# EFFECT OF NEUTRAL SALT ON STABILITY AND DYNAMICS OF HORSE FERROCYTOCHROME *C*

*A*

*Thesis Submitted*

*in partial fulfillment of the requirement for the degree of*

**MASTER OF SCIENCE**

**IN**

**CHEMISTRY**


Submitted by:

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Under the supervision of

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JULY, 2010

# CERTIFICATE

*This is certify that the thesis entitled "**Effect of Neutral Salt on Stability and Dynamics of Horse Ferrocytochrome c**" 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),& **Dr. Amjad Ali, (**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 Neutral Salt on Stability and Dynamics of Horse Ferrocytochrome c"*** *in partial fulfillment 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,&* ***Dr. Amjad Ali****, 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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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 power of their blessings, which has given me the courage, confidence and zeal for hard work.

Karamjeet Kaur

Regards,

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Date: July, 2010

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# Abstract

This work examined the effect of neutral salt (NaCl) on the stability and dynamics of horse ferrocytochrome at pH 7, $25^0$ C. Unfolded CO-liganded ferrocytochrome *c* (UCO) refolds to a native like compact state (NCO), where the non-native $Fe^{2+}$-CO interaction persists. Slow thermal dissociation of the CO molecule from NCO to yields the native state (N). The rate coefficients for thermal dissociation of CO from NCO and association of CO to N decreases by ~2 fold as the NaCl concentration of the reaction medium is increased from 0 to 3.0 M. This has been taken as the evidence for the internal motion constraints and increased stability of the protein in the presence of salt. As [salt] is increased, the fluorescence intensity decrease mono exponentially and plateau at ~0. 5 M NaCl, consistent with stabilization of protein by ionic screening of electrostatic interactions. The unfolding free energy of carbonmonoxycyt *c*, defined as the difference of free energies of unfolded and native states ($\Delta G°$) increases by 2 ( ±0.2) kcal $mol^{-1}$ when the protein is held at 3 M NaCl, which suggest neutral salt increases the global stability of the protein.

# 1 Introduction

There have been rapid advances in structural biology and relating structure to biochemical function and mechanism. However, knowledge of protein structure alone does not ensure accurate prediction of stability, function and biological activity. The complete characterization of any protein requires stability determination and the forces which lead to stability and correct folding. All biological processes depend on proteins being stable and in the appropriate folded conformation. Many factors are responsible for the folding and stability of native proteins, including hydrophobic interactions, electrostatic interactions, hydrogen bonding, and conformational entropy. The roles of electrostatic,[1-5] hydrophobic, [6,7] and hydrogen bonding [8,9] interactions in determining the 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] Ionic interactions in proteins are extremely complex because they can exist on the fully exposed surface of proteins, fully buried in the interior of the protein, or in a partially buried environment with varying degrees of hydrophobicity of the surrounding residues. In addition, buffer conditions (salt and pH) can have dramatic effects on the contributions of ion pairs to stability.

The stability of a protein, as a natural polyelectrolyte, is generally dependent on pH and [salt]. The pH dependence reflects the contribution of electrostatic interactions, which are essential for the conformational stability of globular proteins. The effects of salts on protein stability are generally attributed to electrostatic (Debye-Hückel) screening of Coulombic interactions (i.e., at low to intermediate salt concentration),[9-10] to specific ion binding [11-12] or to increased surface tension of water [13-17] that alter hydrophobic interactions (the Hofmeister effect). The contributions of electrostatic and hydrophobic interactions to protein stability are generally evaluated by determining the stability of the protein in the presence of varying types and concentrations of salts often as a function of pH.[18-20] While the stability and functional properties of many proteins have been studied as a function of ionic strength or [salt], [5,18] few have been studied in sufficient detail to provide insight into the relative contributions of the various mechanisms outlined above by which electrolytes influence protein behavior. The present study provides this analysis for horse cytochrome *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.

## 2 Materials and Methods

Horse heart Cyt c (type VI) was from Sigma. GdnHCl was obtained from Gibco BRL. Other analytical grade chemicals were from Sigma or Merck. All experiments were done in 50 mM sodium phosphate buffer at pH 7, 25 $^0$C.

**2.1 Measurements of equilibrium unfolding.** Samples of cyt *c* (~10 μM) were prepared in the 0-10.0 M range of urea. To prepare carbonmonoxycyt *c*, the solutions containing desired urea concentrations were deaerated and reduced under nitrogen with 2.5 mM sodium dithionite. The dithionite-reduced samples were saturated with CO by passing a gentle stream of the dry gas through the solutions for a minute. Samples were then incubated in tightly capped quartz cuvettes or rubber capped small glass tubes for ~15 minute. The sealed tubes were shaken gently in dark for 5-10 minute at room temperature. Optical absorption measurements were done as usual at pH 7, 25 °C. Tryptophan fluorescence excited at 280 nm was measured at 365 nm. The reported urea concentrations are those determined after measurement**.**

**2.2 Preparation of native carbonmonoxycyt *c* and measurement of CO dissociation kinetics.** Cyt *c*, initially dissolved in 6.35 M GdnHCl, was deaerated and reduced by adding sodium dithionite to a final concentration of 1.8 μM. Unfolded ferrocytochrome *c* (U) thus obtained was liganded with CO. Unfolded carbonmonoxycyt *c* (UCO) was then diluted 101-fold into a degassed and dithionite-reduced CO-free refolding buffer containing a desired concentration of salt. This procedure allows complete refolding of UCO to generate native carbonmonoxycyt c (NCO). The fast UCO→NCO process precedes the slow NCO→N+CO dissociation. Kinetics of CO dissociation was monitored by 550-nm heme absorbance in a conventional UV-visible spectrophotometer.

**2.3 Measurement of CO dissociation kinetics.** The procedure for these measurements has been described earlier. [21, 22] Briefly, cyt *c* (1 mM) dissolved in 50 mM phosphate buffer, pH 7 (±0.1) is deaerated and reduced by adding sodium dithionite (2 mM). 25 μl of this ferrocyt *c* solution is mixed rapidly with 2 ml of 50 mM phosphate buffer containing 2 mM dithionite, and a given amount of NaCl, and 1 mM CO. Association kinetics of CO were followed at 550 nm (dead time

~5 s) at a peltier-controlled temperature of 25°C in a Cary 100 spectrophotometer or in a UV Specord 205 (Analytikjena) spectrophotometer.

## 3. Results and Discussion

**3.1 Thermal Dissociation of CO from NCO.** Unfolded molecules of carbonmonoxycyt c refold extremely fast. This refolding process is essentially the UCO→NCO reaction. Since the concentration of CO in the refolding milieu is substantially reduced, and because the affinity of native ferrocyt *c* for CO is lower relative to that for the intrinsic M80 ligand, the UCO→NCO process leads immediately to the NCO→N+CO conversion yielding to the formation of the $Fe^{+2}$–M80 bond. It is the dynamical events associated with this reaction that form the major thrust of this work.

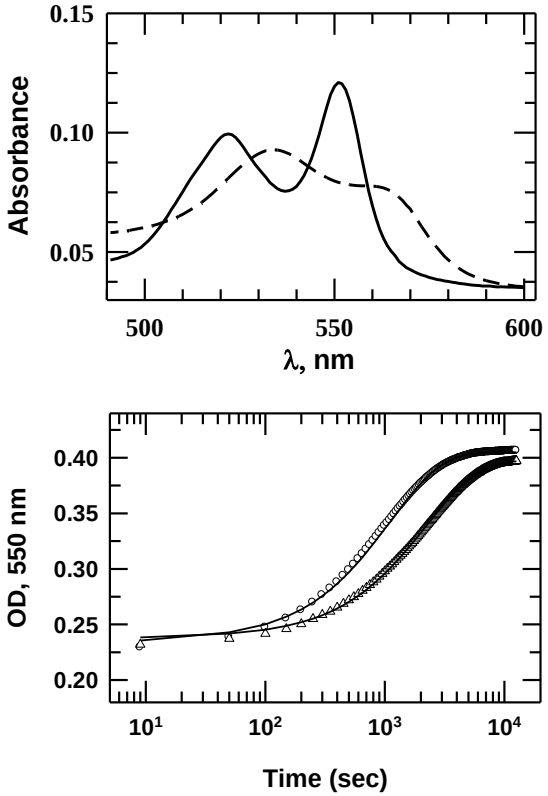


Fig. 2(b) typifies the kinetics of the NCO→N+CO dissociation recorded after diluting the UCO solution 101-fold into a CO-free folding buffer to obtain 0.4 M GdnHCl finally. The increase in absorbance at the heme π→π* α-band (550 nm) in a single exponential is due to dissociation of CO. Because of significantly low concentration of CO in the refolding medium the reverse reaction of CO binding back to N is negligible. Thus the rate constant obtained can be equated to CO dissociation rate coefficient, $k_{diss}$. The slowness of the reaction allows accurate determination of $k_{diss}$ ($\tau_{diss}$ ~ 19.3 min (with no addition); and τ ~ 37 min (2.5 M NaCl), Fig. 2b) by conventional spectrophotometer.

Fig. 1. (a) Steady-state visible absorption spectra of NCO (dashed line) and N (solid line) states. The NCO→N reaction was probed at 550 nm, the $\lambda_{max}$ of the N-state spectrum. The spectra were recorded in 50 mM phosphate buffer, pH 7, containing ~0.35 M GdnHCl at 25°C.(b) Figure 2. Kinetics of CO dissociation from NCO at 25°C, pH~ 7 as monitored by change in absorbance at 550 nm (with (○), no addition; and (Δ) 2.5M NaCl. The solid lines show least-squares fits of the data to a single exponential function (τ ~ 19.3 min (with no addition); and τ ~ 37 min (2.5 M NaCl)).

**3.2 Association of CO with ferrocytochrome *c*.** Ferrocyt c can be driven to bind CO even under native-like conditions when the latter is used in saturating concentration (≈1 mM), and the

same methods used to monitor the dissociation process can be used to extract the association rate coefficients, $k_{ass}$. As an example, Fig. 3 shows the N+CO→NCO reaction in the presence of 0.4 M NaCl. Fig. 2(b) typifies the kinetics of the CO association reaction. The decrease in absorbance at the heme π→π* α-band (550 nm) in a single exponential is due to association of CO. This is essentially the $Fe^{2+}$−M80 →$Fe^{2+}$−CO replacement, the time constant for CO association being ~23 min (0.5 M NaCl, Fig. 3). The $Fe^{2+}$−M80+CO →$Fe^{2+}$−CO+M80 replacement destabilizes the N state of ferrocyt c only marginally, ~2-3 kcal $mol^{-1}$ [23] which approximates the free energy associated with the intrinsic $Fe^{2+}$−M80 interaction. [23] The N and NCO conformations are not different from each other to any considerable extent, [23] and the motional dynamics of the two protein states are not expected to be different either.

The N+CO→NCO process, which is essentially $Fe^{+2}$−M80+CO →$Fe^{+2}$−CO+M80 displacement reactions, can be viewed as bimolecular reactions where the two reacting sites diffuse together to form the encounter complex in which the relevant sites collide with each other at some frequency before either dissociating away or reacting to form products. Under steady-state assumption, $k_{diss/ass}$=Kk, where K is the equilibrium constant for the formation and the breakdown of the encounter complex, and k is the rate coefficient for the barrier-activated reaction. Since the reacting sites forming the reaction volume are uncharged and have no special interaction otherwise, K is simply a constant equal to the volume of the encounter complex. The rate of the barrier-activated reaction depends on the frequency of collisions during an encounter. For both reactions the M80-resident segment of the polypeptide, which is linked to the heme iron through the $Fe^{+2}$−M80 bond in N but is free in NCO, provides a reacting site. The segment of the polypeptide between residues 70 and 85 (Fig. 3) forms a small Ω-loop (24).

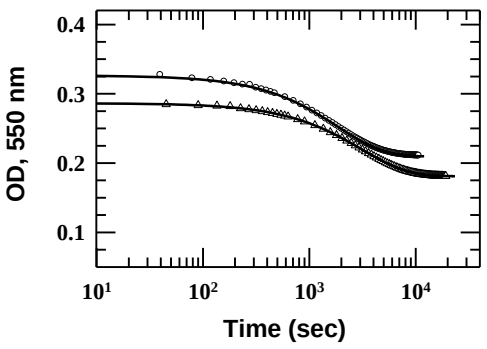


Fig. 2. Kinetics of CO association to ferrocyt *c* at 25°C, pH ~7 as monitored by change in absorbance at 550 nm (with (○), 0.5 M NaCl; and (Δ) 2.5M NaCl. The solid lines show least-squares fits of the data to a single exponential function (τ ~ 23 min (with 0.5 M NaCl); and τ ~ 47 min (3 M NaCl)).

Because the local mobility of the heme ring is suppressed by the intrinsic size and the rigidity of the ring system,[25] and given that the neighboring residues of M80 have significantly

higher thermal factors (25), the collective motion of the Ω-loop is expected to be the leading determinant of the CO association process. Thus the salt concentration modulation of $k_{ass}$ (Fig. 2) reveals the way the collective motion of the loop or of a part of it responds to salt content in the reaction medium. Since atomic fluctuations or high-frequency local motions involve only small spatial displacements, the thermal motion viewed here must be of collective character in which groups of atoms in a part or in the entire Ω-loop move in a correlated manner or as a unit. The observation that it responds to increments of salt concentration implies that it is a local motion, and hence is a low-frequency (τ, millisecond or longer) large-amplitude mode (several Å,) [26]

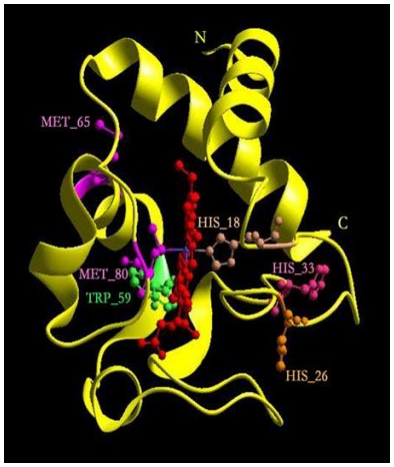


**Figure 3.** A ribbon representation of horse Cyt c; accession code PDB 1HRC. The side-chain of M80 (magenta) and some other key residues are shown explicitly in ball and stick display. Heme atoms are shown in red. The segment of the polypeptide (residues 70-85) containing M80 forms an Ω-loop. The figure has been drawn using Cerius2 Version 4.0 (MSI).

**3.3 NaCl dependence of global stability of carbonmonoxycyt *c*.** To assess the contribution of electrostatic or hydrophobic interactions to the stability of carbonmonoxycyt *c* and to determine the salt dependency of the denaturation free energy, $\Delta G^0$ for unfolding of carbonmonoxycyt *c*, we record a series of fluorescence-monitored (ex: 280 nm) urea-induced denaturation curves for carbonmonoxycyt *c* in the presence of various concentrations of NaCl. Fig. 4 shows a fluorescence-monitored urea-induced unfolding transition curves for carbonmonoxycyt *c* in the presence of 0.0, and 3 M NaCl at 25°C, pH ~7. These transition curves are analyzed by using the standard two-state equation (*26*). The iterated fit parameters are given in the legend to the figure. The unfolding free energy of cytochrome *c*, defined as the difference of free energies of unfolded and native states ($\Delta G°$) increases by 2(±0.2) kcal mol-1 when the protein is held at 3 M NaCl,. Clearly, neutral salt increases the global stability of the protein.

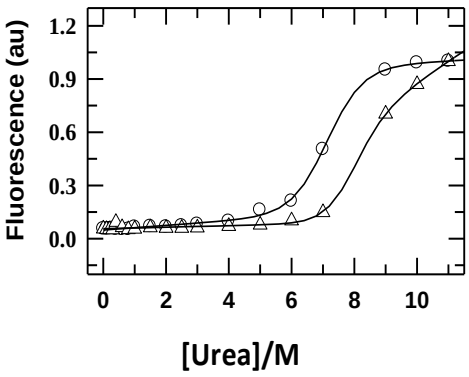


**Fig.4.** Urea–induced equilibrium unfolding curves of carbonmonoxy cyt *c* in the presence of 0 (O) and 3 M NaCl (Δ) at 25°C, pH~ 7.

Iterated fit values for a two-state equilibrium are $\Delta G^0 \approx 7.5$ (±0.3) and 9.5(±0.4) kcal mol$^{-1}$ and $C_m \approx 7.05$, and 7.9 M urea corresponding to 0 and 3 M NaCl, respectively.

**3.4 Salt-induced stabilization and molecular compactness of native carbonmonoxycyt *c*.** Fluorescence emission intensity because of the lone tryptophan (W59) provides a reliable marker for the molecular compactness of carbonmonoxycyt *c*. To determine the extent to which NaCl influences the molecular compactness, we measured fluorescence emission spectra (ex: 280 nm) of carbonmonoxycyt *c* in the presence of 0, 0.015, 0.3, 1.5, 2.5, and 3.0 M NaCl, respectively. As [salt] is increased, the fluorescence intensity decrease monoexponentially and plateau at ~0. 5 M NaCl (Fig. 5(B)), consistent with stabilization of carbonmonoxycyt *c*. by ionic screening of electrostatic interactions.

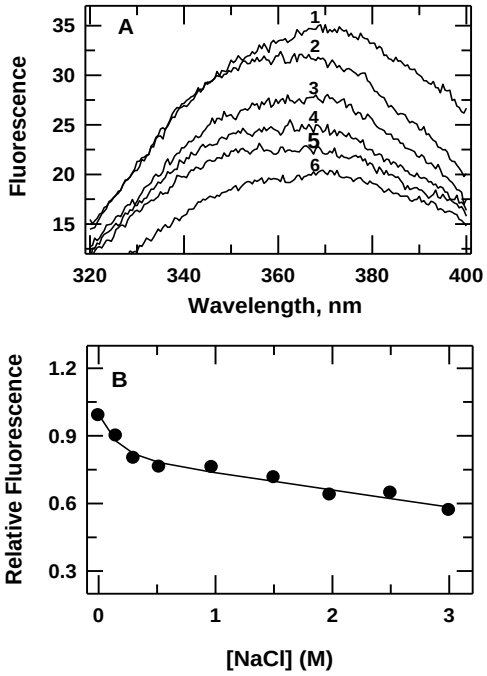


Fig. 5. (A) Fluorescence emission spectra of cyt-CO in the presence of different concentration of NaCl. Curve labels 1 to 6 correspond to 0, 0.015, 0.3, 1.5, 2.5, and 3.0 M NaCl, respectively. (B) Relative quenching of W59 fluorescence as a function of salt (●).

**3.5 NaCl dependence of $k_{diss}$ and $k_{ass}$, and motional dynamics affected by the salt.** Fig. 6 presents the rate coefficients for dissociation /association kinetics of ferrocyt *c*-CO reaction in the 0–3 M range of NaCl. The rate-NaCl data provide an opportunity to analyze the thermodynamic properties of the protein. Data in Fig.6 provide two major results. 1) The congruence of $k_{diss}$ and $k_{ass}$: within experimental error the $k_{diss}$ and $k_{ass}$ values are superimposable under all conditions of protein stability, indicating that it is the same mode of motion that operates in both CO association and dissociation processes. The congruence also implies that the activation barriers for association and dissociation in a given concentration of NaCl have the same height.

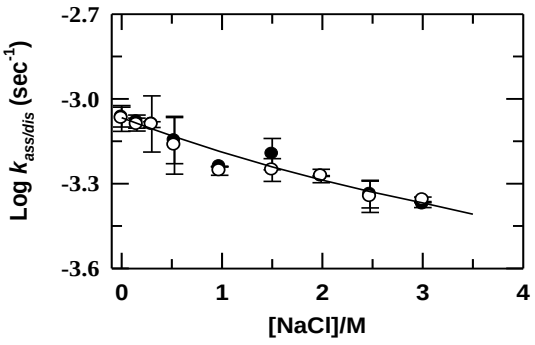


Fig. 6. The [NaCl] dependence of rate coefficients for CO association (•) and dissociation (O) of ferrocyt*c*-CO reaction at 25°C, pH~ 7. The error bars represent the standard deviations of the $k_{diss}$ and $k_{ass}$ values

2) The variation in magnitudes of $k_{diss}$ and $k_{ass}$ with salt: The value of $k_{ass/diss}$ decreases 2 -fold when the concentration of NaCl in the medium is raised to 3 M. The exact functional dependence of $k_{ass/diss}$ on the concentration of the salt additives has not been evaluated, and the solid lines in the figure show apparent trends only. These observations strengthen the implication that the observed decrease in the rate of the CO association and dissociation reaction in the presence of NaCl is mainly due to NCO stabilization by the salt.

Spatial displacement of thermal fluctuations, and hence collisions between different groups of atoms each exhibiting collective motions is dramatically reduced for the native protein in the presence of salt. The inference is drawn from the results of a set of strategic experiments where we observe the rate constant for association of CO with native ferrocyt *c* ($k_{ass}$). This reaction is not diffusion or encounter controlled. It rather involves substantial energy barrier or steric requirements, and hence many collisions between the CO and protein groups, particularly the heme ring, are required before the reaction ensues. The value of $k_{ass}$ under a given solution condition then depends on the frequency of collisions involving the CO and the heme side chains that exhibit highly collective motions.[27] The rate constant for the CO association reaction, $k_{ass}$, decreases dramatically as the NaCl concentration is increased from 0 to 3 M. (Fig. 5). The conclusion that the collective motions involving the side chains of the native protein are relatively restrained is consistent with the indications of organized tertiary structure discussed above.

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