GUANIDINE HYDROCHLORIDE – INDUCED FOLDING AND STABILIZATION OF ALKALI DENATURED HORSE FERROCYTOCHROME *C*

A

thesis Submitted

in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

CHEMISTRY

Submitted By:

Ms. Rumit

(Regd. No. 300802017)

Under the supervision of:

Dr. Rajesh Kumar

Assistant Professor

SCHOOL OF CHEMISTRY AND BIOCHEMISTRY

THAPAR UNIVERSITY

PATIALA- 147004

JULY 2010

CERTIFICATE

*This is certify that the thesis entitled "**Guanidine Hydrochloride – Induced folding and Stabilization of Alkali - Denatured Horse Ferrocytochrome c**" being submitted in 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 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.*

RUMIT 15th July'10

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

Dr. RAJESH KUMAR
Assistant Professor, SCBC,
Thapar University,
Patiala.

Countersigned by:

Dr. SUSHEEL MITTAL 15/7/x
Professor and Head, SCBC,
Thapar University,
Patiala.

Dr. R. K. SHARMA 16.7.10
Dean, Academic affairs,
Thapar University,
Patiala.

CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled, ***"Guanidine Hydrochloride – Induced folding and Stabilization of Alkali – Denatured Horse Ferrocytochrome c"*** *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.

Date: July, 2010
Place: Patiala

RUMIT 15th July 10

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

Dr. RAJESH KUMAR
Assistant Professor,
School of Chemistry and
Biochemistry,
Thapar University,
Patiala.

Submitted 15/7/x

Dr. SUSHEEL MITTAL
Professor & HOD,
School of chemistry and
Biochemistry,
Thapar University,
Patiala.

ACKNOWLEDGEMENT

There is always a sense of gratitude which one expresses to others for helpful services they render during all phase of life. I too would like to thanks all those who helped me directly and indirectly in completion of this work.

It is matter of immense pleasure to acknowledge my debt to my revered teacher and supervisor Dr. Rajesh Kumar, Assistant Professor, School of chemistry and biochemistry, Thapar University, Patiala. It is because of his priceless intellectual guidance, innovative and constructive ideas for having given me complete independence, affectionate encouragement to put my desire and thought, which paved the way for the successful completion of this work. It is indeed my privilege to work under him.

I am also thankful to all the teaching and non-teaching staff members of the department for their invaluable cooperation and help during the entire tenure of my studies in the department.

I take this opportunity to thank the research fellow Mr. Hemant Sharma and my friends Ms. Gurinder Pal Kaur and Ms. Karamjeet Kaur for their help and moral support.

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.

Date: July 2010

Place: PATIALA

Regards, 15th July 10

RUMIT

INDEX

CONTENTS **PAGE NO.**

1.0 INTRODUCTION 1
2.0 MATERIALS AND METHODS
2.1 Equilibrium unfolding 2
2.2 pH titration of ferrocyt *c* 2
2.3 Kinetic measurement of CO 2
3.0 RESULTS 3
3.1 CO binding unfolds the protein 3
3.2 GdnHCl -induced stabilization and unfolding 3
3.3 GdnHCl dependent kinetics of association 4
3.4 Urea dependent kinetics of association 7
4.0 DISCUSSIONS 8
4.1 GdnHCl-induced stabilization of alkali denatured ferrocyt *c* 8
4.2 GdnHCl at low concentrations restricted overall motion of B state 9
REFERENCES 10

ABSTRACT

Guanidine hydrochloride (GdnHCl) and urea are the most commonly used denaturants for proteins. This work examined the effect of these two denaturants on the base-denatured CO-liganded ferrocytochrome *c* (U_B state) at pH ~12.5. As [GdnHCl] is increased in the U_B state, the intensity of fluorescence emission spectra initially decrease, and then increase, displaying a broad minimum around 0.2 M GdnHCl, which indicates molecular compaction of U_B state by addition of guanidinium cations. Strategic experiments involving the measurement of the CO association rate to the ferrocytochrome *c* at pH ~12.5 in the presence of a variable concentration of GdnHCl and urea indicate that guanidinium cations at low concentrations (< 0.2 M) substantially restricted overall motion and stiffen the alkali-denatured protein in the molten globule state (MG), presumably by a charge screening mechanism.

1.0 INTRODUCTION

A compact and largely mobile molecular state containing native-like secondary structure and hydrodynamic radius, but without rigid tertiary structure is recognized as Molten Globule state (MG).[1-3] Structural and kinetic studies have revealed that MG state is a major intermediate in protein folding.[3-9] The MG state corresponds to late folding intermediates, [2, 10-11] which under normal folding conditions at neutral pH are difficult to study because of their transient lifetime.

Evidently, this state has been documented for a large set of proteins.[1-3, 13-14] Test-tube studies of ion-stabilized molten globules require one of the following two transitions, $U_A \rightarrow A$, and $U_B \rightarrow B$, where U_A and U_B are acid and base-denatured proteins, respectively, and A and B are the corresponding MG states. In most cases, the A state has been studied in the presence of anions; the complementary cation-stabilized B state has received meager attention. In fact, the B state has been described for only a very few proteins, including β-lactamase, [15] and barstar, [16] and ferricytochrome *c* (ferricyt *c*).[17]

Realizing the importance of the MG state for protein function in the living cell, [1, 12] we have recently performed a series of experiments with alkali-denatured ferricyt *c*. In the first set of studies, we used the ferricyt *c* at pH 13, and achieved the $U_B \rightarrow B$ transition in the presence of both sodium and guanidinium cations. [17] In this thesis, we present the second set of experiments with the carbonmonoxy derivative of the reduced form of cytochrome *c* (ferrocyt *c*). Ferrocyt *c* hardly denatures even at the extreme of basic pH, and therefore the carbonmonoxy derivative is used. Very interestingly, the guanidinium cations at low concentrations (< 0.2 M) substantially restricted overall motion and stabilize the alkali-denatured protein, presumably by a charge screening mechanism.

2.0 MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI) was from Sigma-Aldrich. Urea and GdnHCl were from Gibco BRL. Sodium Dithionite was purchased from Merck. Other chemicals were from Sigma or Aldrich. Experiments were done in strictly anaerobic atmosphere at 25°C, pH 12.4–13.0 using NaOH with or without 1-2 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid). Solutions contained 0.5-3 mM freshly prepared sodium dithionite, and experiments were completed within 2 hours of exposing the protein to high pH.

2.1 Equilibrium unfolding. Cyt *c* solutions, with or without 1 mM CO, were deaerated and reduced under nitrogen with 1 mM sodium dithionite, and incubated in tightly capped quartz cuvettes or rubber-capped glass tubes for ~30 minutes. Fluorescence emission spectra (ex: 280 nm) were taken in a Perkin Elmer LS-55 fluorescence spectrophotometer. Optical absorption spectra were recorded in a UV Specord 205 (Analytikjena) spectrophotometer or Cary 100 (Varian) spectrophotometer. Data were analyzed using the standard two-state equation for equilibrium unfolding.[18]

2.2 pH titration of ferrocytochrome *c* in the presence 1 mM CO . For pH unfolding studies, a ~10 μM solution of cyt *c*, prepared in an aqueous medium containing 10 mM each of Tris, disodium hydrogen phosphate, and CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), was titrated to different pH values in the 7-13.25 range by the use of NaOH. The titration did not upset the uniformity of the protein concentration in the samples. Samples were deaerated by using nitrogen gas, and reduced by adding a small volume of freshly prepared dithionite to obtain a final concentration of 2-3 mM. The samples were then incubated for ~30 minutes under 1 atm CO gas pressure in tightly capped cuvettes or glass tubes. Fluorescence emission spectra (ex: 280 nm) were taken in a Perkin Elmer LS-55 fluorescence spectrophotometer. Optical absorption spectra were recorded in a Cary 100 instrument. The pH titration curves were analyzed using the following transformed Henderson-Hasselbalch equation,

$$y = \frac{c_u + c_f\left[10^{n(pH - Cm)}\right]}{1 + 10^{n(pH - Cm)}} \qquad (1)$$

where, c_u and c_f are normalized fluorescence signals for the unfolded and the refolded state, respectively, n is the number of OH^- titrated, and c_m is the pH-midpoint for the transition.

2.3 Kinetic measurements of CO. The procedure for these measurements has been described earlier.[19-20] Briefly, cyt *c* (1 mM) dissolved in aqueous NaOH, pH 12.5(±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 NaOH solution containing 2 mM dithionite and 2 mM CAPS, a given amount of GdnHCl or urea, 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.

3.0 RESULTS

3.1 CO binding to the ferrocyt *c* at pH ~12.5 unfolds the protein. Even though ferrocyt *c* in aqueous alkaline solution possesses structural integrity very similar to that at neutral pH, it readily denatures when CO is allowed to bind at pH>12. Notably, CO binding to alkaline ferrocyt *c* was described by Theorell and Åkesson some sixty years ago.[21] Fig. 1(a) shows fluorescence-monitored pH titration curves of ferrocyt *c* in the presence and absence of 1 mM CO. In the absence of CO, the protein remains folded even at pH>13. The pH-midpoint for unfolding in the presence of CO is ≈12.2. Fig. 1(b) shows the same titration monitored by optical density at 550 nm.

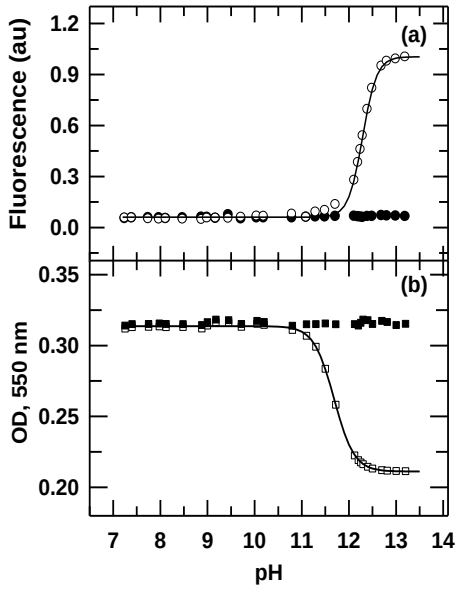


Fig. 1. pH equilibrium profiles: (a) Alkaline pH-induced unfolding of ferrocytochrome *c*. When CO is allowed to bind to the protein, it is fully unfolded at pH>12.5 (O), but in the absence of CO there is no unfolding (●). (b) The same transition is monitored by heme absorption (□).

The results are same, except that the pH-midpoint of the CO-induced unfolding transition appears at ~11.75. The shift may appear to indicate the accumulation of an equilibrium intermediate of ferrocyt *c* stabilized by CO, [22] but it is not clear how the binding of an extrinsic gaseous ligand can do so.

3.2 GdnHCl-induced stabilization and subsequent unfolding of the U_B state: detection of equilibrium intermediate. Fluorescence emission intensity because of the lone tryptophan (W59) provides a reliable marker for the molecular compactness of ferricyt *c*. Native ferricyt *c* is fluorescence-silent (Fig. 2(a)) because of excitation energy transfer from W59 to the heme. Unfolding results in an increase in the heme-tryptophan distance because of molecular expansion and hence, a dramatic increase in the fluorescence (Fig. 2(a)).

To estimate the stability of the MG state we titrated carbonmonoxycyt *c* at pH 12.5 with GdnHCl and monitored the structural changes by fluorescence (Fig. 2). An interesting behavior is observed: as the concentration of GdnHCl is raised, the fluorescence initially decreases in a sharp cooperative manner (Fig. 2(b)), and then increases relatively

monotonously displaying a very weak unfolding transition (Fig. 2(c)). GdnHCl induced folding transitions for the both acid-denatured and base-denatured forms of ferricyt *c* have been reported earlier.[17, 23] In the present case, the initial folding transition (Fig. 2(b)) is attributed to the $U_B \rightarrow B$ reaction brought about by the $GdnH^+$ cations. Assuming that GdnHCl exerts its effect by direct protein-denaturant interaction,[24-26] the number of $GdnH^+$ ions associated with the $U_B \rightarrow MG$ transition can be estimated for a model where the MG state interacts preferentially with the $GdnH^+$ ions and the binding constants for interaction of these cations with different binding sites offered by the MG state are comparable (see 14). The fit of the initial folding transition data to the expression

$$K_{app} = K(1 + K_{bind}a_x)^{\Delta n} \qquad (2)$$

where Δn is the difference of the number of $GdnH^+$ ions bound to U_B and MG states, a_x is the activity of GdnHCl, K_{bind} is the binding constant, and K is the true equilibrium constant for the $U_B \rightleftharpoons B$ transition in the absence of GdnHCl, yields $\Delta n \cong 1.7$, $K_b \approx 118$, and $K \approx 6.8$. At 25°C, the value of K corresponds to the free energy of 1.1 kcal mol^{-1}.

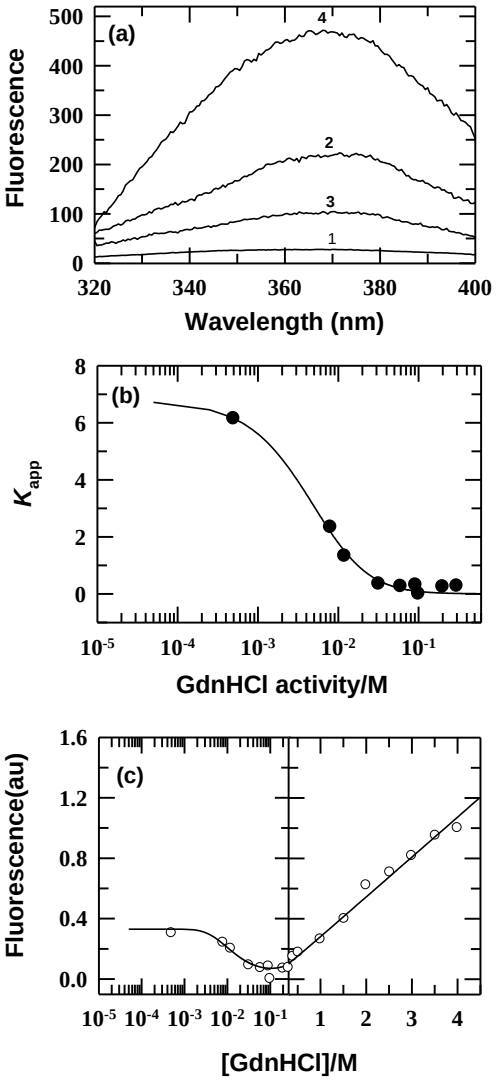


Fig. 2. Stabilization/folding and unfolding of the B state in the presence of GdnHCl, pH 12.5, 25°C. (a) Fluorescence spectra for different states of carbonmonoxycyochrome *c*: 1, pH 7, native; 2, pH 12.5, no salt; 3, pH 12.5, 0.15 M GdnHCl; 4, pH 12.5, 4.0 M GdnHCl. (b) In the presence of low concentrations of GdnHCl, guanidinium ions drive the $U_B \rightarrow B$ transition. The observable equilibrium constant, K_{app}, for the $U_B \rightleftharpoons B$ is plotted against the activity of GdnHCl. The solid line is the fit to data according to eq 2 (see text). (b) The GdnHCl-induced folding-unfolding transition. The continuous line represents the fit to the experimental data by the use of eq 3. The fit yields m= 0.26(±0.01) kcal mol^{-1} M^{-1} and $\Delta G°$=0.4(±0.02) kcal mol^{-1}.

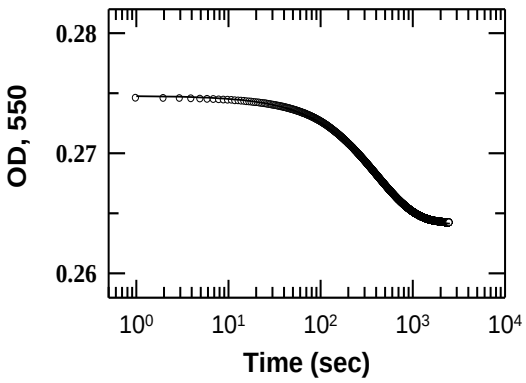
Above 0.4 M GdnHCl, the stabilizing effect of the $GdnH^+$ ions is overrun by its own unfolding action. The unfolding transition of the B state is apparently shallow (Fig. 2(c)), indicating small amount of solvent exposure of amino

acid residues accompanying the unfolding. This suggests absence of a significant hydrophobic core- a generic property of the molten globule state. Perhaps patches of exposed and buried hydrophobic surfaces characterize the alkali MG state. The GdnHCl titration data were fit to, [27]

$$\Delta G = \Delta G° - RT\ln\left(\frac{1+K^{u}_{bind}[GdnHCl]}{1+K^{B}_{bind}[GdnHCl]}\right)^{n} - m[GdnHCl] \qquad 3$$

where m is the equilibrium surface exposure of residues during unfolding, n is the number of $GdnH^{+}$ binding sites, K^{u}_{bind} and K^{B}_{bind} are binding constants of $GdnH^{+}$ to the unfolded and the B state, respectively, and $\Delta G°$ is the free energy of unfolding in the absence of the denaturant. The fit yields m= 0.26(±0.01) kcal mol^{-1} M^{-1} and $\Delta G°$=0.4(±0.02) kcal mol^{-1}. When compared with values reported for the unfolding of the A state (1.61 kcal mol^{-1} M^{-1} and 2.04 kcal mol^{-1}, respectively; ref *23*) and the B state (0.35 kcal mol^{-1} M^{-1} and 1.02 kcal mol^{-1}, respectively; ref 17) of ferricyt *c*, the present results report a relatively less stable B state with largely exposed surface area. The GdnHCl-induced UB→B transition should be verifiable by CD as well. However, alkali solutions containing even small amount of GdnHCl show very large noise signal due to an elevation of the HT voltage; especially, peptide signals are increasingly obscured with GdnHCl content. This difficulty has also been noted in an earlier report of alkaline ferricyt *c*.[28] Nevertheless, the increase of 282-nm CD absorption with increasing concentration of GdnHCl up to 0.3 M is an indication of the U_B→B transition (data not shown).

3.3 GdnHCl dependent kinetics of association of CO to ferrocyt *c* indicates that the MG state is stiff and dynamically constrained. In terms of internal mobility, the MG state is generally defined by increased fluctuations of the aliphatic side chains, although the mobility of aromatic side chains is fairly restricted. The results presented above do not provide evidence for an overwhelming internal mobility of the protein system being studied. To determine qualitatively how dynamic the interior of the MG is, we conducted experiments involving kinetics of association of CO with ferrocyt *c* at pH 12.5 in the presence of variable concentration of GdnHCl and urea. The rationale of the experiment is the following. Destabilized ferrocyt *c* binds CO when the latter is used in saturating concentration (≈1 mM).[29] Since intramolecular thermal collisions provide the energy for barrier crossing in the association reaction, ferrocyt *c* + CO → ferrocyt *c* -CO, the rate coefficient for the reaction (k_{ass}) is expected to decrease if the amplitudes of thermal fluctuations are reduced as a result



of constraints on the collective modes of intramolecular motion.[30-31] The association kinetics are slow, and can be conveniently measured by monitoring the absorbance at 550 nm following the addition of a small volume of the protein solution to a CO-saturated aqueous alkali solution containing the GdnHCl or urea. The representative trace for single-phase CO association kinetics is shown in Fig. 3.

Fig. 3. Kinetics of CO association to ferrocytochrome *c* at 25°C, pH~ 12.4 as monitored by change in absorbance at 550 nm. The solid line show least-squares fits of the data to a single exponential function (τ_{obs} ~ 7 min., 0.06 M GdnHCl).

Fig. 4(b) shows the rate coefficients for association of CO to ferrocyt *c* at pH ~ 12.5 under conditions of varying protein stability in the 0-3.5 M range of GdnHCl concentration. Given the midpoint of GdnHCl-induced unfolding transition of ferrocyt *c*, C_m≈2 M at 25°C (Fig. 4(a)), these data provide an opportunity to analyze the dynamical behavior associated with the transitions from $U_B \rightarrow$ B state and B→U state.

The variation in magnitudes of k_{ass} with denaturant is of considerable interest. As [GdnHCl] is increased in the U_B state, k_{ass} initially decrease and then increase, displaying a broad minimum around 0.3 M GdnHCl. In going from 0.0 to 0.3 M GdnHCl, the value of k_{ass} decreases 3.6(±0.3)-fold, and increases thereafter. A sharp increase from 0.3 to ~3.5 M GdnHCl is followed by apparently stronger dependence on the denaturant concentration. The decrease suggests that at low GdnHCl concentrations (< 0.3 M), Gdn^+ tends to block association of CO to ferrocyt *c* (i.e., U_B→B transition). The increase in rate coefficients above 0.3 M GdnHCl can be interpreted to arise from MG state destabilization and structural unfolding (i.e., B→U transition) that would facilitate CO association process.

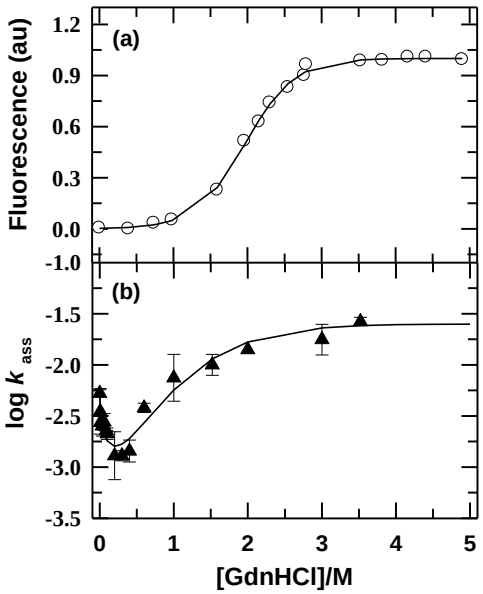


Figure 4. Equilibrium stability and rate-denaturant spaces. (a) GdnHCl-induced equilibrium unfolding of ferrocyt *c* (O) at 25(±1)°C, pH~ 12.5. The continuous curve is fit to the data using

procedures described (32). Values of $\Delta G(H_2O)$ and m_g, respectively, obtained are 3.53(±0.4) kcal mol^{-1} and 1.8 kcal mol^{-1} M^{-1}. (b) GdnHCl dependence of k_{ass} (▲, manually measured $OD_{550\ nm}$). The line through the data, based on empirical equation, has been drawn to guide eye only.

Clearly, the internal dynamics of the MG state is substantially constrained relative to that of the U_B and U states. We attribute this to reduced motional freedom in the MG state relative to that in the U_B and U states. In terms of internal dynamics, the MG state closely matches the native state.

3.4 Urea dependent kinetics of association of CO to alkaline ferrocyt *c*: If the decrease in the value of k_{ass} with increasing concentration of GdnHCl, as exhibited by left limbs of the logk_{ass}-GdnHCl chevron (Fig. 4(b)), is indeed due to the charge screening effect of counterions which generally reduces the fluctuations of groups of atoms, we conducted experiments involving kinetics of the association of CO with ferrocyt *c* at pH 12.5 in the presence of a variable concentration of urea. Figure 5b shows the log k_{ass}-urea space at 25 °C, pH 12.5. Given the midpoint of urea-induced unfolding transition of ferrocyt *c*, C_m≈2.7 M at 25°C (Fig. 5(a)), these data provide an opportunity to analyze the dynamical behavior associated with the transitions from $U_B \rightarrow$ U state.

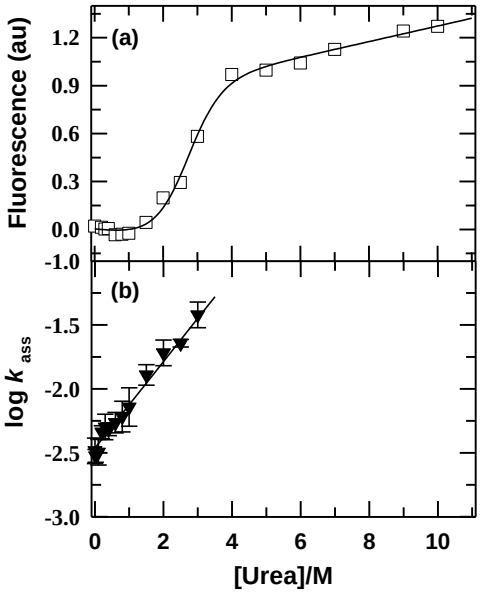


Figure 5. Equilibrium stability and rate-denaturant spaces. (a) Urea-induced equilibrium unfolding of ferrocyt *c* (□) at 25(±1)°C, pH~ 12.5. The continuous curve is fit to the data using procedures described (32). Values of $\Delta G(H_2O)$ and m_g, respectively, obtained are 3.28(±0.7) kcal mol^{-1} and 1.23 kcal mol^{-1} M^{-1}. (b) Urea dependence of k_{ass} (▼, manually measured $OD_{550\ nm}$). The line through the data, based on empirical equation, has been drawn to guide eye only.

The rate for CO association does not show any decrease in the rate versus-urea concentration plots (Fig. 5 (b)). The linear increase in rate coefficients from 0 to 4.0 M urea can be interpreted to arise from base-denatured protein destabilization and structural unfolding (i.e., $U_B \rightarrow U$ transition) that would facilitate CO association process. As noted earlier the charge screening effect of ionic GdnHCl add on to the stability of native proteins.[33-34] Indeed, both entropic and electrostatic effects contribute toward the stability of native ferrocyt *c*.[30] Purely entropic stabilization can be isolated by using nonionic urea as reported earlier.[30] The linear

increase in rate coefficients from 0 to 4.0 M urea (Fig. 5(b)) indicates that an entropic effect not contributes significantly to the alkaline MG state.

4.0 DISCUSSION

4.1 GdnHCl-induced stabilization of alkali denatured ferrocyt *c*. In our earlier studies, we achieved the $U_B \rightarrow B$ transition for ferricyt *c* in the presence of guanidinium cations.[17] Here, we notice the $U_B \rightarrow B$ transition for ferrocyt *c* in the presence of low concentrations of GdnHCl (Fig. 2(b)). The complementary $U_A \rightarrow A$ transition for ferricyt *c* is also driven by GdnHCl.[23] In both cases, the ions dissociated from GdnHCl influence the stability. The possible mechanism of interaction of $GdnH^+$ with the protein +ve charges in each case need not be the same, however. In acid medium (pH<2), where the net charges of ferricyt *c* is +24, Cl^- can shield the +ve protein charges by Debye-Huckel or ion-pair or both types of interactions. In the basic medium (pH>12.4), where the net protein charge is –17, $GdnH^+$ ions are more likely to form ion-pairs with the anionic sites of the protein. Formation of Debye-Huckel type spheres will perhaps be unfavorable due to large size of the $GdnH^+$ ions. Crystal structures of proteins with low concentrations of GdnHCl do show direct interaction of $GdnH^+$ ions with proteins.[30, 35-36] This argument is advanced only in the context of GdnHCl stabilization of base-denatured ferricyt *c* and ferrocyt *c*.

Stabilization of native or native-like states by subdenaturing concentrations of GdnHCl at neutral pH has also been shown for a few proteins.[30, 37-39] Although ionic interactions between the ions of GdnHCl and the protein are implicated in all cases, the structural, dynamical, and thermodynamic consequences of protein-denaturant interactions are more involved than the simple charge screening argument.[24-25, 30] The interactions of the denaturant molecules with different groups of the protein can establish non-specific networks of intramolecular interactions, as observed in crystal structures of proteins with low concentrations of denaturants.[36] These cross-linking interactions may serve to decrease the motional freedom in different parts of the protein, but increase the barriers to motions in the more compact conformers of the protein. Existing X-ray data for proteins do show a significant decrease in the B-factor of side chain and backbone atoms in the presence of low concentrations of GdnHCl.[36] In this perspective, $GdnH^+$-induced stabilization of the alkali denatured protein ($U_B \rightarrow B$ transition; Fig. 2(b)) would appear to originate largely from a reduction in fluctuations in the positions of individual or clusters of atoms around their

average. As noted earlier the charge screening effect of ionic GdnHCl add on to the stability of native proteins.[33-34] The relative contribution of these two effects- electrostatic shielding and conformational entropy- to the free energy of stabilization of the B state is remains to be assessed. Indeed, both entropic and electrostatic effects contribute toward the stability of native ferrocyt *c.*[30] Purely entropic stabilization can be isolated by using nonionic urea as reported earlier.[30] This work results demonstrates that an entropic effect do not contribute significantly to the stabilization of the B state.

4.2 GdnHCl at low concentrations restricted overall motion of the B state

Spatial displacement of thermal fluctuations, and hence collisions between different groups of atoms each exhibiting collective motions is dramatically reduced in the B state. The inference is drawn from the results of a set of strategic experiments where we observe the rate constant for association of CO with alkali-denatured 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.[40] The rate constant for the CO association reaction, k_{ass}, decreases dramatically as the base-denatured polypeptide undergoes transition to the B state in the presence of GdnHCl (< 0.3 M) (Fig. 4(b)). Because the local mobility of the heme ring is suppressed by the intrinsic size and the rigidity of the ring system, [40] and given that the neighboring residues of M80 have significantly higher thermal factors, [41] the collective motion of the Ω-loop is expected to be the leading determinant of the CO association process. Thus the denaturant modulation of k_{ass} (Fig. 4(b)) reveals the way the collective motion of the loop or of a part of it responds to solvent 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 denaturant concentration implies that it is a local motion, and hence is a low-frequency (τ, millisecond or longer) large-amplitude mode (several Å, ref 42). The conclusion is that the collective motions involving the side chains of the B state are relatively restrained.

Indeed, the polypeptide backbone in the A state is more mobile with respect to both native and unfolded states.[1] Small but recognizable increase in the amplitude of local backbone fluctuations characterize the transition from the acid denatured state to the molten globule state of ∝-lactalbumin.[43] Values of NMR measured backbone N−H order parameter, which provides an indication of the rigidity of the protein main chain, decreases significantly in the A state.[44] Molecular dynamics simulations also show a substantial increase of the fluctuations in the main-chain dihedral angles φ and ψ[45] A molecular interpretation of the chain stiffness in the B state is unclear at present.

REFERENCES

1. Ptitsyn, O. B. 1995. *Adv. Protein Chem.* **47**, 83- 229.
2. Kuwajima, K., and Arai, M. 2000. In *Mechanism of Protein Folding* (Pain, R. H., Ed.) **2**nd ed., pp 138-174, Oxford University Press, New York.
3. Arai, M., and Kuwajima, K. 2000. *Adv. Protein Chem.* **53**, 209-271.
4. Ptitsyn, O.B. 1987. *J. Protein Chem.* **6**, 273-293.
5. Ohgushi, M. and Wada, A. 1983. *FEBS Letters* **164**, 21-24.
6. Baldwin, R.L. 1991. *Chemtract* **2**, 379-389.
7. Kuwajima, K. 1989. *Proteins: Struct. Funct. Genet.* **6**, 87-103.
8. Kuwajima, K. 1989. *Curr. Opin. Biotechnol.* **3**, 462-467.
9. Christensen, H. and Pain, R. H. 1991. M*Eur. Biophys. J.* **19**, 221-229.
10. Colón, W., and Roder, H. 1996. *Nat. Struct. Biol.* **3**, 1019-1025.
11. Mok, K. H., Nagashima, T., Day, I. J., Hore, P. J., and Dobson, C. M. 2005 .*Proc. Natl. Acad. Sci. USA* **102**, 8899-8904.
12. Bychkova, V. E., Pain, R. H., and Ptitsyn, O. B. 1988. *FEBS Lett.* **238**, 231-234.
13. Goto, Y., Calciano, L. J., and Fink, A. L. 1990. *Proc. Natl. Acad. Sci. USA* **87**, 573-577.
14. Goto, Y., Takahashi, N., and Fink, A. L. 1990. *Biochemistry* **29**, 3480-3488.
15. Goto, Y., and Fink, A. L. 1989. *Biochemistry* **28**, 945-952.
16. Rami, B. R., and Udgaonkar, J. B. 2002. *Biochemistry* **41**, 1710-1716.
17. Kumar, R., Prabhu, N.P., Rao, D.K., and Bhuyan, A.K. 2006. *J. Mol. Biol.* **364**, 483-495.
18. Santoro, M. M., and Bolen, D. W. 1988. *Biochemistry* **27**, 8063-8068.
19. Kumar, R., Prabhu, N. P., Yadaiah, M., and Bhuyan, A. K. 2004. *Biophys. J.* **87**, 2656-2662.
20. Mayr, L. M., and F. X. Schmid. 1993. Biochemistry. **32**, 7994–7998.
21. Theorell, H., and Åkesson, A. 1941. *J. Am. Chem. Soc.* **63**, 1812-1827.
22. Pabit, S. A., Roder, H., and Hagen, S. J. 2004. *Biochemistry* **43**, 12532-12538.
23. Hagihara, Y., Aimoto, S., Fink, A. L., and Goto, Y. 1993. *J. Mol. Biol.* **231**, 180-184.
24. Schellman, J. A. 1978. Solvent denaturation, *Biopolymers* **17**, 1305-1322.
25. Schellman, J. A. 1987. *Annu. Rev. Biophys. Biophys. Chem.* **16**, 115-137.
26. Makhatadze, G. I., and P. L. Privalov 1992. *J. Mol. Biol.* **226**, 491-505.
27. Hagihara, Y. Tan, Y., and Goto, Y. 1994. *J. Mol. Biol.* **237**, 336-348.
28. Hoang, L., Bédard, S., Krishna, M. M. G., Lin, Y. and Englander, S. W. 2002. *Proc. Natl Acad. Sci. USA,* **99**, 12173–12178.
29. Theorell, H., and Åkesson, A. 1941. *J. Am. Chem. Soc.* **63**, 1812-1827.
30. Bhuyan, A. K. 2002. *Biochemistry* **41**, 13386-13394.
31. Kumar, R., Prabhu, N. P., Yadaiah, M., and Bhuyan, A. K. 2004. *Biophys. J.* **87**, 2656-2662.
32. Santoro, M. M., and Bolen, D. W. 1988. *Biochemistry* **27**, 8063-8068.
33. Mayr, L. M., and F. X. Schmid. 1993. Biochemistry **32**, 7994–7998.

34. Monera, O. D., C. M. Kay, and R. S. Hodges. 1994. Protein Sci. **3**, 1984–1991.
35. Hibbard, L. S., and A. Tulinsky. 1978. *Biochemistry* **17**, 5460-5468.
36. Dunbar, J. H. P. Yennawar, S. Banerjee, J. Luo, and G. K. Farber. 1997. *Protein Sci*. **6**, 1272-1733.
37. Pace, C. N., D. V. Laurents, and J. A. Thomson. 1990. *Biochemistry* **29**, 2564-2572.
38. Mayr, L. M., and F. X. Schmid. 1993. *Biochemistry* **32**, 7994-7998.
39. Kumar, R., Prabhu, N. P., Yadaiah, M., and Bhuyan, A. K. 2004. *Biophys. J.* **87**, 2656-2662.
40. Morgan, J. D., and McCammon, J. A. 1983. *Biopolymers* **22**, 1579-1593.
41. Berghuis, A. M., and Brayer, G. D. 1992. *J. Mol. Biol.* **223**, 959-976.
42. Petsko, G. A., and Ringe D. 1984. *Ann. Rev. Biophys. Bioeng*. **13**, 331-371
43. Chakraborty, S., Ittah, V., Bai, P., Luo, L., Haas, E., and Peng, Z. 2001, *Biochemistry* **40**, 7228-7238.
44. Redfield, C., Smith, R. A. G., and Dobson, C. M. 1994. *Nat. Struct. Biol.* **1**, 23-29.
45. Daggett, V., and Levitt, M. 1992. *Proc. Natl. Acad. Sci. USA* **89**, 5142-5146.