Effect of Neutral Salt on the Stability of Horse Cytochrome *c* at Acidic pH

A

Thesis Submitted

In partial fulfillment of the requirement for the degree of

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IN

CHEMISTRY

Submitted by:

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Under the supervision of

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PATIALA- 147004

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CERTIFICATE

*This is to certify that the thesis entitled "**Effect of Neutral Salt on the Stability of Horse Cytochrome c at Acidic pH**" being submitted in 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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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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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled, ***"Effect of Neutral Salt on the Stability of Horse Cytochrome c at Acidic pH"*** *in partial fulfilment of the requirement 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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Place: Patiala

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This is to certify that the above declaration made by the student concerned is correct and true to the best of my knowledge.

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In the end, I wish to express my deep sense of gratitude to my family, for supporting and encouraging me at every step of my work. It is the power of their blessings, which has given me the courage, confidence and zeal for hard work.

Dhiraj

Date: 14 July, 2011
Regards,

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Abstract

Horse ferricytochrome *c* (ferricyt *c*) at mildly acidic pH (~3.8) behaves as a two state folding protein. This work examined the effect of salt (NaCl) on the stability of horse ferricyt *c* at pH~ 3.8 (25^0 C). Chemical unfolding studies in urea (based on fluorescence; excitation: 280 nm, and emission: 365 nm) show that NaCl significantly increases the chemical stability of the protein. On the other hand, thermal unfolding studies (based on absorbance at 399 nm) show that NaCl significantly decreases the thermal stability of ferricyt *c*. As [NaCl] is increased, the midpoint transition temperature, T_m, for unfolding of ferricyt *c* decrease mono-exponentially, plateau at ~0.5 M NaCl consistent with destabilization of ferricyt *c* by ionic screening of electrostatic interactions.

1.0 Introduction:

Protein misfolding and its pathogenic consequences have become a significant issue over the last three decades. According to the prion researcher Susan Lindquist, half of all human diseases could be the result of protein misfolding and aggregation.[1] Protein misfolding is believed to be the primary cause of Alzheimer's disease, Parkinson's disease, Huntington's disease, Creutzfeldt–Jakob disease, cystic fibrosis, Gaucher's disease and many other degenerative and neurodegenerative disorders. Protein misfolding is also responsible for various p53-mediated cancers. To function proteins fold[2], whereas misfolding is linked to a number of conformational diseases[3-4], thus making it imperative to determine the factors that control the stability of proteins.[2] Protein stability characterization is a critical element of biopharmaceutical and vaccines development.

The complete characterization of any protein requires stability determination and the forces which lead to stability and correct folding. Several factors, such as electrostatic interactions, hydrophobic interactions, hydrogen bonding, and conformational entropy are accountable for the folding and stability of proteins. The roles of these non-covalent interactions in determining the thermal stability of proteins are well recognized, 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.[5-7] At low to intermediate concentrations of salt (<0.5M NaCl), generally electrostatic effect is dominant[8-9], while at higher concentrations (> 0.5M NaCl) hydrophobic effect is dominant. Reduction of the net charge of proteins, via salts acting as counterions (screening effect), is an additional important effect of salts on protein stability.[10-11]

Indeed, the effect of various anions on protein stability usually follows their ranking in the Hofmeister series: $HPO_4^{2-} \approx SO_4^{2-} > CH_3COO^- > Cl^- > NO_3^- > ClO_4^-$. Anions at the beginning of this series (kosmotropes or promoters of water structure formation) stabilize proteins, whereas those towards the end of the series (chaotropes or disrupters of water structure) destabilize proteins.[12-15] The species to the left of Cl^- are referred to as kosmotropes, while those to its right are called chaotropes. A useful summary of Hofmeister ion interactions and how they affect protein stability is given by Jencks.[16] The question of how Hofmeister ions affect the structure and hydrogen-bonding properties of water was reviewed by Collins and Washabaugh.[17]

The contribution of electrostatic interactions to protein thermal stability is generally evaluated by determining the thermal stability of the protein in the presence of varying types and concentrations of electrolytes, often as a function of pH.[18-20] Although the thermal stability and functional properties of numerous proteins have been studied as a function of [salt] or ionic strength[18] few have been studied in adequate detail to offer insight into the relative contributions of the various mechanisms discussed above by which electrolytes influence protein behavior. The present thesis research work provides this analysis for horse cytochrome *c* (cyt *c*), a heme protein that transfers electron in the mitochondrial electron transport chain by virtue of the redox equilibrium $Fe^{3+} \rightarrow Fe^{2+}$, where the oxidized protein (Fe^{3+}) accepts an electron from the donor, undergoes reduction, and donates the electron to the acceptor.

In the native state, the heme iron of ferricyt *c* is axially coordinated by H18 and M80. The Fe^{3+}-M80 bond in ferricyt *c* readily ruptures even under mild perturbation of conformation produced by denaturants, facilitating non-native ligation of the heme iron with other potential intrapolypeptide ligands, such as histidines and lysines. Under unfolding conditions at neutral pH, the non-native heme iron-intrapolypeptide contacts (Fe^{3+}−H26 and Fe^{3+}−H33) are stable, so the dissociation rates of these two ligands from the iron are far smaller than the protein refolding rate. Consequently, the non-native ligands and parts of the polypeptide, they belong to are trapped in the misfolding ensemble. The problem of kinetic trap or chain misorganization in the folding run of ferricyt *c* can be mitigated by allowing refolding at low pH or in the presence of an extrinsic heme ligand that blocks non-native histidine ligation. Folding kinetics are then modeled as a two-state reaction[21-22] or by invoking intermediates.[23] This thesis work examined the effect of salt (NaCl) on the thermodynamic stability of horse ferricyt *c* at pH~ 3.8 (25^0 C). Under these mildly acidic conditions, the protein behaves in a two state manner.

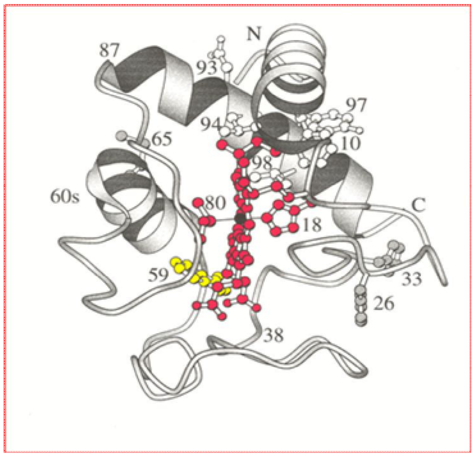

Figure 1. A ribbon representation of horse Cyt c; accession code PDB 1HRC. The side-chain of M80 and some other key residues are shown explicitly in ball and stick display. Heme atoms are shown in black. The figure has been drawn using Cerius2 Version 4.0 (MSI).

2.0 Materials and Methods

Horse heart Cyt *c* (type VI) was purchased from Sigma and used without further purification. NaCl was obtained from Sigma. Urea was purchased from USB. Other analytical grade chemicals were from Sigma or Merck. All experiments were done in 10 mM sodium acetate buffer at pH~3.8, 25^0 C.

2.1 Measurements of urea-induced unfolding of ferricyt *c* at different [NaCl]:

Samples of ferricyt *c*, prepared in the 0-8 M range of urea, contained ~10 μM protein, ~10 mM sodium acetate, and a desired NaCl concentration. Samples were incubated for ~30 minutes at 25°C. Fluorescence emission at 365 nm (excitation: 280 nm) was measured at 25 °C using a Perkin Elmer Fluorescence Spectrometer setting the excitation and emission slits to 2.5 and 5 nm, respectively. Data were analyzed using the standard two-state equation for equilibrium unfolding as described in earlier studies.[24]

2.2 Measurements of thermal-induced unfolding of ferricyt *c* at different [NaCl]:

These experiments involved temperature- dependent absorbance change of ferricyt *c* at 399 nm (Heme environment changes). Ferricyt *c* concentration was ~0.8 μM. The protein was held at pH ~3.8, contained ~10 mM sodium acetate in the presence of variable NaCl concentrations. Peltier-controlled heating rate was 1°C per minute.

3.0 Results:

3.1 Effects of salt on urea-induced unfolding of ferricyt *c*:

Fluorescence-detected (excitation 280 nm, emission 365 nm) urea titrations were employed to measure the effects of NaCl on the stability of the ferricyt *c* (Figure 2). The normalized data were fitted to a standard two-state equilibrium unfolding equation 1.[24]

$$\mathrm{S_{obs}} = \frac{(c_\mathrm{f} + m_\mathrm{f}[\mathrm{D}]) + (c_\mathrm{u} + m_\mathrm{u}[\mathrm{D}])\exp(\frac{-\Delta G_\mathrm{D} + m_\mathrm{g}[\mathrm{D}]}{RT})}{1 + \exp(\frac{-\Delta G_\mathrm{D} + m_\mathrm{g}[\mathrm{D}]}{RT})} \qquad (1)$$

where S_{obs} is the observed signal, c_f and c_u, and m_f and m_u represent intercepts and slopes of native and unfolded baselines, respectively, $[D]$ is the concentration of urea in M, R, the gas constant, ΔG_D, the free energy associated with the transition, and m_g, the surface area of the protein exposed by the solvent. The iterated fit parameters are given in the Table 1. Both ΔG_m and m_g, decrease mono-exponentially with increase in NaCl concentration, and saturates at ~ 1 M NaCl (Figure 2. and Table 1). The C_m, the transition midpoint of urea concentration, was calculated from equation (equation 2).

$$C_m = \frac{-\Delta G_D}{m_g} \qquad (2)$$

The C_m value increases in a cubic fashion with NaCl concentration (Figure 2d).

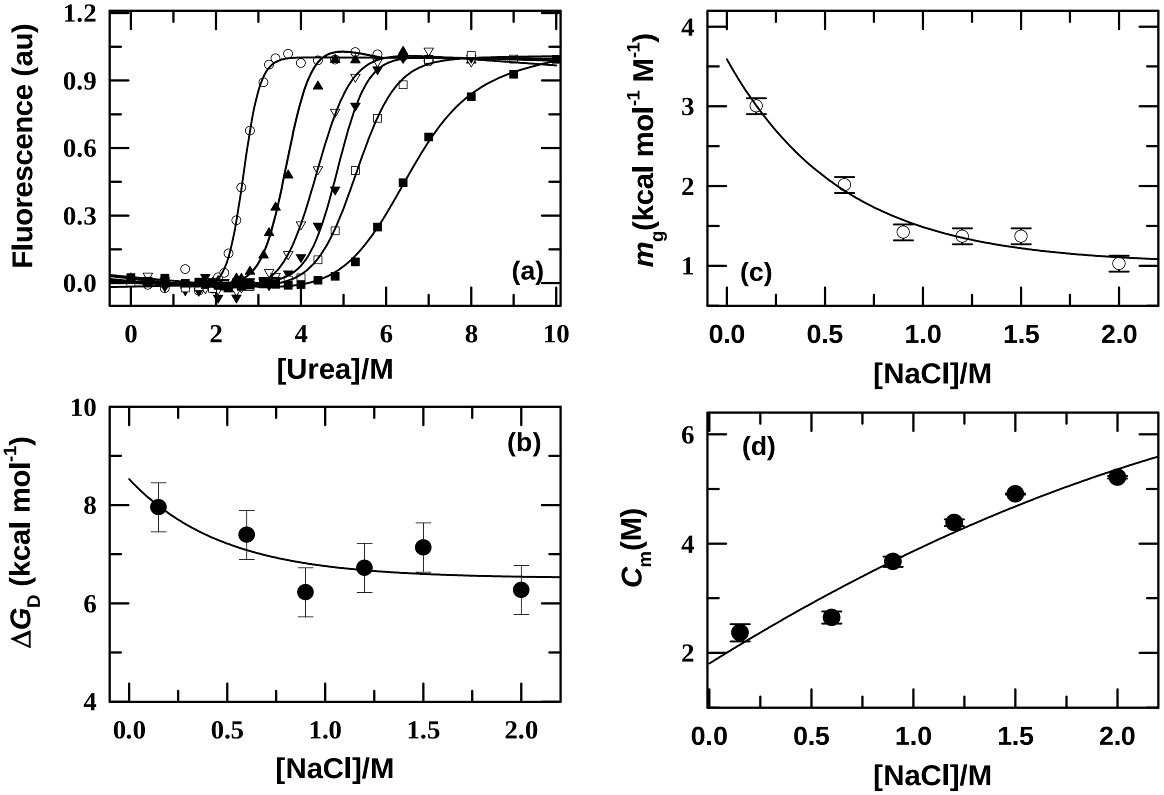

Figure 2(a) Urea–induced denaturation of ferricyt *c* in the presence of different concentration of NaCl (0.15 (O), 0.6 (▲), 0.9 (▽), 1.2 (▼), 1.5 (□), 2.0 (■) M NaCl) at 25(±1) °C, 0.01 M sodium acetate, pH~ 3.8. The solid curves represent non-linear least–squares fits to the the standard two state equation (equation 2).[24] (b) Variation of change in unfolding free energy, $\triangle G_D$ with NaCl concentration. (c) Surface area exposed by solvent of ferricyt c as a function NaCl concentration. Panel (d) shows urea-midpoints of unfolding (C_m) in different NaCl concentration.

3.2 Effects of salt on thermal unfolding of ferricyt *c*:

To assess the contribution of electrostatic interactions to the thermal stability of ferricyt *c*, the thermal dependence of ferricyt *c* unfolding was evaluated as a function of NaCl concentrations at pH~ 3.8 (Figure 3). The data presented were normalized according to

$$A(T) = \frac{A_{\mathrm{obs}} - (m_{\mathrm{pre}}T + c_{\mathrm{pre}})}{(m_{\mathrm{post}}T + c_{\mathrm{post}}) - (m_{\mathrm{pre}}T + c_{\mathrm{pre}})} \tag{2}$$

where, A_{obs} is the observed absorbance, 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 NaCl.

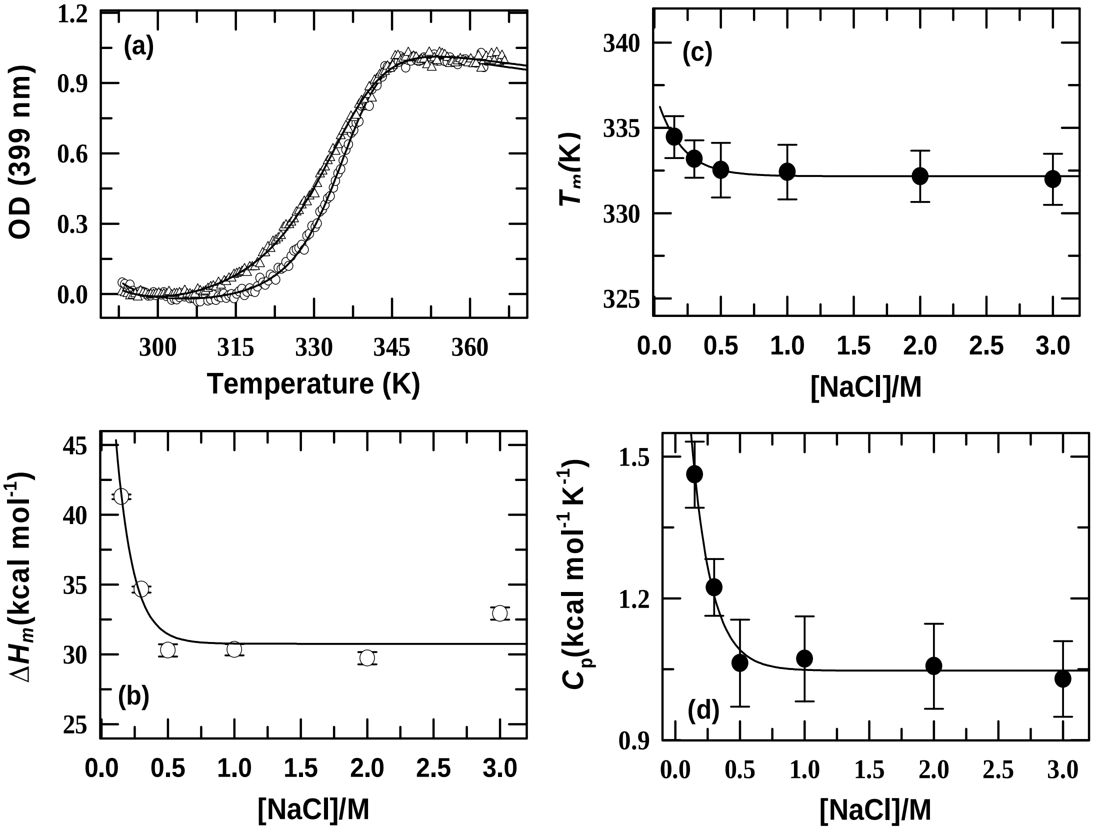

Figure 3. (a) The representative thermal unfolding transitions of ferricyt *c* at pH~ 3.8, 0.15 (Δ) and 3 M NaCl (○), respectively. The other thermal unfolding transitions (0.3, 0.5, 1.0, and 2.0 NaCl) were not shown in panel (a) to show the clarity of thermal data. Transitions were monitored by the decrease in optical density at 399 nm. The solid curves represent non-linear least–squares fits to the two-state unfolding model (*i.e.,* Gibbs-Helmoltz equation, equation 3). (b) Variation of $\triangle H_{\mathrm{m}}$ with NaCl concentration. Panel (c) shows temperature midpoints of unfolding, T_{m} in different NaCl concentration. Panel (d) shows heat capacity change, $\triangle C_{\mathrm{p}}$ with [NaCl].

Thermal unfolding of ferricyt *c* is reversible, so the normalized thermal transitions of ferricyt *c* were analyzed by non-linear, least-squares fitting of the data to the Gibbs-Helmoltz equation (equation 3).[24-25]

$$A(T)=\frac{(c_{\mathrm{pre}}+m_{\mathrm{pre}}T)+(c_{\mathrm{post}}+m_{\mathrm{post}}T)\exp\left[\frac{\Delta H_{\mathrm{m}}\left(\frac{T}{T_{\mathrm{m}}}-1\right)+\Delta C_{\mathrm{p}}\left[T_{\mathrm{m}}-T+T\ln(T/T_{\mathrm{m}})\right]}{RT}\right]}{1+\exp\left[\frac{\Delta H_{\mathrm{m}}\left(\frac{T}{T_{\mathrm{m}}}-1\right)+\Delta C_{\mathrm{p}}\left[T_{\mathrm{m}}-T+T\ln(T/T_{\mathrm{m}})\right]}{RT}\right]} \qquad (3)$$

where, ΔH_{m} is the enthalpy at the transition midpoint, T_{m}, and ΔC_{p} is the change in heat capacity. Table 2 lists the T_{m}, ΔH_{m} and ΔC_{p} values. At pH ~3.8, the values of T_{m} ¸ΔH_{m}, and ΔC_p for unfolding of ferricyt *c* decreases mono-exponentially with increasing salt concentration from 0.0 to 1.2 M NaCl (Table 2 and Figure 3). The decrease in ΔH_{m}, T_{m}, and ΔC_p values are more pronounced at millimolar salt concentrations and plateaus at ~0.5 M NaCl (Figures 3 b, c, and d).

4.0 Discussion:

4.1 Stability of ferricyt *c* in the presence of neutral salt

The present work provides direct evidence that neutral salt such as sodium chloride significantly affect the thermal and chemical stability of ferricyt *c*. Chemical unfolding studies in urea show that NaCl significantly increases the stability of the protein. On the other hand, thermal unfolding studies (based on absorbance at 399 nm) show that NaCl significantly decreases the thermal stability of ferricyt *c*. The effects of electrolytes on protein stability are generally attributed to electrostatic (Debye-Hückel) screening of Coulombic interactions,[8-9] to specific ion binding[26-28] or to increased surface tension of water[13,15,29-30] that alters hydrophobic interactions (the Hofmeister effect). Debye-Hückel screening can be considered to involve both co-solute screening effects (resulting from the presence of electrolyte in solution) and ion binding to charged residues (e.g., ion pair formation) at the protein surface at higher concentrations than required for specific ion binding.

The Hofmeister series characterizes some properties of ions in protein solutions. This series has consistent effects on the stability of the proteins. Anions have more pronounce effect than cations, and typically follow this order: $F^{-} \approx HPO_4^{2-} > SO_4^{2-} > CH_3COO^{-} > Cl^{-} > NO_3^{-} > Br^{-} > ClO_3^{-} > I^{-} ClO_4^{-} > SCN^{-}$. [12-14] The species to the left of Cl^{-} are referred to as kosmotropes, while those to its right are called chaotropes. Sodium chloride typically affects protein stability by modifying the ionic strength of the solution, which can be slightly stabilizing or destabilizing depending on nature of the charge distribution within the protein.[15]

As [NaCl] is increased, the conformational stability, ΔG_m and surface area of protein exposed by solvent, m_g decrease mono-exponentially and plateau at ~1 M NaCl. On the other hand, the urea-unfolding midpoint, C_m increases as the [NaCl] is increased. This may be due to the m_g values varied greatly with [NaCl] as compared to the ΔG_m. As shown in Figure 2c, the m_g value becomes larger at low salt concentration, suggesting that more surface area is exposed to solvent upon unfolding at salt concentration. The m_g value is generally believed to be proportional to the change in the solvent-accessible surface area between the unfolded state and the folded state.[31-32]Assuming that the structure of the folded state does not change significantly, the larger m_g values suggest a larger solvent-accessible surface area in the unfolded state at low salt concentration. Deviations from two-state folding can lead to lower m_g values.[33] Thus, another possibility is that folding is not two-state at high salt concentration values and becomes two-state at lower salt concentration values.

Thermal unfolding of ferricyt *c* at pH~3.8 shows that NaCl significantly decreases the thermal stability of ferricyt *c*. The values of midpoint transition temperature, T_m, and enthalpy change, ΔH_m, for unfolding of ferricyt *c* decreases mono-exponentially with increasing salt concentration from 0.0 to 1.2 M NaCl (Table 2 and Figure 3). The decreases in ΔH_m and T_m, values are more pronounced at millimolar salt concentrations and plateaus at ~0.5 M NaCl (Figures 3 b, c, and d). Although the decrease in thermal stability of proteins other than ferricyt *c* by increasing NaCl concentration is unusual, several examples are well documented. At low to intermediate concentrations of salt (<0.5M NaCl), ionic screening of electrostatic interactions often destabilize halophilic proteins,[5, 34-36] human plasma lipoproteins,[37-38] human prion protein,[39] an archaebacterial carboxypeptidase,[18] GCN_4 leucine zipper,[40] and human serum transferrin.[41]

The decrease in thermal stability of ferricyt *c* induced by salt indicates that destabilization of the protein by salt is not solely attributable to the salt-induced destabilization of hydrophobic effect but may involve substantial contributions from a salt-induced decrease in Coulombic interactions at the protein surface. Several lines of evidence indicate that the decrease in thermal stability of ferricyt *c* by salt results from ionic screening of electrostatic interactions. First, the range of salt concentration over which ferricyt *c* (0.01-0.5 M, Figure 3) is destabilized, is characteristic of the concentration range at which the effects of salts on protein stability are dominated by ionic screening. In contrast, specific ion binding usually occurs at lower salt concentrations (<0.001 M)[26-27] while hydrophobic effects of salt become predominant at higher concentrations (>0.5 M).[5, 8-9, 34, 42] Exponential saturation of the salt effect on ferricyt *c* (Figure 2) is also consistent with Debye-Hückel screening by diffusive counterions (*e.g.*, co-solute screening).[9, 37]

4.2 Effect of neutral salt on heat capacity of ferricyt *c*.

The value of heat capacity change, ΔC_p for unfolding of ferricyt *c* decreases mono-exponentially with increasing salt concentration from 0.0 to 1.2 M NaCl (Table 2 and Figure 3).The decreases in ΔC_p values are more pronounced at millimolar salt concentrations and plateaus at ~0.5 M NaCl (Figures 3d). In general, the ΔC_p values mainly reflecting the extent of hydrophobic interactions in a given protein. The larger ΔC_p values of ferricyt *c* suggest a higher contribution of hydrophobic interactions at low salt concentration. The value of ΔC_p for ferricyt *c* in the absence of salt reported here is at least 0.5 kcal mol^{-1} K^{-1} higher than that for the ferricyt *c* in the presence of 2 M NaCl (Table 2), suggesting that the hydrophobic residues in the protein are relatively more shielded from water.

5.0 Conclusion:

Ferricyt *c* has been studied as a function of [NaCl] and temperature to assess the effects of these factors on the stability of protein. Urea-induced unfolding of ferricyt *c* at different [NaCl] indicates that the salt significantly increases the stability of the protein. As [NaCl] is increased, the urea-unfolding midpoint, C_m is also increased, indicating an increase of chemical stability of the protein in the presence of salt. On the other hand, the surface area of protein exposed by solvent, m_g decreases mono-exponentially as the [NaCl] is increased from 0.0 to 2 M. The large decrease in m_g value with increasing [NaCl] indicates that the folding of ferricyt *c* is not remained two-state at higher salt concentrations. The temperature-induced unfolding of ferricyt *c* at variable [NaCl] indicates that the NaCl reduces the thermal stability of ferricyt *c*, contrary to the usual stabilizing influence of NaCl for most other proteins. The decrease in thermal stability of ferricyt *c* by NaCl indicates that the thermo-destabilization of the protein by salt is mainly attributable due to the electrostatic effect. As [NaCl] is increased, the thermal denaturation midpoint, T_m, for unfolding of ferricyt *c* decreases mono-exponentially and plateau at ~0.5 M NaCl consistent with destabilization of ferricyt *c* by ionic screening of electrostatic interactions. Further, the value of heat capacity change, ΔC_p for unfolding of ferricyt *c* also decreases mono-exponentially with increasing [NaCl]. The value of ΔC_p for ferricyt *c* in the absence of salt is at least 1.5 fold higher than that for the ferricyt *c* in the presence of 2 M NaCl (Table 2), suggesting that the hydrophobic residues in the protein are relatively more shielded from water.

6.0 Tables

Table1. Dependence of the urea unfolding free energy, ΔG_D (kcal mol^{-1}), surface area of protein exposed by solvent, m_g (kcal mol^{-1} M^{-1}), and urea unfolding midpoint, C_m (M) on salt (pH 3.8) as monitored by Trp fluorescence (ex: 280; em: 365 nm).

[NaCl] (M)	**ΔG_D (kcal mol^{-1})**	**m_g (kcal mol^{-1}M^{-1})**	**C_M (M)**
0.15	7.95	3.0	2.4
0.6	7.5	2.1	2.6
0.9	6.3	1.6	3.6
1.2	6.8	1.5	4.4
1.5	7.2	1.6	4.8
2.0	6.4	1.1	5.3

*The uncertainties associated with ΔG_D, m_g, and C_m are ± 0.5 (kcal mol^{-1}), 0.1 (kcal mol^{-1} M-^{1}), and *0.5* (M), respectively.

Table 2. Dependence of the transition temperature (K), enthalpy change, ΔH_m (kcal mol^{-1}), and heat capacity change, ΔC_p (kcal mol^{-1} K^{-1}) for unfolding of ferricyt *c* on salt (pH 3.8) as monitored by absorbance at 399 nm.

[NaCl] (M)	T_m **(K)**	ΔH_m **(kcal mol^{-1})**	ΔC_p **(kcal mol^{-1}K^{-1})**
0.15	334.0	41.5	1.48
0.3	333.0	34.8	1.23
0.5	332.7	30.1	1.06
1.0	332.5	30.2	1.07
2.0	*332.5*	30.0	1.06
3.0	*332.0*	32.7	1.04

*The uncertainties associated with T_m, ΔH_m, and ΔC_p are ± 1.0 (K), 2.0 (kcal mol^{-1}), and 0.2 (kcal mol^{-1} K^{-1}), respectively.

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