EFFECT OF SOLVENT VISCOSITY ON THE MOTIONAL DYNAMICS OF NATIVE CARBONMONOXYCYTOCHROME *C*

A

thesis submitted

in partial fulfillments of requirements

for the degree of

Master of Science (Chemistry)

Submitted By

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Journey becomes easier when you are with people who are supportive. I experienced same kind of atmosphere during completion of my thesis work. It is a pleasant aspect to have the privilege to evince a word of thanks to everyone who made this journey smooth.

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I whole heartedly thanks to my parents who are building blocks of my life.

Date: July 15, 2010

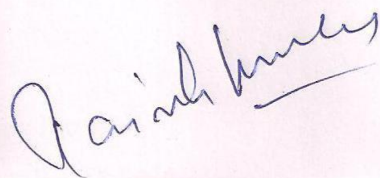
Place: Patiala

Gurinder Pal Kaur 15/7/2010

Gurinder Pal Kaur

CERTIFICATE

This is to certify that the thesis entitled "**Effect of solvent viscosity on the motional dynamics of native carbonmonoxycytochrome *c*"** being submitted by **Ms. Gurinder Pal Kaur** in partial fulfillment of requirement for the award of degree of **Masters of Science in Chemistry** submitted in the **School of Chemistry and Biochemistry (SCBC), Thapar University Patiala** is a bonafide work carried out under the supervision of **Dr. Rajesh Kumar** and that no part of this project has been submitted for the award of any other degree.



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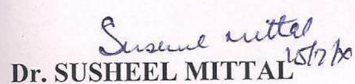

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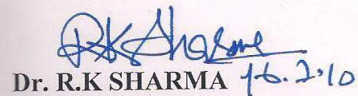

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled **"Effect of solvent viscosity on the motional dynamics of native carbonmonoxycytochrome *c*"** in partial fulfillment of the requirements for the award of the degree of **Masters of Science in Chemistry** submitted **in School of Chemistry and Biochemistry (SCBC), Thapar University,** Patiala, is an authentic record of my own work under the supervision of **Dr. Rajesh Kumar.** I have not submitted the matter embodied in this thesis for the award of any other degree.

Place: Patiala

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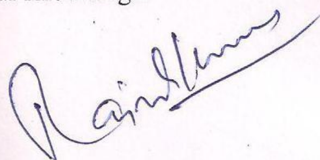
This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.



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Abstract

To examine the solvent viscosity effect on the motional dynamics of molten globule-like trapped intermediates, optical absorption spectroscopy has been used to measure the CO dissociation kinetics of a natively folded state of CO-liganded ferrocytochrome *c* (NCO state) in the 0.89-175 centipoises range of glycerol viscosity at pH 7.0, 25 ^{0}C. The single rate coefficient for thermal dissociation of CO from NCO decreases initially when the solvent viscosity is low (<6 cP), but saturates at higher viscosity, indicating that the rate-viscosity data for NCO→N+CO transition do not fit Kramer's model. Analysis based on the phenomenological expression due to Beece et.al[1] can account for the observed non-Kramer behaviour.

List of Figures

Figure 1(a) Steady state of visible absorption spectra of NCO

Figure 1(b) Kinetics of CO dissociation from NCO

Figure 2 GdnHCl-induced equilibrium unfolding curves of oxidised cyt *c* in the presence of different concentration of glycerol

Figure 3 Ribbon representation of cyt *c*

Figure 4 Glycerol dependence of rate coefficient for dissociation of CO

Figure 5 Non- Kramer scaling observed for CO dissociation

INDEX

CONTENTS **PAGE NO.**

1. Introduction 2
2. Materials and methods 5
   2.1 Preparation of native carbonmonoxycytochrome *c* and measurement of CO dissociation. 5
3. Results and discussions 7
   3.1 Thermal dissociation of CO. 7
   3.2 Challenges in viscosity studies of the CO dissociation dynamics of the NCO. 7
   3.3 The structural fluctuations of the M80-containing Ω-loop. 8
   3.4 Phenomenological description of viscosity dependence of the NCO→N+CO. 9

References 11

Section 1.0

INTRODUCTION

1.0 INTRODUCTION

Protein structural dynamics plays a significant role in the control of protein reactivity and thus its function. Protein undergoes a broad range of structural fluctuation including a large-amplitude collective global motion as well as a smaller and more local internal motion. Either type of motions depends in some way on environmental factors such as solvent composition, pH, ionic strength, and viscosity. A popular approach for investigating the importance of protein dynamics in chemical reactions is to study the effect of the solvent viscosity. Kramer's theory [2] models the kinetics of chemical reactions and relates the reaction rate to the viscosity of solvent. Kramer's Theory assumed that the reactions rates were inversely proportional to the solvent viscosity, η_s.[2] The validity of Kramer's' law has been confirmed experimentally for polymer dynamics,[3] and by computer simulations.[4] The viscosity-dependent exchange of energy between the protein and the solvent has been proposed to be a source of energy for the protein structural fluctuations determining its function.[5,6]

Indeed, many investigators have considered the phenomenal Kramer's model to examine the exact effect of solvent viscosity on several types of protein reactions, including enzyme kinetics, protein folding reactions, and the binding of ligands to heme proteins.[1, 7-23] For most of such protein reactions investigated, the observed rate constant has been found to be inversely proportional to the fractional power of the viscosity, $k \propto \eta_s^{-n}$ ($0<n<1$).[24-25] There could be two possible origins for this fractional power dependence- breakdown of Kramer's theory, and violation of Stokes law.[1,16,25-30] In this perspective, we wish to examine the influence of solvent viscosity on the CO dissociation kinetics of a natively folded state of CO-liganded ferrocytochrome *c* (NCO state). The NCO state resembles the generic molten globule-like states and can be driven to the native state by pulsed laser photolysis of the CO ligand.[31-32] Slow thermal dissociation of the CO molecule from NCO also yields the native state (N).[33-34] The N and NCO conformations are not different from each other to any considerable extent, [35] and the motional dynamics of the two protein states are not expected to be different either. The NCO→N+CO conversion occurs because of its instability due to greater affinity of M80 sulfur for Fe^{+2} relative to that of CO. In this work, we are employing the same system to investigate the viscosity effect on the experimentally measured rate coefficient for thermal dissociation of CO from NCO. The NCO→N+CO rate-viscosity data are inconsistent with Kramer's theory.[1] The rate coefficient varies linearly at low viscosities (< 6cP), but saturates at high viscosities, exhibiting deviation from $k \propto \eta_s^{-1}$ relation. Analysis

based on the phenomenological expression due to Beece et.al[1] can account for the observed non-Kramers behaviour.

Section 2.0

MATERIALS AND METHODS

2.0 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. The composition of glycerol is expressed in (w/w) %. The absolute viscosity (η) of glycerol solutions at 25^0 C was taken from literature.[36]

2.1 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 3.5 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 composition of glycerol. 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.

Section 3.0

RESULTS AND DISCUSSION

3.0 RESULTS AND DISCUSSION

3.1 Thermal Dissociation of CO from NCO.

Within the limit of the stopped-flow resolution (3 ms dead-time), unfolded 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 ferrocytochrome *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. Figure1b 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 $\pi\rightarrow\pi^*$ α-band (550 nm) in a single exponential is due to dissociation of CO.

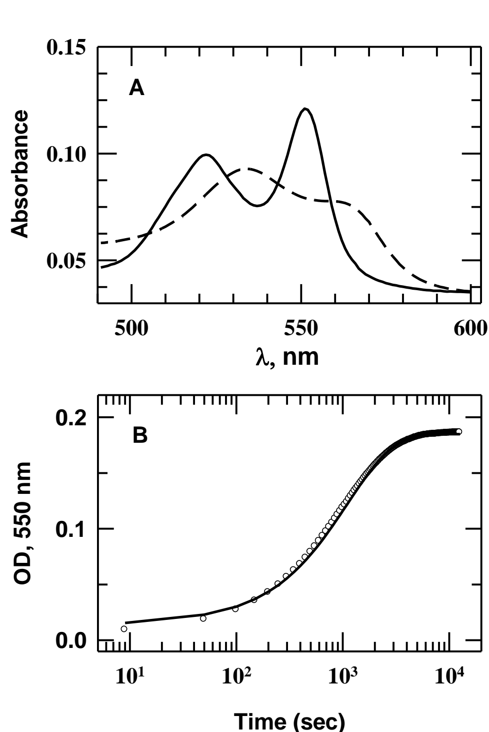


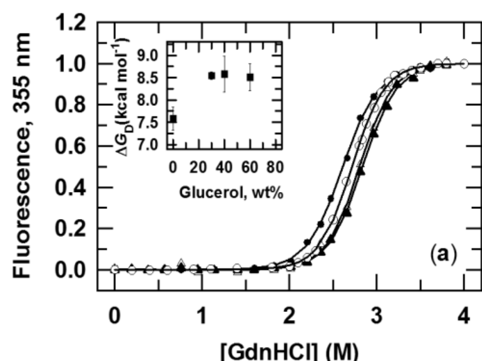
Figure1. (a) Steady-state visible absorption spectra of NCO (dashed line) and N (solid line) states. The NCO→N reaction was probed at 550 nm, the λ_{max} of the N-state spectrum. (b) Kinetics of CO dissociation from NCO at 25°C, pH~ 7 as monitored by change in absorbance at 550 nm (0.4 M GdnHCl). The solid lines show least-squares fits of the data to a single exponential function (τ ~ 20 min (0.4 M GdnHCl)).

3.2 Challenges in viscosity studies of the CO dissociation dynamics of the NCO.

A major experimental challenge in the study of the viscosity dependence using cosolvents such as glycerol is the proper accounting for the accompanying changes in protein stability and other solvation effects. In fact, the CO dissociation rate of NCO may decrease if the protein gains stability with increments of glycerol. The challenge is to solely alter the viscosity while otherwise maintaining the same energy of activation between the initial state and the transition state. A simple approach to the problem is not available at the present. To determine the extent to which glycerol influences the stability, we measured the GdnHCl-induced unfolding curves of ferricytochrome *c* in the presence of 0, 30, 40, and 60% (w/w) glycerol at 25 °C. The unfolding free energy of ferricytochrome *c* ($\Delta G°$) increases by only

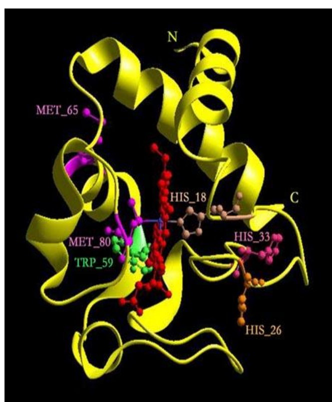
1(±0.5) kcal mol^{-1} when the protein is held at 40% glycerol, and remains unchanged in higher concentration of glycerol (Figure 2).

Figure 2. GdnHCl–induced equilibrium unfolding curves of oxidized cyt *c* in the presence of 0 (•), 30 (○), 40 (Δ), and 60% (Δ) of glycerol solution at 25°C, pH~ 7. Iterated fit values for a two-state equilibrium are ΔG^0≈ 7.5 (±0.3), 8.54 (±0.2), 8.6 (±0.4), and 8.5(±0.4) kcal mol^{-1} and C_m ≈ 2.6, 2.73, 2.81, and 2.84 M GdnHCl corresponding to 0, 30, 40, and 60% glycerol composition, respectively. Inset shows the plot of ΔG^0 as a function of glycerol composition (w/w) %.



These data were taken using ferricytochrome *c* which is ~11 kcal mol^{-1} less stable than the ferrocytochrome *c* used in this study.[37] However, since the two oxidation states are nearly identical both structurally and conformationally,[38-41] the degree of stability offered by glycerol is expected to be the same. Further, as discussed earlier the NCO-state is structurally native-like, although the value of its Gibbs free energy is larger than the latter by ~2 kcal mol^{-1},[42] suggesting that the glycerol-induced changes in the free energies of the NCO and N states would be parallel. Thus glycerol would affect the reactant and the product to the same extent. We consider these suppositions based on our work in this area. The instability of the NCO-state toward denaturing concentrations of GdnHCl hardly renders possible the direct determination of the free energy of the NCO-state. For the present nonetheless, the use of glycerol as a viscogen does not appear to influence the CO dissociation rate of NCO considerably.

3.3 The structural fluctuation of the M80-containing Ω-loop. The NCO→N+CO process, which is essentially Fe^{+2}–CO+M80→Fe^{+2}–M80+CO 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, $k_{\text{diss/ass}}$=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.[43]

Figure 3. A ribbon representation of horse Cyt *c*; accession code PDB 1HRC.[39] 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).

Because the local mobility of the heme ring is suppressed by the intrinsic size and the rigidity of the ring system,[44] and given that the neighboring residues of M80 have significantly higher thermal factors,[45] the collective motion of the Ω-loop is expected to be the leading determinant of the CO dissociation process. Thus the solvent composition modulation of k_{diss} (Figure 4) reveals the way the collective motion of the loop or of a part of it responds to solvent content in the reaction medium.

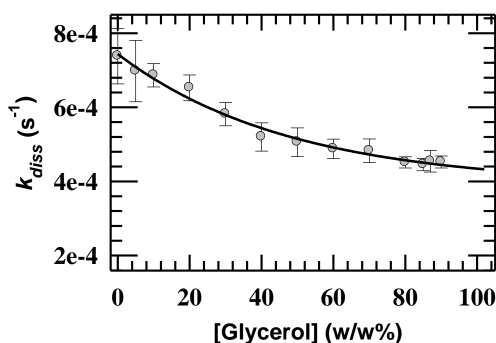


Figure4. The [glycerol] dependence of rate coefficients, k_{diss} for CO dissociation of NCO at 25°C, pH~ 7. The error bars represent the standard deviations of the k_{diss} values.

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 solvent composition implies that it is a local motion, and hence is a low-frequency (τ, millisecond or longer) large-amplitude mode (several Å,).[46]

3.4 Phenomenological description of viscosity dependence of the NCO→N+CO dissociation rate. Experimentally, the slow thermal dissociation of CO molecule from NCO yields the native state (N). The NCO→N+CO conversion does not involve any major conformational adjustment. It is the dynamical events associated with this reaction that form the major thrust of this work. A representative kinetic trace generated after thermal dissociation of CO from NCO is shown in Fig. 1b. 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} (τ=20 min in Fig. 1b) by conventional UV-visible spectrophotometer.

Values of k_{diss} determined at several concentrations of glycerol holding the GdnHCl constant (0.4 M) are plotted in Figure 5 as a function of solvent viscosity, η_s. The rate coefficient decreases linearly at lower viscosities (< 6 cP), but saturates at high viscosity. This is obviously inconsistent with Kramer's theory,[2] indicating that the general inverse dependence of rate on viscosity does not explain the experimentally measured CO dissociation rates of NCO in the range of 0.89-175 cP.

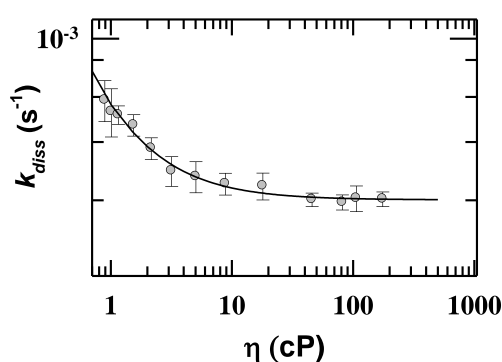


Figure 5. Non-Kramers scaling observed for CO dissociation (NCO→N+CO) rate constant of NCO as a function of glycerol viscosity in 50 mM phosphate buffer, 0.4 M GdnHCl, pH 7, 25°C. The fit according to Eq. (1) is obtained with $\omega_1\omega_2$ ~6.5×10^{31} s^{-2}, A ~4.4×10^{-4} s^{-1}, n~0.72 (±0.05), and E_a = 23.4 (±1.4) kcal mol^{-1}.

The data can nonetheless be fitted to the fractional viscosity dependence relation, a phenomenological expression due to Beece et al.[1]

$$k_f = \left(A + \frac{\omega_1\omega_2}{2\pi \frac{\eta_s^n}{m}} \right) \exp\left(\frac{-E_a}{RT} \right), \qquad (1)$$

where the adjustable parameter A describes a finite rate for η_s >6 cP, n is an exponent in the 0-1 bound, and E_a is the size of the energy barrier. ω_1 and ω_2 are the frequencies of the parabolic wells at the bottom and top of barrier, respectively, and m is the effective molecular mass of the protein. Here, we have used m=20.6×10^{-21} g for the molecular mass (molecular weight divided by Avogadro's number) of cytochrome c. Fits of the data according to this equation yield n~0.72 (±0.05), A ~4.4×10^{-4} s^{-1}, $\omega_1\omega_2$ =6.5×10^{31} s^{-2}, and E_a = 23.4 (±1.4) kcal mol^{-1}. In earlier studies, the fractional exponent, n, was found to decrease as the energy barrier was less accessible to the solvent,[1] and the A factor was hypothesized to result from weak coupling between solvent viscosity and diffusive chain motions within a protein at high viscosity.[1,16,26-27] These interpretations qualitatively agree with the molecular organization of the NCO state, which nearly simulates the N state in terms of compactness, dimension, and solvent-accessible surface area. However, it is of interest as to how the local structural diffusion in the protein interior and

solvent-coupled global fluctuations participates to produce finite rates at infinite solvent viscosities, and thus non-Kramers viscosity scaling. [26-30]

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