RT}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right]}{1 + exp\left[\frac{-H_m}{RT}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right]} \qquad (1)$$

Where, $y(T)$ is the observed variable parameter, y_N and y_U, and m_N and m_U, represent intercepts and slopes of the native (pre-transition) and unfolded (post-transition) baselines respectively, ΔH_m is the enthalpy at the transition temperature T_m, R is the gas constant and T is the absolute temperature. Both transition temperature (T_m) and van't Hoff enthalpy (ΔH_m) decrease linearly with an increase in GdnHCl concentration (Figure 3a). One of the reliable methods for obtaining an accurate value of ΔC_p is to measure the temperature dependence of the van't Hoff enthalpy, ΔH_m of the transition at different denaturant concentrations [17-18].

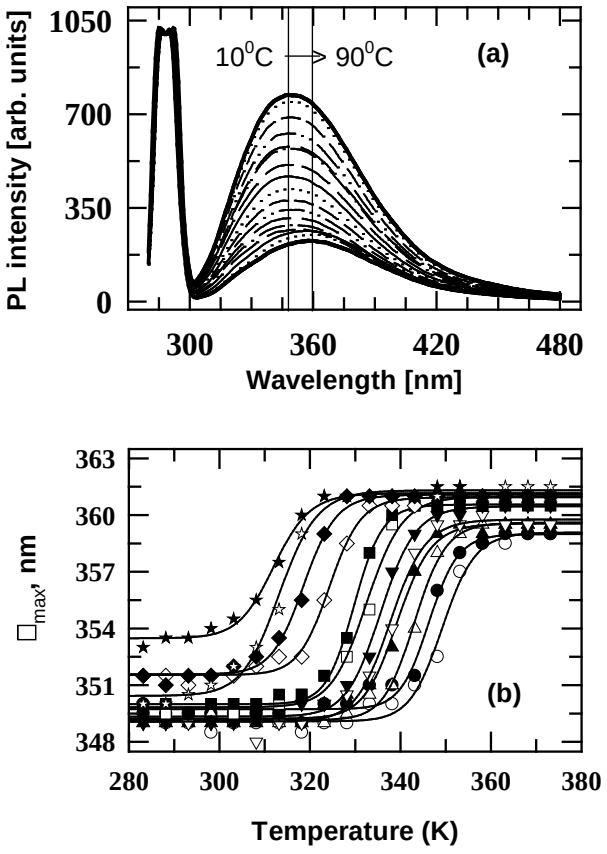


Figure 2.(a) Steady state PL spectra of lysozyme excited at 290 nm and measured at different temperatures. The bold black lines indicate the spectra at the lowest (10 ºC) and the highest (90 ºC) temperature. The vertical solid lines indicate the position of the band peaks as a guide to the eyes. b) Temperature-induced denaturation of lysozyme in the presence of different concentration of GdnHCl (0.0(o), 0.25(●), 0.50(Δ), 0.75(▲), 1.0(▽), 1.3(▼),1.6(□), 2.0(■), 2.5(◊), 3.0(♦), 3.5(★),4.0(☆) M GdnHCl at 25 ºC, 50mM glycine and 25mM sod. acetate buffer,pH~5. The solid curves represent non-linear least–squares fits to the the standard two state equation.

Figure 3.(b) shows the dependence of the van't Hoff enthalpy, ΔH_m on the transition temperature, T_m. The ΔC_p value (1.21 ± 0.1 kcal K^{-1} mol^{-1}) was determined by linear least-squares fit of the equation 2,

$$\Delta H_m = \Delta C_p T_m + b \qquad (2)$$

Where, b is the y-intercept. Using ΔC_p=1.21 ± 0.1 kcal K^{-1} mol^{-1}, we determined the free energy of thermal denaturation (ΔG_u) at 295.15 K from the integrated form of the Gibbs-Helmholtz relation (Equation 3).

$$\Delta G_u = \Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p \left(T - T_m - T \ln\left(\frac{T}{T_m}\right)\right) \qquad (3)$$

Where ΔG_u is the free energy associated with the transition, T_m is the midpoint temperature for the transition, ΔH_m is the enthalpy change of the transition and ΔC_p is the change in heat capacity for the transition which is assumed to be temperature dependent. The thermal denaturation free energy data are plotted in the Figure 4b.

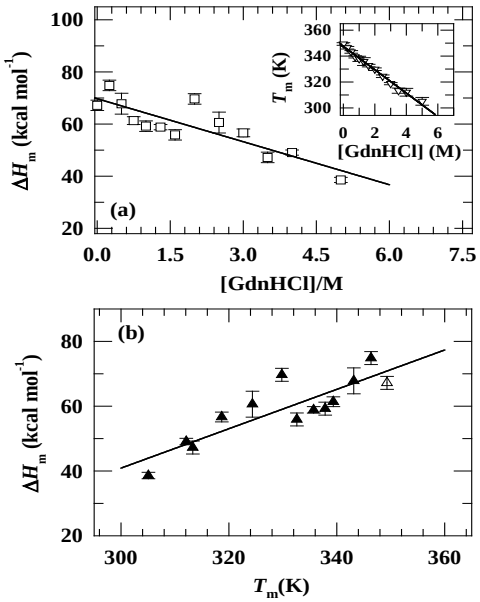


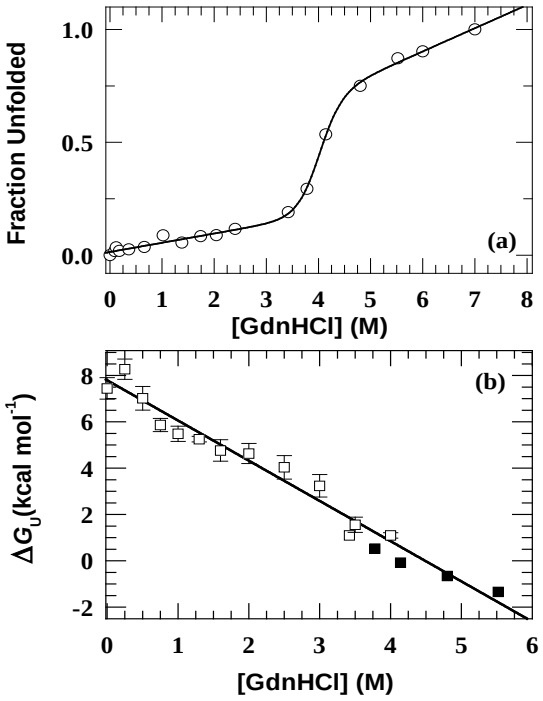
Figure 3.(a) Variation of ΔH_m with denaturant concentration. Temperature midpoints of unfolding (T_m) in different GdnHCl concentrations are shown in the inset. (b) Enthalpies of the thermal transition of lysozyme as a function of transition temperature in the presence of different GdnHCl concentrations. The line represents a linear least-square fit.

3.2. Protein stability: functional dependence of denaturational energy on GdnHCl

Figure 4a present the GdnHCl induced equilibrium unfolding transition of lysozyme at pH~5. The unfolding transitions were analyzed by assuming a two state transition between the folded (N) and unfolded (U) conformation by using the procedure of Santoro and Bolen [19].

$$S_{obs} = \frac{\left(y_N + m_N[D]\right) + \left(y_U + m_U[D]\right) exp\left(\frac{-\Delta G_D + m_g[D]}{RT}\right)}{1 + exp\left(\frac{-\Delta G_D + m_g[D]}{RT}\right)} \qquad (4)$$

Where S_{obs} is the observed signal, y_N and y_U, and m_N and m_U represent intercepts and slopes of native and unfolded baseline respectively, [D] represent denaturant (GdnHCl) concentration in molar, R is the gas constant, ΔG_D the free energy associated with transition,



and m_g is the surface area of the protein exposed by the solvent. All parameters were floated during fitting.

Figure. 4 (a) GdnHCl-induced equilibrium unfolding transition of lysozyme at pH~5, 25 °C. The data were normalized and fitted to the standard two state equation (19) to obtain ΔG°_{u} = 7.66±0.2 kcal mol^{-1} and m_g= 1.63±0.1 kcal mol^{-1} M^{-1}.(b) Evaluation of unfolding free energy changes as a function of GdnHCl. The denaturation free-energy below 4 M GdnHCl (□) were calculated from the thermal denaturation data in the presence of different concentrations of GdnHCl, at 25 °C. Points above 4 M GdnHCl (■) were calculated from equilibrium curve shown in Figure 4a (ΔG_D=-RTln K_D, where K_D=F_U/F_N is the equilibrium constant, and F_U and F_N are the unfolded and folded fractions). The solid line represents the results of the application of the linear extrapolation model.

To test the validity of linear functional dependence of Gibbs-free energy of unfolding on GdnHCl, the values of ΔG_D (ΔG_D = -RT lnK_D), where K_D=F_U/F_N is the equilibrium constant, and F_U and F_N are the unfolded and folded fractions of the protein) determined from GdnHCl unfolding curves are plotted in figure 4b together with the values of ΔG_u calculated from equation (3). A linear least squares fit of the data gives ΔG_D° = 7.66±0.2 k cal mol^{-1} and m_g = 1.63±0.1 k cal mol^{-1} M^{-1}. These values of ΔG_D° and m_g are in excellent agreement with those obtained from the equilibrium curve Figure 4a. The results thus validate the linear functional dependence of ΔG_D on GdnHCl in the full range of the denaturant concentration.

4.0 Discussion

4.1 Effect of GdnHCl on the structure and thermal stability of lysozyme

The present work provides direct evidence that denaturant such as GdnHCl significantly affects the structure and thermal stability of lysozyme. In the temperature induced unfolding of lysozyme a thermal red shift is observed which starts at a temperature value which strongly depends upon the GdnHCl concentration. A cooperative conformational transition, likely due to the aperture of the cleft between the α and the β domains and consequent exposure to the solvent of the buried tryptophan 108, is identified in this sigmoidal red-shift. There is a good agreement of the transition temperatures with the already found values of thermal unfolding calculated by calorimetric experiments [20-21].

The GdnHCl-induced red shift, the PL λ_{max} clearly shows a sigmoidal red shift as a function of temperature, with a transition temperature which decreases progressively by increasing GdnHCl concentration. At the end of the transition, *i.e.,* at high temperature the λ_{max} remains slightly red-shifted as a function of increasing GdnHCl content, which indicates that the preferential solvation of GdnHCl molecules is maintained.

4.2 Two state folding of lysozyme: analyses of linear free energy relationship

The equilibrium unfolding transition curve, monitored by the change of intrinsic fluorescence, fits well with the two-state model, indicating that the transition could have taken place without the formation of any detectable intermediates, which would have altered the emission spectra. It is possible that the intermediate species might accumulate during the unfolding pathway; however, those non-native states were not distinct in terms of their

microenvironment around the tryptophan residues. As a result, detectable intermediate species could not be traced while monitoring the GdnHCl-induced unfolding process of lysozyme using intrinsic fluorescence spectroscopy [22].

The basic experimental test to ascertain the operation of a protein according to the two-state equilibrium, N↔U, is the coincidence of unfolding transitions for multiple spectroscopic probes [23], although observation of distinct transition curves of two different properties for the denaturation does not necessarily provide a proof of the existence of an intermediate [24]. In the present case the GdnHCl dependence of unfolding free energy (ΔG_D) is linear over the full range of the denaturant concentration, which suggest an absence of equilibrium intermediates in the U↔N folding of lysozyme.

4.3 Linear free energy model (LFEM) *vs* binding model

There has been considerable debate about how chemical denaturants unfold proteins. Although the most popular 'linear free energy model' (LFEM) that holds that the unfolding free energy can be linearly extrapolated from the transition region to the absolute native condition, has been theoretically formalized by Schellman [25-26]. Unfolding data can also be treated by invoking stoichiometric binding of denaturant molecules to protein groups [27]. While the LFEM has been experimentally validated by numerous studies [28-30 for example], some of the most detailed solution-state studies of the interaction of chemical denaturants with proteins validate the binding model [31-32 for example]. Protein structures determined from crystals soaked in denaturants [33-34] also lend substantial support to the binding model. However, the results for lysozyme presented here validate LFEM rather than the binding model. The observation that the ΔG_D-denaturant plot not deviates from LFEM, suggests an absence of equilibrium intermediate in the U↔N folding of lysozyme.

5.0 Conclusion

In this work, we studied the structure and thermal stability of lysozyme at different concentrations of Guanidine hydrochloride (GdnHCl). Thermal denaturation of lysozyme depends upon the GdnHCl amount, and it is accompanied by a red-shift of the PL emission band (Figure 2), associated with the solvent exposure of tryptophan 108. The addition of GdnHCl to the system progressively decreases the transition temperature reducing the protein thermal stability.

The ΔG_u dependence on GdnHCl is linear, suggesting the validity of the linear free energy model in the analysis of GdnHCl denaturation data. It has been shown further that the ionic nature of GdnHCl, and therefore the electrostatic screening effect, has no influence on the applicability of one or the other model. The linear dependence of ΔG_u as a function of GdnHCl concentrations has also been shown for lysozyme, which suggests an absence of equilibrium intermediates in the U↔N folding of lysozyme. Thus lysozyme folds *via* a two state folding mechanism without an accumulation of intermediate at pH 5.

6.0 References

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