Effect of Amino Acids on the Thermal Stability of Hen Lysozyme both in the Absence and Presence of Denaturant

Thesis Submitted

In partial fulfillment of the requirement for the degree of

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Submitted by:

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Under the supervision of

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PATIALA- 147004

JULY, 2013

Certificate

This is to certify that the thesis entitled ***"Effect of amino acids on the thermal stability of hen lysozyme both in absence and presence of denaturants"*** *being submitted in the partial fulfillment 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 out 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 declare that the work presented in this thesis entitled ***"Effect of amino acids on the thermal stability of hen lysozyme both in absence and presence of denaturants"*** *submitted in the partial fulfillment 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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This is to certify that above statement made by the student concerned is correct and true to the best of my knowledge.

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Rajwinder Kaur

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Regards,

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1.0 Introduction

For many decades, the kinetic, thermodynamic, and structural aspects of the protein folding reaction *in vitro* have been studied by using the various physical, chemical, and biological methods. These studies have surely provided some insight, but the basic questions – how the unfolded polypeptide chain folds to a native three dimensional conformation within the biologically relevant folding time remains enigmatic. To perform biological function, the unfolded polypeptide chain needs to fold correctly. However, some time it do mistakes and misfold, which can produce a number of conformational diseases [1-2].

The complete characterization of a protein requires stability determination and the forces which lead to stability and correct folding. There are several factors responsible for correct folding and stability of a protein. These include electrostatic interactions [3-7], hydrophobic interactions [8-9], and hydrogen bonding [10-11]. However, the relative contributions of each of these factors vary from one protein to another and with the solution conditions under which the protein is exposed [3, 9]. Protein stability characterization is a necessary element for biopharmaceutical and vaccines development and has an extreme importance for a number of applications ranging from protein-based pharmaceutical formulation to protein misfolding diseases.

A large number of cosolvents are widely used in the studies of protein folding, and in determining the molecular interactions that stabilize or destabilize the protein structures. The native conformation of proteins under external osmotic stress such as dehydration, temperature variations, variable pH, freezing, high salinity, and internal stress such as high concentrations of denaturants can be stabilized by the accumulation of low-molecular-weight organic molecules, termed as osmolytes [12-15]. Naturally occurring organic osmolytes can be grouped into three

categories, namely, polyols; amino acids and their derivatives; and methylamines and urea [16-18]. The protein functions that have been lost because of the presence of denaturants can be regained by osmolytes such as methylamines through restoring the protein to its native structure [19-25]. Amino acids that serve as osmolytes are, proline, serine, arginine, glycine, alanine, threonine etc. An earlier study by Taneja et al (1994) has shown that arginine and histidine destabilize the native cytochrome *c*; isoleucine, leucine, and phenyalanine have no effect on the stability of this protein; and valine stabilizes this protein [26]. However, the mechanism by which these amino acids affect the native protein structure is not clearly understood.

When present at higher concentrations, chaotropic denaturant such as guanidine hydrochloride (GdnHCl) typically unfold the proteins through direct binding to peptide groups [27-29]. Though, the effects of amino acids and denaturants on the heat perturbation of proteins are extensively studied [26-29], however, the effect of amino acids on the heat perturbation of native protein in the presence of denaturants is rarely studied. In the present work, we analyzed the effect of various amino acids (proline, serine, arginine, glycine, alanine, threonine) on the thermal stability of hen egg white lysozyme both in the absence and presence of denaturant by monitoring the wavelength shift with temperature increase in the fluorescence emission spectrum (ex: 290) of lysozyme at pH 4.5. The thermal denaturation experiments revealed stabilizing effect of all amino acids used except arginine. It is also observed that among the stabilizing amino acids, the thermal stability of lysozyme is increased more for proline and least for alanine. The present work also examines the effect of various amino acids on the heat perturbation of native protein in the presence of GdnHCl. The inclusion of stabilizing amino acids in the presence of GdnHCl results in the counteraction of the destabilizing effect of the GdnHCl.

Hen egg-white lysozyme is a 129-residue enzyme used to catalyze the hydrolysis of the linkage between N-acetylmuramic acid and N-acetyl glucosamine subunits in the peptidoglycan polymers of many bacterial cell walls. Native form of hen egg-white lysozyme is cross-linked by four disulfide bridges and adopts mainly helical conformation (~30 % of α helix; and ~6 % of beta sheet). Furthermore, hen egg-white lysozyme is structurally homologous to human lysozyme (60% sequence homology), which has been found to be responsible for hereditary non-neuropathic systemic amyloidosis [30-31]. Given the aforesaid features, hen egg-white lysozyme therefore serves as a nice model system to investigate the *in vitro* protein folding process.

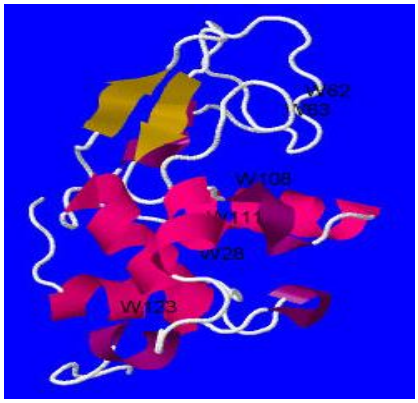

Figure 1. The Structure of native hen egg-white lysozyme (PDB accession ID: 1AKI). The tryptophan residues are marked according to their location in one letter symbol.

2.0 Material and methods

Hen egg white lysozyme was purchased from calbiochem and was used without further purification. GdnHCl was purchased from USB. Amino acids (proline, glycine, alanine, arginine, serine and threonine) were purchased from high media. Sodium acetate was purchased from Sigma. All experiments were done in 50 mM sodium acetate buffer at pH~4.5.

2.1 **Thermal unfolding of native lysozyme (pH 4.5) in the presence of different concentrations of amino acids**

To determine the effect of amino acids on thermal unfolding of lysozyme, the protein samples (~6μM) were prepared in 50 mM sodium acetate buffer that contained different concentrations of amino acids (arginine, alanine, glycine, proline, threonine, and serine). PL measurements were performed on fluorescence spectrometer (PerkinElmer LS 55) equipped with a temperature controlled cell holder and an optical cuvette of 1 cm path length. Thermal unfolding of lysozyme was followed by measuring intrinsic emission fluorescence (excitation, 290 nm) in the wavelength range of 280 to 480 nm and temperature from 10 to 90 °C. Pelteir controlled heating rate was 1°C/min. Slits were set as 6 nm for both excitation and emission.

2.2 Thermal unfolding of native lysozyme (pH 4.5) as a function of GdnHCl both in the absence and presence of amino acids

To investigate the effect of denaturant on thermal unfolding of lysozyme, the protein samples (~6μM) were prepared in 50 mM sodium acetate buffer that contained different concentrations of GdnHCl. To determine the effect of amino acids on the GdnHCl dependent thermal stability of protein, the protein samples were prepared in 50 mM sodium acetate buffer that contained 0.5 M amino acid (arginine, alanine, glycine, proline, threonine, and serine) and varying concentrations of GdnHCl. Thermal unfolding measurement parameters are same as described in section 2.1.

3.0 Result

3.1 Effect of amino acids on the thermal denaturation of native lysozyme

To determine the effect of amino acids on the thermal denaturation of native lysozyme, a series of intrinsic tryptophan PL-monitored thermal-denaturation curves for lysozyme in the presence of diffrent concentrations of amino acids (arginine, alanine, glycine, proline, threonine, and serine) were recorded at pH 4.5. Figure 2 shows the representative temperature dependent fluorescence emission spectra of lysozyme in the presence of ~3.6 M glycine at pH~4.5.

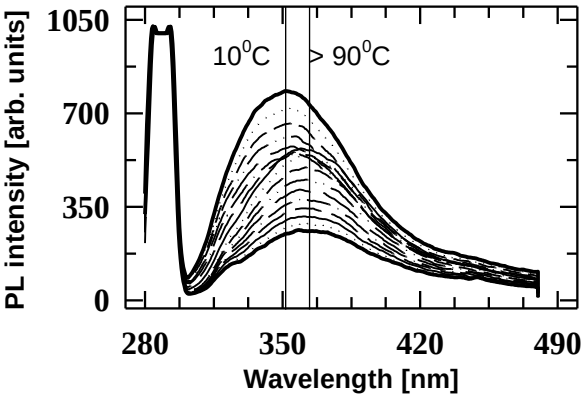

Figure 2. Steady state PL spectra of lysozyme excited at 290 nm and measured at different temperatures. The bold black lines indicate the spectra at the lowest (10 °C) and the highest (90 °C) temperatures. The vertical solid lines indicate the position of the band peaks as a guide to the eyes.

Figure 3a shows the PL-monitored temperature-induced λ_{max} shift of lysozyme in the absence and presence of 0.5 M amino acid (arginine, alanine, glycine, proline, threonine, and serine) at pH~4.5. At low temperatures, the λ_{max} of these thermal unfolding curves does not change significantly. However, when temperature is increased above 60°C, the tryptophan luminescence band shows a progressive red shift which ends at a temperature depending on the concentration of amino acid used. Furthermore, with increasing the temperature, the signal intensity decreases. This is probably due to the thermal activation of nonradioactive channels, which quench the luminescence from the excited electronic state [32]. The data presented in Figure 3b were normalized according to equation (1)

$$\text{Fraction Unfolded} = \frac{\lambda_{\text{max, (obs)}} - (m_{\text{pre}}T + c_{\text{pre}})}{(m_{\text{post}}T + c_{\text{post}}) - (m_{\text{pre}}T + c_{\text{pre}})} \quad (1)$$

where, $\lambda_{max\ (obs)}$ is the observed λ_{max}, T is the temperature, m_{pre} and c_{pre} are slope and intercept, respectively, of the pre-transition baseline, and m_{post} and c_{post} are slope and intercept of the post-transition baseline in the presence of a given concentration of amino acid.

Figure 3c clearly indicates that as the concentration of arginine is increased, the thermal unfolding curve shift towards lower temperatures. However, with the increase in concentration of other amino acids (alanine, glycine, proline, threonine, and serine), the thermal unfolding curves shift toward higher temperatures (Figure 3b). The thermodynamic parameters (T_m and ΔH_m) associated with the temperature-induced unfolding of lysozyme in the presence of amino acids were obtained by nonlinear least squares analysis of the temperature dependence of the λ_{max}.

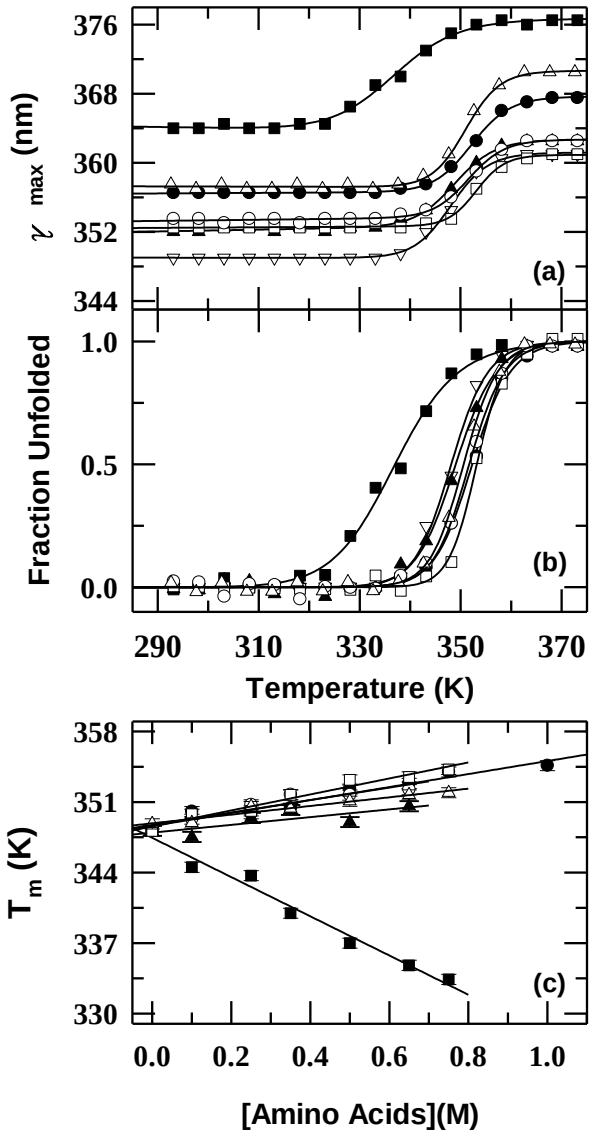

Figure 3 (a) PL-monitored temperature-induced λ_{max} shift of lysozyme in the absence (∇) and presence of 0.5 M amino acid (threonine (o), serine (●), glycine (Δ), alanine (▲), proline (□), arginine (■)), in 50 mM sodium acetate buffer, pH ~4.5. (b) Panel (b) shows the normalized temperature-induced denaturation curves of lysozyme in the absence (∇) and presence of 0.5M amino acid (threonine (o), serine (●), glycine (Δ), alanine (▲), proline (□), arginine (■)). The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation 2. (c) Variation of T_m with amino acid concentration, threonine (o), serine (●), glycine (Δ), alanine (▲), proline (□), arginine (■). The solid lines in panel (c) represent the linear fit to the data.

In these analyses, a two-state denaturation process was assumed when fitting the data to the van't Hoff equation [33]:

$$\lambda_{\max,\,(obs)}\,(\mathrm{T}) = \frac{\left(m_{pre}T + c_{pre}\right) + \left(m_{post}T + c_{post}\right) exp\left[\dfrac{-\Delta H_{\mathrm{m}}}{RT}\left(\dfrac{1}{T_{\mathrm{m}}} - \dfrac{1}{T}\right)\right]}{1 + exp\left[\dfrac{-\Delta H_{\mathrm{m}}}{RT}\left(\dfrac{1}{T_{\mathrm{m}}} - \dfrac{1}{T}\right)\right]} \qquad (2)$$

where, $\lambda_{\max\,(obs)}$ (T) is the observed variable parameter, H_{m} is the enthalpy at the transition temperature T_{m}, R is the gas constant and T is the absolute temperature. The resulting midpoint transition temperature, T_{m}, for unfolding of lysozyme was plotted as a function of amino acid concentration in Figure 3c. As amino acid concentration is increased, the T_{m} decreases linearly in case of arginine while it increases linearly for other amino acids (alanine, glycine, proline, threonine, and serine). These findings suggest that the arginine decrease the thermal stability of the protein while the other amino acids (alanine, glycine, proline, threonine, and serine) increase the thermal stability of the lysozyme. Further, among the stabilizing amino acids, the thermal stability is increased more for proline and least for alanine (Figure 3c).

3.2 Effect of GdnHCl on thermal denaturation of native lysozyme

Figure 4(a) shows the results of λ_{max} analysis on the steady-state PL of lysozyme in the presence of different concentrations of GdnHCl (0.0, 0.75, 1.3, 2.0, 2.5, 3.0, 3.5 M) at pH 4.5. The data shown in Figure 4a were normalized by using the equation (1). The fraction of unfolded protein was plotted as a function of temperature in Figure 4b. Figure 4b clearly shows that as GdnHCl concentration is increased, the thermal denaturation curve shift toward lower temperature. The T_{m} and ΔH_{m} for the thermal unfolding of lysozyme at different GdnHCl concentration were determined by fitting the data to the van't Hoff equation (2). The resulting, T_{m}, for unfolding of lysozyme was plotted as a function GdnHCl concentration in Figure 4c. As GdnHCl concentration is increased, the value of T_{m} decrease linearly, which suggests that GdnHCl decrease the thermal stability of native protein.

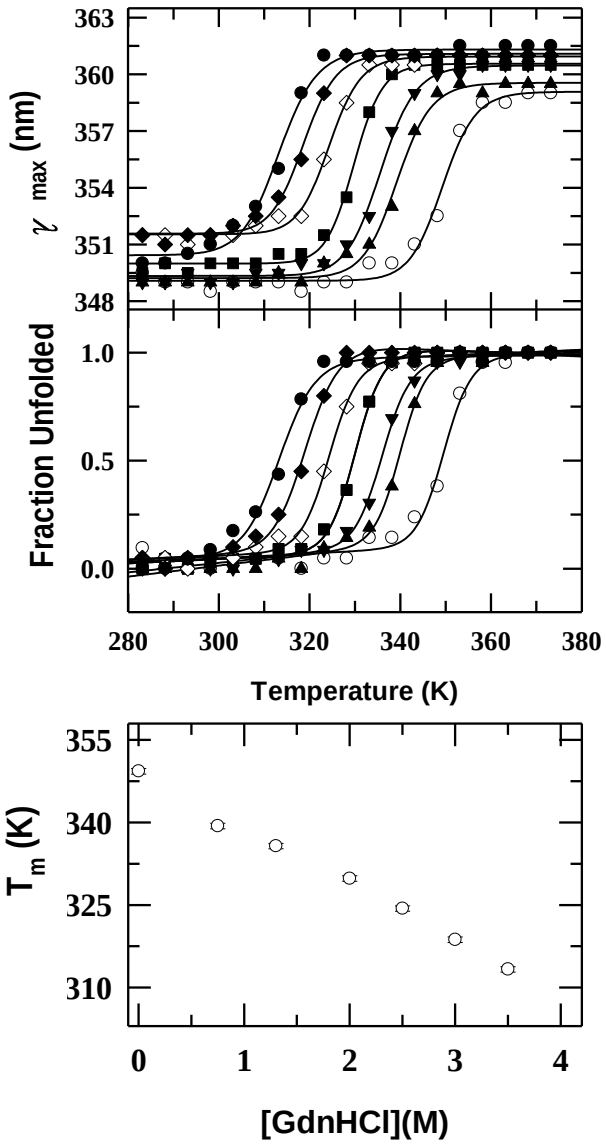

Figure 4. (a) PL-monitored temperature-induced λ_{max} shift of lysozyme in the presence of different concentrations of GdnHCl (0.0 (○), 0.75 (▲), 1.3 (▼),acetate buffer, pH~4.5. (b) Panel (b) show the normalized thermal-induced denaturation curves of lysozyme in the presence of different concentrations of GdnHCl (0.0 (○), 0.75 (▲), 1.3 (▼), 2.0 M (■), 2.5 (◊), 3.0 (♦), 3.5 (●)). The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation (2). (c) Variation of T_m with the GdnHCl concentration at pH 4.5. The solid line represents the linear fit to the data.

3.3 The effect of amino acids on the GdnHCl-dependent thermal stability of native lysozyme

The results of λ_{max} analysis on the steady-state PL of lysozyme in the absence of GdnHCl or amino acids at pH 4.5 are shown in Figure 5a. The results of λ_{max} analysis on the steady-state PL of lysozyme in the presence of 2.0 M GdnHCl with and without 0.5 M of amino acid (arginine, alanine, glycine, proline, threonine, and serine) are also shown in Figure 5a. The data presented in Figure 5a were normalized according to equation (1) and are plotted as fraction of unfolded protein with temperature in Figure 5b. Figure 5b clearly showed that the thermal unfolding curve shift toward lower temperature in the presence of GdnHCl. However, when about 0.5 M of stabilizing amino acids (alanine, glycine, proline, threonine, and serine) is included, the shift of thermal unfolding curve in the presence of GdnHCl toward lower temperature become less pronounced (Figure 5b). On the other hand, with inclusion of 0.5 M arginine the shift of the thermal unfolding curve toward

lower temperature becomes more pronounced (Figure 5b). The T_m and ΔH_m associated with thermal unfolding of lysozyme (Figure 5a and Figure 5b) were obtained by fitting the data to the van't Hoff equation (2).

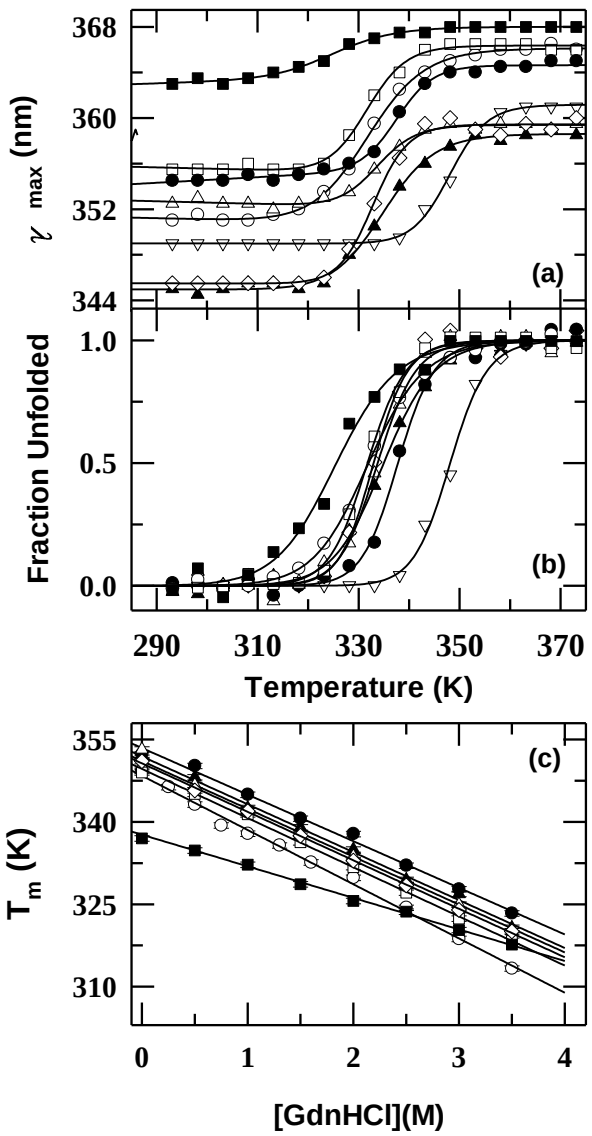

Figure 5 (a) PL-monitored temperature-induced λ_{max} shift of lysozyme in the presence of 0.0 M GdnHCl (∇), 2.0 M GdnHCl (o) and 2.0 M GdnHCl with 0.5 M amino acid (arginine (■) alanine (□), glycine (◊), proline (Δ), threonine (▲), and serine (●)), in 50 mM sodium acetate buffer, pH~4.5. (b) Panel (b) show the normalized thermal-induced denaturation curves of lysozyme in the presence of 0.0 M GdnHCl (∇), 2.0 M GdnHCl (o) and 2.0 M GdnHCl with 0.5 M amino acid (arginine (■) alanine (□), glycine (◊), proline (Δ), threonine (▲), and serine (●)). The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation (2). (c) The variation of T_m with GdnHCl concentration both in the absence (o) and presence of 0.5 M amino acid (arginine (■) alanine (□), glycine (◊), proline (Δ), threonine (▲), and serine (●)). The solid lines in panel (c) represent the linear fit to the data.

The variation of T_m with the GdnHCl concentration both in the absence and presence of 0.5 M amino acid (arginine, alanine, glycine, proline, threonine, and serine) is shown in Figure 5c. When the concentration of GdnHCl is increased, the T_m decreases linearly (Figure 5c). However, when 0.5 M of the stabilizing amino acid (alanine, glycine, proline, threonine, and serine) is included, the decrease in T_m with increase in the GdnHCl concentration becomes less pronounced, which indicates that the stabilizing amino acids (alanine, glycine, proline, threonine, and serine) counteract the destabilizing effect of denaturants. However, when destabilizing amino acid, arginine is included, both GdnHCl and arginine showed additive effect on T_m (Figure 5a).

4.0 Discussion

The present work discusses the effect of amino acids (arginine, alanine, glycine, proline, threonine, and serine) on the thermal stability of lysozyme both in the absence and presence of GdnHCl. The possible explanations for the observed effect of amino acids on the thermal stability of lysozyme in the presence of GdnHCl are also discussed.

4.1 Amino acids affect the thermal stability of lysozyme

The results obtained indicate that the amino acids modulate the thermal stability of native lysozyme. Among the amino acid used, arginine decreases the thermal stability of native lysozyme. An earlier report has also shown that arginine decreases the thermal stability of native cytochrome *c* [26]. The current study showed that alanine, glycine, proline, threonine, and serine increase the thermal stability of protein. It is also observed that the thermal stability of native protein is increased more for proline and least for alanine. The stabilizing effect of amino acids on the thermal stability of protein can be explained on the basis of two facts [34], (i) the preferential exclusion of amino acids from the folded protein domain, and (ii) preferential interaction of amino acid with the unfolded protein [26]. In general, the preferential exclusion of amino acids causes the preferential hydration of the protein. This increases the surface tension of water and tends to oppose denaturation which results in an increase in surface area of the protein [26]. On the other hand, the preferential interaction of amino acid with the unfolded protein tends to favour denaturation. This tendency increases with increasing hydrophobicity [26]. This is presumably because denaturation results in the exposure of buried hydrophobic side chains in the protein. Thus the observed effect depends on the fine balance between these two opposing forces. Clarke and Zounce [35] reported that the arginine behave as protein denaturant because part of its side chain is structurally similar to $GdnH^+$ ions. Timasheff has suggested that the

nature of interaction of denatured proteins with arginine is similar to that of $GdnH^+$ ions, which binds to peptide groups and aromatic side chains [36].

4.2 GdnHCl decreases the thermal stability of lysozyme

The present work also indicates that GdnHCl significantly decreases the thermal stability of lysozyme. It has been suggested that the denaturing action of denaturants is due to their migration into the interior of the protein and also due to the formation of hydrogen bonds to the atoms in the backbone [37]. The preferential interaction of the protein with the solvent components in the presence of high concentrations of GdnHCl also leads to the structural destabilization of protein.

4.3 Stabilizing amino acids counteracts the destabilizing action of the denaturant

The destabilizing action of GdnHCl is mainly due to their ability to bind to the protein [27-29]. The thermal stability of lysozyme is decreased in the presence of GdnHCl (Figure 4c, and Figure 5c). The inclusion of stabilizing amino acids (alanine, glycine, proline, threonine, and serine) results in the counteraction of the destabilizing action of the GdnHCl (Figure 5c). On the other hand, arginine shows an additive effect on the destabilizing action of GdnHCl (Figure 5c). It has been reported that addition of osmolytes alters the balance between preferential binding of denaturants and preferential exclusion of osmolytes resulting in the increased hydration of the protein and hence counteract the denaturing effect of denaturants [39-40]. Similarly, it can be assumed that the addition of stabilizing amino acids alters the balance between the preferential binding of GdnHCl and preferential exclusion of these amino acids, resulting in the increased hydration of the protein and hence counteracts the destabilizing action of the denaturant. Among the stabilizing amino acids, the counteraction effect on the destabilizing action of the denaturant is more pronounced for serine and least for alanine.

5.0 Conclusion

The present work examines the effect of amino acids (arginine, alanine, glycine, proline, threonine, and serine) on the thermal stability of native lysozyme (pH 4.5) both in the absence and presence of GdnHCl. It has been found that the thermal stability of lysozyme decreases in the presence arginine while it increases in the presence of alanine, glycine, proline, threonine, and serine. Among the amino acids used, the extent of the increase in the thermal stability of native lysozyme is more for proline and least for alanine. The stabilizing effect of amino acids is mainly due to preferential hydration of protein or the preferential exclusion of the amino acids [26].The destabilizing effect of arginine can be attributed to its side chain similarity with $GdnH^+$ ions [35]. Both $GdnH^+$ and arginine bind to peptide groups and aromatic side chains of protein [36]. As usual, GdnHCl decreases the thermal stability of native protein and the inclusion of stabilizing amino acids results in the counteraction of the destabilizing effect of the GdnHCl. This may be due to the fact that the addition of these amino acids alters the balance between the preferential binding of GdnHCl and preferential exclusion of amino acids, resulting in the increased hydration of the protein and thus helps in the counteraction of the destabilizing action of the GdnHCl. The counteraction effect of stabilizing amino acids on the destabilizing action of the denaturant was found to be more for serine and least for alanine.

6.0 References

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