Photoacoustic Calorimetry Studies of the Earliest Events in Horse Heart Cytochrome-c Folding

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Photoacoustic Calorimetry Studies of the Earliest Events in Horse Heart Cytochrome-c Folding

by

Tarah Arica Word

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Chemistry College of Arts and Sciences University of South Florida

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Keywords: Protein Folding, Chloramine-T Cytochrome-c, Ferric Cytochrome-c, Ferrous Cytochrome-c, Carbon Monoxide-bound, Nitrogen Monoxide-bound

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DEDICATION

This dissertation is dedicated to my (maternal) grandmother who did not live to see this day, but whose prayers have kept me strong and helped me to endure thus far! I love you and miss you dearly.

R.I.H.

(Rest in Heaven)

“Eleanora Word Guerrant”
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PREFACE

The work in Chapter 4 was done in collaboration with Christi L. Whittington (Dr. H. Lee Woodcock’s group, USF). Christi ran MD simulations of modified versus native ferrous Cc (CO-bound) using NAMD software. Christi used VMD to obtain the trajectories and figures for each system analyzed.

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ABSTRACT

The protein folding problem involves understanding how the tertiary structure of a protein is related to its primary structure. Hence, understanding the thermodynamics associated with the rate-limiting steps for the formation of the earliest events in folding is most crucial to understanding how proteins adopt native secondary and tertiary structures. In order to elucidate the mechanism and pattern of protein folding, an extensively studied protein, Cytochrome-c (Cc), was chosen as a folding system to obtain detailed time-resolved thermodynamic profiles for the earliest events in the protein folding process. Cytochrome-c is an ideal system for understanding the folding process for several reasons. One being that the system can unfold and refold reversibly without the loss of the covalently attached heme group. A number of studies have shown that under denaturing conditions, ferrous Cc (Fe$^{2+}$Cc) heme group in the presence of carbon monoxide (CO) results in a disruption of the axial heme Methionine-80 (Met80) bond ultimately unfolding the protein. CO-photolysis of this ferrous species results in the formation of a transient unfolded protein that is poised in a non-equilibrium state with the equilibrium state being that of the native folded Fe$^{2+}$Cc complex. This allows for the refolding reaction of the protein to be photo-initiated and monitor on ns - ms timescales. While CO cannot bind to the ferric form, nitrogen monoxide (NO) photo-release has been developed to photo-trigger ferric Cc (Fe$^{3+}$Cc) unfolding under denaturing conditions. Photo-dissociation of NO leaves the Fe$^{3+}$complex in a conformational state that favors unfolding thus allowing the early unfolding events of Fe$^{3+}$Cc to be probed. Overall the results presented here involve the use of the ligands CO and NO along with photoacoustic calorimetry (PAC) to photo-trigger the folding/unfolding
reaction of Cc (and modified Cc). Thus, obtaining enthalpy and molar volume changes directly associated with the initial folding/unfolding events occurring in the reaction pathways of both Fe$^{2+}$ and Fe$^{3+}$Cc systems that are most essential to understanding the driving forces involved in forming the tertiary native conformation. The PAC data shows that folding of proteins results from a hierarchy of events that potentially includes the formation of secondary structures, hydrophobic collapse, and/or reorganization of the tertiary complex occurring over ∼ns – tens of μs time ranges. In addition, the PAC kinetic fits presented in this work is the first to report Cc folding exhibiting heterogeneous kinetics (in some cases) by utilizing a stretched exponential decay function.
CHAPTER 1: INTRODUCTION

1.1 The Protein Folding Problem

In general, protein folding is defined as a process through which a protein assumes its native conformation. The structural descriptions of proteins are traditionally described in terms of four levels of protein structure: primary (1°), secondary (2°), tertiary (3°), and quaternary (4°) (Figure 1.1). The 1° structure is the amino acid sequence while the 2° structure is the local spatial arrangement of a polypeptide organized into regular structure elements such as α helices, β sheets, loops, turns and coils. The 3° structure is the 3-D arrangement of the 2° structure and, finally, the 4° structure involves the assembly of its subunits. While it is clear that the amino acid sequence has all the essential information for a protein to achieve its native conformation, the way in which such information is encoded to the protein is not fully understood. Hence, the protein folding problem is in understanding how the 3° structure of a protein is related to its 1° structure (see Figure 1.1).

Questions that remain unclear and may enhance our understanding of the protein folding problem would include: in what order do the structural motifs form? What are the kinetic barriers that set the rate in which the motifs are formed? What are the energetics associated with those barriers? Does the 3° structure fold prior to complete formation of the 2° structure or does the 2° structure template the 3° structure, how does a random coil form a compact 3° structure in which performs its physiological function?[1,2] Each question is equally significant in addressing the protein folding problem and most likely the answers differ from one protein to another. None the
less, one thing to note from different protein systems is that it is the initial events that drive the folding of the protein to its native conformation. While this exact folding mechanism is not known, the rates of formation associated with the hierarchy of protein folding are, to some extent, resolved. Specifically, the formation of $2^\circ$ structures are associated with the fastest folding events occurring on nanoseconds – microsecond (ns – $\mu$s) timescales$^{[3,4]}$ and depending on the conditions and size of the protein, the tertiary structure can fold to its native conformation on timescales ranging milliseconds – seconds (ms – sec) (Figure 1.1). Since the initial events are a key component in resolving the protein folding problem, it is necessary to be able to examine these events on sub-$\mu$s timescales while obtaining thermodynamic information that corresponds to each kinetic event.

![Diagram of protein structures and folding](image)

**Figure 1.1:** General Levels of Protein Structures and the Protein Folding Problem scheme (Protein structures: PBD 10EL, 1PRB, and 1C52).
1.2 Protein Misfolding/Errors and Biological Relevance

Proteins perform their physiological function only when in their correct native structure (most thermodynamically stable conformation). However, a key event associated with protein folding that has increased interest in research is proteins mis-folding. Such errors in protein folding have been linked to a number of human diseases such as: Alzheimer’s, some forms of cancer, and other degenerative diseases (Table 1.1). To understand the cause of such diseases as well as potentially develop advance therapeutic agents, a molecular level understanding of the mechanism of protein folding into its physiological conformation is of utmost importance. In particular, Alzheimer’s disease will kill ~100,000 Americans a year, and cost society billions to care for its patients. One pathway to Alzheimer’s disease is the excessive build-up of a protein known as β-amyloid. Accumulation of the protein on human neural tissue forms deposits (neuritic plaques), eventually causing the formation of fibrils. This build up is believed to be toxic and ultimately interferes with the functioning of the brain. Consequently, it is the folding preceding the accumulation of the β-amyloid aggregates that inhibits certain enzyme functions that affects the brain. Hence, it is extremely important to have a detailed understanding of the folding process, as results from protein folding studies will have a long term impact that could lead to treatments for diseases caused by inhibited pathways.
Table 1.1: Examples of Diseases linked to abnormalities in protein folding

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<td>Cancer</td>
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<td>Mutated forms of this protein folds slowly, allowing its target, Clastase, to destroy lung tissue</td>
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*Table adapted from Reference 5.

1.3 Paradigms of Folding

The simplest way in which to think about how a protein fold into its native conformation was first described by Levinthal.\(^\text{10}\) This description involves a peptide sampling every possible amino acid contact until a native contact is made and continues to search its conformational space until the native protein is formed.\(^\text{10-12}\) For example, a small protein composed of 130 amino acid residues that has 10 conformations per side chain can fold \(10^{130}\) times.\(^\text{10-12}\) However, in order to sample each conformation, even in femtoseconds (fs), would take thousands of years. Since a protein folds on the order of seconds, it is clear that the folding process must fold by some ordered pathway. Two paradigms have been advance to describe possible pathways for protein folding known as the multi-state folding model and energy landscape model.

The multi-state folding model assumes to be a homogenous population of unfolded protein molecules that have a short lifetime with the relaxation occurring as a uni-molecular
process $\text{(A} \rightarrow \text{B}, \frac{d[A]}{dt} = -k[A])$.\textsuperscript{[13-16]} The model suggest that the unfolded protein population proceeds through a specific pathway accumulating distinguishable folding intermediates that ultimately crosses over activation barriers, leading to the folded native conformation (Figure 1.2).\textsuperscript{[13-16]}

In contrast, the energy landscape model assumes that the unfolded protein begins with an ensemble of unfolded conformations that are high in energy relative to the native structure.\textsuperscript{[4]} Thus, folding is assumed to be a many-state kinetic process $\frac{dP_a}{dt} = \sum_b k (b \rightarrow a)P_b - \sum_b k (a \rightarrow b)P_a$, with $P_a$ and $P_b$ being fractions of protein in a conformational state “a or b”).\textsuperscript{[17]} The model suggest that the protein does not follow a specific folding path through the configurational space, but instead, may travel through a number of pathways taking on various partially folded structures that form down the free energy landscape to its native conformation (that is lowest in energy).\textsuperscript{[18]} Specifically, the (heterogeneous) unfolded population is rapidly interconverting between conformations as the funnel is going down a smooth energetic pathway unless trapped in a non-native energy well creating an irreversible trap.\textsuperscript{[19,20]} This is interpreted as being a “frustrated pathway” creating a “rough” energy landscape. As the unfolded molecule makes a native contact the system reduces in energy as well as in the number of accessible conformations until it reaches a single low energy, native conformation (Figure 1.2). However, the debate within each model is if the nature of the protein folding pathway best described as a well-defined pathway with sequential intermediates or does folding follow multiple pathways without passing through a unique transition state.\textsuperscript{[3]}

5
1.4 Experimental Studies Triggering the Fast Folding/Unfolding of Small Peptide/Protein Systems Coupled with Time-Resolved Photo-thermal Methods

The early events of protein folding are a crucial part of the folding pathway, yet our understanding towards the process remains very limited.\cite{4} This is due to experimental limitations such as limited time windows of traditional methods (i.e. stopped-flow, etc.).\cite{21,22} Information such as the thermodynamics of the initial events would assist in completing an entire picture of the protein folding process. Specifically, the energetics associated with the steps leading to the native structure is critical, as energetic pathways ultimately drive the folding process.\cite{4}

To date, there is an extensive database of folding systems that have been used to describe the mechanism of protein folding. A number of these studies started with simple peptide folding systems as a model in order to understand the folding of more complex systems. In addition, this kinetic data is available for small peptides and proteins folding into 2° structures and, more recently, corresponding thermodynamic data for such processes. This is due to the recent advances in photo-thermal methods including photoacoustic calorimetry (PAC). One of the
advantages of using PAC is the fact that triggering the reaction with light allows for the resolution of the earliest folding process in time (on ns to 10’s of µs timescales). A second advantage is that PAC has the ability to accurately measure direct enthalpy and molar volume changes for reactions, which are important thermodynamic parameters. A third advantage of PAC is that it allows for the detection of optically silent conformational changes and, finally, structural information on molar volume changes, enthalpy, and kinetics of protein folding/unfolding can be obtained in a single experiment. The enthalpy constitutes the heat component of the free energy whereas molar volume changes describe changes in solvation, and protein:solvent interaction. Thus, coupling PAC together with fast triggering techniques (i.e. pH jump and “caged” photo-cleavable groups) has been successful in elucidating energetics involved with the folding mechanism of systems examined.

1.4.1 Poly-L-Glutamic Acid and Apomyoglobin Fast Folding

An example of a fast triggering method used in conjunction with photo-thermal techniques is the laser induced pH jump unfolding/folding reaction of two previously studied systems conducted by Abbruzzetti et al: horse heart apomyoglobin (apoMb) and Poly-L-glutamic acid (PLG).\textsuperscript{[23,24]}

In the case of PLG (82 Glu residues), the system is known to change its structure from a random coil to a α-helix as the pH decreases from pH ~7 to <5.\textsuperscript{[24-26]} Abbruzzetti et al.\textsuperscript{[24]} took advantage of this by utilizing PAC together with laser induced pH jump to probe the kinetics and thermodynamics of the coil-to-helix transition. Briefly, the proton concentration is rapidly increased using a ns UV laser pulse to photolyze o-nitrobenzaldehyde (oNBA, caged proton).\textsuperscript{[24]} Photolysis of oNBA is irreversible and the magnitude of the pH jump depends on the pre-pulse
pH. The protons released (within ~10 ns) react with proton acceptors through diffusion mediated processes.\textsuperscript{[24]} Due to the large excess of acceptors, the pH returns to the pre-pulse value before the next laser flash\textsuperscript{[24]} and the refolding events of the system is monitored using PAC (see Figure 1.3). The data obtained indicated that at a pH near the pK\textsubscript{a} of PLG (5.4), protonation of the Glu side chains was followed by a kinetic process of ~100 ns.\textsuperscript{[24]} The ~100 ns process was not thermally activated and was suggested to be the result of local hydrogen bond formation near the site of protonation. This kinetic phase resulted in a change in enthalpy (\(\Delta H\)) \(-1.5\) kcal mol\(^{-1}\) and activation energy (\(E_a\)) = 1.6 kcal mol\(^{-1}\). In addition, a molar volume change (\(\Delta V\)) of ~7 mL mol\(^{-1}\) was observed that was suggested to be inconsistent with the formation of hydrogen bonds only.\textsuperscript{[24]} The authors concluded that additional local \(\alpha\)-helical structures formed that were rate-limiting in the formation of \(\alpha\)-helical PLG.

![Figure 1.3: Schematic representation for laser induced jump of \(\alpha\)-helical PLG (*Similar figure can be found in Reference 27).](image)

The second system, apoMb, is a heme free form of the small oxygen binding naturally occurring protein, Myoglobin (Mb). Native Mb contains eight \(\alpha\) helices designated as A-H that form a hydrophobic pocket around the heme.\textsuperscript{[23]} Removal of the heme group from Mb results in a peptide retaining most of the structural topology as the native form including a compact hydrophobic core and helices A, B, E, G and H (designated native (N) state for apoMb) (see Figure 1.4).\textsuperscript{[23]} At pH 4 and 3 apoMb is described as a molten globule (MG) conformation.
(referred to intermediate (I) and (E) state, respectively) and at an even lower pH (<3) is characterized as being consistent with a random coil conformation (unfolded (U) state). Thus, apoMb represents a versatile protein folding system in which to study the mechanism and kinetics of folding through well-defined (folding) intermediates. Abbruzzetti et al. utilized laser induced pH jump techniques together with PAC to probe the N → I unfolding transition. In this experiment, the rapid acidification upon photolysis of oNBA resulted in a shift of the equilibrium towards the I state and allowed for the kinetic and thermodynamic parameters for the unfolding reaction to be obtained. Upon photolysis two kinetic reactions were observed. The first process occurred with a lifetime of ~100 ns and was accompanied by a ΔV of −82 mL mol⁻¹ and an ΔH of 8 kcal mol⁻¹ that was attributed to protonation of His24 and/or His119. The second process was attributed to neutralization of carboxylic acid residues occurring with a lifetime of ~2.4 μs but was accompanied by a volume expansion of 3.4 mL mol⁻¹ and an ΔH of only 2 kcal mol⁻¹. The Eₐ for the protonation of His24/119 and the γ-carboxylates was 16 kcal mol⁻¹ and 9 kcal mol⁻¹, respectively. It was suggested that the high Eₐ and large volume contraction for the His protonation represented the rate-limiting step in the N → I transition.

A more recent study by the Larsen group utilized PAC as well as PBD to study apoMb unfolding (N → I state). The PAC data reveal two kinetic phases. The first phase occurred faster than the resolution time of the PAC instrument in that study (<50 ns) with a expansion in ΔV = −9 mL mol⁻¹ and a heat release (Q) of −24 kcal mol⁻¹ (where ΔH = (Eₕv − Q)/Φ). In addition, a slower phase (~600 ns) was resolved with a ΔV and ΔH of −22 mL mol⁻¹ and 77 kcal mol⁻¹, respectively (Eₐ = 6.2 kcal mol⁻¹). The PAC data differ in reference to the results obtained by Abbruzzetti et al. The main reason was due to Abbruzzetti et al. utilizing guanidine hydrochloride (GdnHCl) to destabilize the N conformation, whereas Larsen et al. did not use
chemical denaturants. Hence, in the absence of denaturants the fast phase involved ion formation and solvation (from α-NBA photolysis) and potentially protonation of protein carboxylic acids.[28] The slower phase was suggested to arise from global protein conformational changes. In addition, the PBD results showed no conformational changes on longer timescales. Thus, all the transitions associated with the N → I transition in apoMb appear to be complete in <10 ms.[28]

![Figure 1.4](image)

**Figure 1.4:** Structure of horse heart ApoMb (PBD 1AZ1) with the location of disordered helices of “I” state shown as ribbons and the AGH core helices are shown as cartoon. (*Similar Figure can be found in Reference 27).*

### 1.4.2 Caged Peptide Fast Folding Systems

The experiments described above rely on the use of a perturbant (pH) to initiate the folding and unfolding process. It is unclear the extent to which the folding/unfolding reactions represent physiologically relevant folding processes, which do not involve denaturants bound to the protein. An alternative method to initiate protein folding (without the use of denaturants) is to use a photo-cleavable protecting groups or a “cage” on a specific segment of the protein and/or peptide in such a way that the caged derivative of the complex is either folded or unfolded and the photo-product shifts the unfolded/folded equilibrium, depending on which way the system is being studied.
To date, a number of research groups have explored the use of caged peptides and proteins to probe protein folding.\cite{29-33} In these systems, a small synthetic peptide/protein is modified using a photo-cleavable group in such a way that the caged system disrupts the native conformation and unfolds the complex. Photo-cleavage of the linker (from an unfolded system) spontaneously folds into a well-defined 2° or 3° structure.\cite{29-33} Specifically, refolding was achieved by cleavage of the “cage” within $10^{-9}$ s by irradiation pulses from a UV laser. Chan’s group pioneered the synthesis of several small synthetic peptide systems designed in such a way that a portion of the peptide was constrained with a photo-cleavable linker.\cite{29-33} The folding systems developed include basic 2° structural motifs found in proteins. Specific systems include a modified α-helical villin headpiece\cite{30,31} as well as a β-sheet model system, 19-merE11C.\cite{29,31} Both synthetic peptide systems thermodynamics were probed on fast timescales upon photo-cleavage of the linker utilizing PAC in collaboration with Larsen’s group.

In the case of the modified villin headpiece, the peptide is best characterized as a three helical segment (35 residues) that surrounds a hydrophobic core (see Figure 1.5).\cite{30,31} The peptide contains a Met12 that is mutated to a Cys12 in order to covalently link the N-terminus of the villin headpiece to the benzoin-based linker (3’, 5’-dimethoxy benzoin).\cite{30,31} The small helical segment between the Cys12 and the N-terminus adopts a random coil-like conformation in the modified peptide. Photo-cleavage of the linker allowed for kinetics and energetics to be probed through PAC. The results from PAC revealed two kinetic phases, one with a rate (<40 ns) faster than the resolution time of the PAC instrumentation and the second with a lifetime of ~100 ns.\cite{31} The first event is associated with photochemistry of the linker with an endothermic $\Delta H$ of 41 kcal mol$^{-1}$ and a $\Delta V$ of −2.6 ml mol$^{-1}$.\cite{31} The second event was described as the N-terminal
The second synthetic system, 19merEC11, is the β-hairpin sequenced VFIIVDGOTYTCVDPGOKILQ.\textsuperscript{[29,31]} In addition, the synthetic peptide is an analogue of a Gellman β-sheet where the first hairpin turn sequence has been replaced with “VDGO” turn sequence.\textsuperscript{[29,31]} The 19merEC11 peptide contains a Glu11 residue that is mutated to Cys11 and cyclized with a photo-labile linker, bromoacetyl-carboxymethoxybenzoin (BrAcCMB), on the N-terminal of the peptide chain (see Figure 1.6).\textsuperscript{[29,31]} Refolding kinetics and thermodynamics were obtained through PAC following photo-cleavage of the linker. The PAC data revealed a refolding event with a lifetime of 600 ns following the first kinetic phase that occurred faster than the resolution time of the authors PAC instrument (<40 ns).\textsuperscript{[29,31]} The <40 ns phase was associated with a volume expansion of ~7 ml mol\textsuperscript{-1} and an corresponding endothermic $\Delta H$ of ~50 kcal mol\textsuperscript{-1}, where as the slower process exhibited a volume contraction of ~6.3 ml mol\textsuperscript{-1} and an exothermic $\Delta H$ of ~36 kcal mol\textsuperscript{-1} ($E_a = -1.8$ kcal mol\textsuperscript{-1}).\textsuperscript{[29,31]} The fast phase photochemistry represented the breakage of the linker and, in addition, initial folding events (potentially the nucleation step) occurring within 40 ns. The authors indicated that the 600 ns phase
corresponded to the refolding of the peptide in which the volume decrease reflects contraction arising from a structural collapse and reburial of hydrophobic residues, and included the turn amino acid sequence.\cite{29,31}

![Figure 1.6: Representation of c-19merE11C cyclized with a photo-labile linker (Br-AcCMB) and the thio-glutamate residue produced after UV photolysis (*Similar figure can be found in Reference 31).](image)

In principle, it is possible to extend the “caging” strategy to larger protein systems beyond just structural motifs using multiple photo-labile linkers suitably placed in the protein structure. However, the synthesis is ideal in protein systems consisting of \( \leq 30 \) amino acid residues and becomes more difficult with larger systems. As a result, the larger proteins systems becomes challenging to constrain with photo-cleavable protecting groups as well as accurately interpret the data from photo-thermal studies. Hence, there is a need to be able to study the folding process of more complex and larger systems.

### 1.4.3 Cytochrome-c Fast Folding

Photo-triggering the folding of a larger protein system was accomplished by Jones and co-workers. The authors utilized the photo-triggerable heme protein, Cytochrome-c (Cc) as a model system for folding.\cite{34} Cytochrome-c is a 12 KDa (104 amino acids) heme protein whose primary role in aerobic organisms is in the transport of electrons between cytochrome-bc1 to cytochrome-oxidase. The heme group of Cc is covalently attached to the polypeptide via two Cys thioether linkages and is 6-coordinate low spin with His18/Met80 serving as axial ligands.
that occupy the heme iron 5\textsuperscript{th} and 6\textsuperscript{th} coordination sites, respectively (Figure 1.7).\textsuperscript{[35,36]} The protein is known to unfold and refold reversibly while the heme group remains covalently attached to the protein and possesses a single Tryptophan residues, hence a spectroscopic probe. Therefore, the system is amenable to measurements by a variety of methods allowing for native and non-native interactions to be identified along the folding pathway.

**Figure 1.7:** Ribbon Structure of Horse Heart Cytochrome-c (PBD 1HRC) with key residues Met80 and His18 around the heme pocket highlighted.

The idea is to utilize CO as a photo-trigger to initiate the relatively fast folding of ferrous Cc. Native ferrous Cc (Fe\textsuperscript{2+}Cc) does not bind other ligands to the heme group since the heme iron is six-coordinate. However, in the presence of a denaturant, the axial heme Met80 bond is destabilized and can be displaced by CO leading to the unfolding of the protein (Figure 1.8, top). Upon photolysis, a transient species is formed in which the unfolded protein is poised in a non-equilibrium state with the equilibrium state being that of the native folded Fe\textsuperscript{2+}Cc complex\textsuperscript{[34]} (Figure 1.8, bottom).

Jones and co-workers demonstrated that in the presence of 4.5 M guanidine hydrochloride (GdnHCl) in pH~7 buffer solution, Fe\textsuperscript{2+}Cc destabilizes in the presence of CO
allowing photo-initiation of the folding process to occur.\textsuperscript{[34]} Using time-resolved optical absorption (TROA) and kinetic modeling, the authors concluded that the early events associated with Fe\textsuperscript{2+}Cc folding were due to the binding of Met80 or 65 and His18 or 33 binding to the heme before folding of the 3\textdegree{} structure occurred.\textsuperscript{[34]} In addition, their model suggested that the Met ligand coordinates to the heme ten times faster than that of the His ligand contrary to the fact that both His residues are positioned closer to the heme.\textsuperscript{[34]} Overall, this study was a major advance for initiating the folding process of Fe\textsuperscript{2+}Cc folding. In addition, CO photo-initiation method has led to a number of Fe\textsuperscript{2+}Cc folding studies that have provided information on the kinetics and structural assignments associated with the early events occurring in Cc folding process discussed below.

\textbf{Figure 1.8}: Schematic of Fe\textsuperscript{2+}Cc: (Top) Met80 residue destabilized under denaturing conditions and in the presence of CO (Bottom) fast folding using CO a photo-trigger.
A study later conducted by Chen and co-workers[37] followed up Jones et al. work using near/far UV time-resolved circular dichroism (TRCD) in order to probe the 2° structural changes that occurred upon CO photolysis. The data showed that only ~8% of a native-like formation took place within ~2 µs and was interpreted as the N-/C-terminal helices being formed.[37] This native-like formation occurs following a hydrophobic collapse (burst phase) versus being a distinctive folding intermediate.[37,38] Kumar et al.[21] termed this collapse phase U* → U’ (a phase between U → I of the U → I →N stages of protein folding) but could not directly characterized this process due to the event occurring during the dead-time of their experimentation. In any case, the authors suggested that this phenomenon proceeds in which the protein contracts or expands until it crosses over a rate-limiting barrier in order to attain its native conformation.[21] In addition, they were able to contribute additional kinetics that occurred during the refolding process of ferrous Cc. Specifically, using stopped-flow and laser flash photolysis (0 – 4 M GdnHCL, pH ~12.8) Kumar et al. were able to resolve, in time, four processes involving a Met80/65 coordination to the heme with a lifetime of (τ₁) 300 ns, a His26/33 coordination with a much slower lifetime of (τ₂) 2.5 µs, followed by a (τ₃) 700 µs phases associated with CO rebinding to the heme, and finally a (τ₄) 2.5 ms phase assigned to the protein achieving its native conformation (Figure 1.9).[21]
A study conducted by the Terazima group\textsuperscript{39} utilized laser-induced transient grating (TG) techniques to measure the change in diffusion coefficient (\(D\)) for Fe\textsuperscript{2+}Cc refolding process (U \(\rightarrow\) N). Refolding of the system was triggered by photo-injection of electrons from \(\beta\)-nicotinamide adenine dinucleotide (NADH) to unfolded ferric Cc (Fe\textsuperscript{3+}Cc) in the presence of denaturant concentrations ranging from 2.5 M – 4.25 M GdnHCl and temperatures of \(\sim14^\circ\text{C} – \sim35^\circ\text{C}\).\textsuperscript{39} The protein stability is different in its reduced form versus oxidized form against various concentration of GdnHCl. For example, at 3.5 M GdnHCl Fe\textsuperscript{2+}Cc is relatively folded versus Fe\textsuperscript{3+}Cc at this same concentration which resembles more of denatured structure. Thus photo-injection of the electrons to the unfolded ferric system initiated Fe\textsuperscript{2+}Cc folding. The authors reported that at 3.5 M GdnHCl \(D\) increases with the protein refolding process.\textsuperscript{39-41} The increase was interpreted as the hydrogen bonding network changing in the protein, including inter-molecular to intra-molecular bonding, movement of the amino acid residues, and water molecules controlling the folding dynamics.\textsuperscript{39-41} In addition, the diffusion change was consistent with a two-state model over a wide time range (~ms) and what the author considered were from
two conformations. The two conformations (a local structural change and a global conformational change) were proposed to differ in rate constants but have similar transition states and activation energies (values not reported) with an $m^+$-value of $\sim -1.9^{[39]}$.

On the other hand, Choi et al.$^{[42,43]}$ also utilized TG but used CO as a photo-trigger to probe Fe$^{2+}$Cc folding in various concentration of denaturant. The TG signals showed changes in $D$ supporting Cc folding as a three-state folding mechanism with detectable intermediates instead of a two-state mechanism proposed by Terazima’s group$^{[39]}$ (that involved U $\rightarrow$ N without any detectable intermediates). The mechanism involved an initial collapse phase that included CO dissociation and Met80 coordination followed by additional folding dynamics of Cc that proceeded to the native conformation. In addition, the authors obtained rates and activation energies for the U $\rightarrow$ I and I $\rightarrow$ N transition for Cc folding by utilizing quantitative global analysis of the TG signals. The U $\rightarrow$ I transition occurred with a rate of $\sim$290 $\mu$s $-$ $\sim$940 $\mu$s and with an $E_a$ of 8.7 kcal mol$^{-1}$. For the I $\rightarrow$ N transition, the observed rates were $\sim$10 ms - $\sim$50 ms with an $E_a$ of 7.1 kcal mol$^{-1}$$^{[42,43]}$.

Up to this point, data that contains mainly the kinetic contributions have been acquired for most of the events occurring in the Fe$^{2+}$Cc folding process. However, direct enthalpy and molar volume changes associated with the earliest events in folding have not been well characterized due to the limitation of the techniques used in those specific studies.$^{[44-46]}$

A significant body of research is also available on the folding of Fe$^{3+}$Cc.$^{[47-60]}$ Overall, studies have determined that folding/unfolding in Fe$^{3+}$Cc is guided by a hierarchy of specific domains referred to as “foldons”.$^{[53]}$ Foldons are characterized as five folding units in which folding of the protein proceeds by a stepwise assembly of these units progressively building the native structure. Unfolding of the native structure is achieved in this same way but the units go in
opposite direction.\textsuperscript{[53]} In addition, nearly all of the studies on Fe\textsuperscript{3+}Cc folding have concluded that the system folds to near-native conformation on a timescale on the order of seconds, with bis-His coordination being the rate-limiting step. The studies also concluded that reorganization of the ferric complex did not bind with either Met ligands (80 or 65) during the initial stages of folding (as seen in the ferrous form).\textsuperscript{[51]} The authors suggested that this is likely due to the slow dissociation rate of the non-native His (~100 s\textsuperscript{-1}) that is trapped into a frustrated folding intermediate.\textsuperscript{[48]} Dissociation of His ultimately led to the completion of the folding process to its native conformation at a rate of ~10 s\textsuperscript{-1}.\textsuperscript{[48,52]} However, information regarding the thermodynamics associated with ligand rebinding as well as early events occurring in Fe\textsuperscript{3+}Cc folding/unfolding process are less well characterized. One main reason is due to methods not yet available to initiate Fe\textsuperscript{3+}Cc folding on sufficiently fast timescales.

1.5 Dissertation Research/Objectives

The objective of this dissertation was two-part. The first part of the work presented here, utilizes PAC to obtain enthalpy and molar volume changes directly associated with the ferrous form of Cc (and modified Fe\textsuperscript{2+}Cc) under denaturant conditions that allow refolding of the complex subsequent to CO photo-dissociation. The data obtained from these studies have been used to construct a detailed thermodynamic profile for the folding pathway and allow for a linkage between the energetics and structural assignments of kinetically identifiable intermediate states in Fe\textsuperscript{2+}Cc (see Figure 1.5).

The second part of this study focuses on ferric Cc (and modified Fe\textsuperscript{3+}Cc) unfolding/folding. Here modification of the Jones et al. method was done by using NO as a photo-trigger to initiate the unfolding/folding process of the oxidized forms. Direct measurement
of the kinetics, enthalpy, and molar volume changes associated with each transition state that occur within the response time of the PAC detector (20 ns – 20 µs) are probed in order to construct a detailed thermodynamic profile for the fast unfolding or folding pathway of Fe$^{3+}$Cc.

It is the initial folding/unfolding events occurring in the reaction pathways of both Fe$^{2+}$ and Fe$^{3+}$Cc systems that are most crucial to understanding the driving forces involved in forming the 3° native structure in the protein folding process. Hence, the overall results will provide a more detail understanding of the energetics involved with the steps leading to the native conformation.

1.6 References


CHAPTER 2: EXPERIMENTAL METHODOLOGY

2.1 Photo-thermal Methods

As previously stated in Chapter 1, PAC is a powerful technique for determining the timescales and magnitude of enthalpy and molar volume changes associated with physiological events such as the protein folding process. The technique takes advantage of photo-excited molecules that results from a transition from the ground electronic state to some higher excited state governed by Fermi’s Golden Rule.\[1\] Following excitation, the molecules may relax via radiative decay resulting in emission of a photon, intersystem crossing to an excited triplet state or non-radiative resulting in heat deposition to the solvent (see Figure 2.1). In addition, excited state molecules may undergo photo-chemical processes that can alter the molecular dimensions of molecules (i.e., changes in van der Waals volume), as well as alter the charge distribution (electrostatic effects) of the sample in question.\[2,3\]

![Figure 2.1: Perrin-Jablonski diagram.](image)
2.2 Photoacoustic Calorimetry

In this work, PAC is the method utilized for elucidating the fast folding events occurring subsequent to CO or NO photo-dissociation in each Cc folding system investigated. The set-up, theory and analysis for PAC has been extensively described in detail elsewhere.[2-22] The physical principle behind PAC is that the photo-excited molecules dissipate excess energy via vibrational relaxation (non-radiatively) to the ground state which is accompanied by thermal heating to the surrounding solvent[5,14,23]. For solvents such as water, this excess energy causes a rapid volume expansion within the illuminated volume resulting in a pressure wave (acoustic wave) which can be expressed as:

\[ P = -k_T \frac{dV}{V} \]  \hspace{1cm} (2.0)

where \( k \) is the isothermal bulk modulus, \( dV \) is the change in volume subsequent to excitation, and \( V \) is the unperturbed volume. In addition, volume changes in the system of interest (resulting from a photo-initiated reaction) also contribute to the acoustic wave and the overall volume change \( \Delta V_{\text{Overall}} \) is expressed as:

\[ \Delta V_{\text{Overall}} = \Delta V_{\text{th}} + \Delta V_{\text{con}} \]  \hspace{1cm} (2.1).

This acoustic wave can be detected with a piezo-electric crystal (2 MHz transducer). The resulting acoustic signal can be written as:

\[ S = KE_a(\Delta V_{\text{th}} + \Delta V_{\text{con}}) \]  \hspace{1cm} (2.2)

where \( K \) is an instrument response parameter (unique with every experiment), \( E_a \) is the number of Einsteins absorbed, \( \Delta V_{\text{th}} \) is the volume change due to thermal expansion, and \( \Delta V_{\text{con}} \) is the
change in volume due to ligand binding/release, protein conformational changes, van der Waals
volumes, electrostatic contributions, etc. The thermal expansion term can be described by:

$$\Delta V_{th} = Q*(\beta/Cp)$$

where $Q$ is the heat released to the solvent ($\text{kcal mol}^{-1}$), $\beta$ is the thermal expansion coefficient of
the solvent ($\text{K}^{-1}$), $Cp$ is the solvent's heat capacity ($\text{kcal g}^{-1} \text{ K}^{-1}$), and $\rho$ is the solvent density ($\text{g}
\text{mL}^{-1}$). In order to determine the $Q$ and associated $\Delta V_{con}$ for the sample being investigated
subsequent to excitation, a calorimetric reference was employed. The acoustic wave of the
reference is measured under identical experimental conditions as the sample to eliminate $K$. As
the reference molecules do not undergo any photochemistry and are non-fluorescent (i.e., $\Delta V_{con} =
0$) the energy of the photon absorbed, $E_{hv}$, is converted into heat with a unity quantum yield ($Q =
E_{hv}$).\[5\] Thus, the amplitude of the PAC reference acoustic wave ($R$) can be expressed as:

$$R = KE_a (\beta/Cp)E_{hv}$$

Taking a ratio of the sample and reference acoustic amplitudes ($S/R = \phi$) and scaling to the
energy of absorbed photon gives:

$$(S/R)E_{hv} = \phi E_{hv} = Q + (\Delta V_{con})/(Cp/\beta)$$

Plotting $\phi E_{hv}$ versus $Cp/\beta$ (temperature dependent for water and weakly buffered solutions)
gives a line allowing for the determination of $Q$ (intercept) and $\Delta V_{con}$ (slope). Since $Q$ is the
amount of heat released to the solvent associated with a reaction step, $(E_{hv} - Q)/\Phi = \Delta H$ for
reactions taking place within the integrated time ($< 20 \text{ ns}$) resolution of the instrument and $\Phi$ is
the quantum yield for the photochemical process.

### 2.2.1 PAC Deconvolution: Homogenous Decay Model

The acoustic transducer is also sensitive to the frequency of the acoustic signal. Hence, if a photo-initiated process has multiple kinetic events occurring slower than the response time of the piezoelectric crystal (between ~20 ns - ~20 μs), the resulting sample acoustic wave is shifted in frequency relative to the reference waveform (See in Chapter 3). Thus, the observed acoustic signal is composed of a series of acoustic signals each having a distinct amplitude and lifetime. This is due to the fact that the observed acoustic signals are a convolution of the instrument response function (an under damped oscillator) and exponential heat decay functions. The individual contributions of $\Delta V_{\text{con}}$ and $Q$ for each step can be resolved using deconvolution methods. In order to extract the relevant $Q_i$, $\Delta V_{\text{cons}}$, rate constants ($k_i$) corresponding to the observed kinetic processes, the sample acoustic wave, $E(t)_{\text{obs}}$, is treated as a convolution of an instrument response function, $T(t)$, and time-dependent heat generating function, $H(t)$, according to

$$E(t)_{\text{obs}} = H(t) \otimes T(t) \quad (2.6)$$

where

$$H(t) = \sum (\phi_i \exp(-t/t_i)) \quad (2.7).$$

In practice, the instrument response function $T(t)$ is taken to be the calorimetric reference waveform. The amplitudes, $\phi_i$, and lifetimes, $\tau_i$, of the resolvable kinetic processes are extracted using a simplex parameter estimation algorithm within software developed in our laboratory (LarsenWare2009V1). Subtracting $Q_p$ (obtained from the <20 ns phase) from $E_{hv}$ and scaling to
the reaction quantum yield ($\Phi$) gives the reaction enthalpy ($\Delta H_p$) for the initial (prompt) phase of the reaction while $Q_i = -\Delta H_i$ for each additional step resolved in the deconvolution. The corresponding $\Delta V_{\text{con}}$ values are obtained from the slopes of the plots described in Equation 2.5 (with $\Delta V_{\text{con}}$ for the prompt phase being the slope/$\Phi$).

In nearly all PAC applications reported in the literature, the exponential decay model is applied to the heat source function. The heat release is assumed to be a uni-molecular process and give rise to homogenous relaxation (chemical processes that are separated by energy barriers well above $k_bT$, i.e. uni-molecular or pseudo-first order processes).

### 2.2.2 PAC Deconvolution: Heterogeneous Decay Model

Many biological processes (i.e., protein folding) may display heterogeneous kinetics and, therefore, cannot be expressed as a single exponential decay model. In this case, the system has low energy barriers (similar to or just below $k_bT$) between energetic states that give rise to an ensemble of species or rapidly interconverting states that lead to a heterogeneous population\(^{[24]}\) (i.e. energy landscape model).

A different approach for modeling kinetic events occurring slower than the resolution time of the piezoelectric crystal (between 20 ns - 20 $\mu$s) is that deconvolution is treated as a probability lifetime distribution convoluted with the instrument response function $T(t)$, with the lifetime distribution being:

$$P(t) = A \exp[-(t/\tau)^\beta]$$  \hspace{1cm} (2.8)

where

$$A = \beta t^{\beta-1}(\phi/t_i)^\beta$$  \hspace{1cm} (2.9)
where $\tau$ is the lifetime of the process, and $\beta$ ($<$1) is the stretching factor that is related to the degree of heterogeneity of the folding system being investigated.\textsuperscript{[24]} Extraction of the kinetic processes and amplitudes occurring slower than the instrument response is accomplished using Equation 2.6 and 2.8 using a simplex algorithm within software developed in our laboratory (LarsenWare2009V1). Finally, the parameters are deconvoluted with the reference, $\chi^2$ and autocorrelation calculated. This process is repeated until a good fit is obtained as judged by the autocorrelation.

2.2.3 PAC Instrumentation Setup

The sample is placed in a temperature-controlled cell holder (Flash 200, Quantum Northwest) with a 1 MHz transducer (Panametrics V103) mounted to the side of the cuvette and facilitated by a thin layer of vacuum grease. Photolysis of CO or NO was initiated by excitation with a frequency doubled Nd:YAG laser (Continuum Minilite I, 532 nm, 7 ns FWHM pulse, $\sim$100 $\mu$J/pulse). The photochemistry is initiated using 15 – 30 lasers pulses (on average) over temperatures ranging from 6 – 24 °C. The signal generated by the output of the transducer is amplified (Panametrics 5678) and recorded using a digital oscilloscope (25 MHz PicoScope 2105). The calorimetric reference consisted of black ink (Pelikan 4001), which is photochemically inert and non-fluorescent. The reference data were collected under identical experimental conditions as the Cc sample analyzed.
2.2.4 Quantum Yield Measurements

The quantum yield, $\Phi$, for CO and NO photolysis from each Cc sample investigated was determined by the “pulsed” method described by Brunori et al. The quantum yields were determined using laser flash photolysis techniques with instrumentation described later in this Chapter. The output of the frequency doubled Nd:YAG laser was used as the excitation source (Continuum Minilite I, 532 nm 7 ns pulse width, $\sim$50 mJ/pulse). The laser intensity was attenuated using 5 neutral density filters with varying optical density and CO-bound Mb was used as the reference ($\Phi_{COMb} = \sim$1). Carbon monoxide recombination following laser flash photolysis was monitored at 440 nm for COMb and the monitoring wavelength was the maximum absorbance at the Soret for the Cc sample. The quantum yield was calculated by plotting the amplitude of the absorbance change at the monitored wavelength as a function of

\[ \text{Figure 2.2: (Top) Schematic of PAC experiment “in practice” and (Bottom) PAC setup.} \]
photon density. The ratio of the slopes is then used to obtain $\Phi$ according to:

$$\Phi_{\text{sample}} = \Phi_{\text{COMb}} \left( \frac{[\text{Slope}_{\text{sample}}]}{[\text{Slope}_{\text{COMb}}]} \right) \quad (2.10).$$

### 2.2.5 Calculating Activation Parameters

In order to obtain a complete thermodynamic profile (see Figure 2.3), the activation parameters for each step in the reaction are required. The activation parameters of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ can be obtained from temperature dependent rate constants (between 20 ns - 20 $\mu$s) extracted from the PAC fits. As a result, the enthalpy and entropy of activation for ligand binding and intermediates along the folding pathway were calculated based upon the equation:

$$\ln(k_{\text{obs}}h/k_BT) = -\frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \quad (2.11)$$

where $k_B$ is the Boltzmann constant, $h$ is Planck’s constant, and $R$ is the gas constant. A plot of $\ln(k_{\text{obs}}h/K_BT)$ versus $1/RT$ generates a straight line with a slope that yields the activation of enthalpy, $\Delta H^\ddagger$, and a intercept that is proportional to the activation of entropy, $\Delta S^\ddagger$.

**Figure 2.3:** Schematic of thermodynamic profile parameters for $\Delta H$. The schematic illustrates the thermodynamics associated with conversion between various intermediates along a reaction pathway from initial reactants (A) to final products (C).
2.2.6 Calculating $\frac{C_p}{\beta}$ for Buffer:Denaturant Solutions: Where the Denaturant Environment Alters the Thermal Elastic Properties of Aqueous Solution

In order to obtain $\Delta H$ and $\Delta V$, the magnitude of the photo-generated acoustic wave must be temperature dependent by way of the solvent terms, $C_p/\beta$. For water and weakly buffered solutions, this term is significantly temperature dependent and has been determined for a wide range of temperatures.\textsuperscript{[26]} However, in some cases of buffer:denaturant solutions (i.e. HEPES (pH ~7):GdnHCl (4.5 M)), water looses its thermal elastic properties and no longer exhibits a temperature dependence. Thus, in this study, ideal buffer:denaturant conditions that 1) allowed for refolding of the protein to be photo-triggered and 2) where $C_p/\beta$ still demonstrated a temperature dependence were found. The exact values of the unknown $C_p/\beta$ parameters (due to the solvent properties being altered) are determined by comparing the acoustic signals of a calorimetric reference ($S_{\text{Reference}}$) in water to the corresponding buffer:denaturant solution ($S_{\text{Sample}}$) as a function of temperature ($^\circ\text{C}$) and using the equation:

$$(C_p/\beta)^{\circ\text{C}}_{\text{sample}} = (C_p/\beta)^{\circ\text{C}}_{\text{H}_2\text{O}}([S_{\text{Reference}}/S_{\text{sample}}]) \quad (2.12).$$

2.3 Transient Absorption Spectroscopy

Transient absorption spectroscopy (TA) monitors the changes in absorption versus time of photo-chemical reactions following excitation via a pulsed laser.\textsuperscript{[27,28]} Consequently, this allows for the difference absorption ($\Delta A$) spectrum to be calculated, (i.e., the absorption spectrum of the excited sample minus the absorption spectrum of the sample in the ground state).\textsuperscript{[29]} Here, TA is utilized as a tool for probing ligands binding along the folding pathway as well as NO or CO recombination to the heme iron of the Cc folding systems investigated. In addition, TA has the potential to reveal if the refolding reaction results in transient or long-lived intermediate states.
due to conformational changes that affect the heme electronic state.

### 2.3.1 TA Instrumentation Setup

Transient absorption measurements for CO and NO rebinding were performed using a homebuilt instrument that has previously been described in detail.\(^{[28]}\) In summary, the output of the frequency doubled Nd:YAG laser was used to obtain a 532 nm excitation pulse (Continuum Minilite I, 7 ns pulse width, ~50 mJ/pulse) directly to the sample of interest, thus allowing photo-dissociation of the ligand and triggering the reaction. Single wavelength transient absorption kinetics was obtained using a 150 W Xenon arc lamp (Thermo Oriel). The probe beam is centered onto the sample in a temperature-controlled sample holder and the emerging light is focused on the entrance slit of a monochromator (H10, Yvon Jobin) and detected by a photo-multiplier tube (Hamamatsu). The signal is then amplified twice using a difference amplifier (Melles Griot) and 300 MHz amplifier then digitized (Tektronix TDS 7404 4GHz). Kinetic traces were fitted using Origin Pro 8.0\(^{TM}\) software.

![Figure 2.4: Transient Absorption Setup](image-url)
2.4 References


CHAPTER 3: FERROUS SYSTEM I
Surfactant-Induced Protein Folding in Ferrous Horse Heart Cytochrome-c

3.1 Background

As stated in Chapter 1, Cc is known to unfold and refold reversibly while the heme group remains covalently attached to the protein. Jones and co-workers took advantage of this after revealing that, under certain experimental conditions, the Fe-Met80 coordination is destabilized to the extent that CO can displace Met and bind to the Fe$^{2+}$ heme. The ligand replacement triggers the unfolding of the protein and upon CO photolysis a transient species is formed in which the unfolded protein is poised in a non-equilibrium state with the equilibrium state being that of the native folded Fe$^{2+}$ Cc complex. To date, numerous studies have reported the kinetic contributions for most of the events occurring in the folding process of Fe$^{2+}$ Cc, under various experimental conditions. However, energetics directly associated with the earliest folding events have not been well characterized. Hence, in this study, PAC has been employed to obtain the magnitude of the kinetics, molar volume, and enthalpy changes associated with CO-Fe$^{2+}$Cc subsequent to CO photolysis. The folding studies were conducted under solution conditions thermodynamically favoring the folded conformation of Fe$^{2+}$Cc. The experiments described in this work utilize a denaturant, sodium dodecyl sulfate (SDS), together with CO as a photo-trigger to probe the fast refolding events of Fe$^{2+}$Cc occurring from a surfactant-induced partially unfolded state. Specifically, the complex environmental conditions are in the presence of ~0.74 mM SDS 50 mM HEPES pH ~7.5 buffer solution.
The data obtained from PAC along with TA have been used to construct a detailed thermodynamic profile for the early events that are most crucial in understanding how the protein adapt its native structure along the folding pathway. In addition, we have also discovered the possibility of heterogeneous kinetics involved in Cc folding by applying the PAC kinetic fits to a stretched exponential decay model.

### 3.2 Materials and Methods

Horse heart Cc and SDS were all purchased from Sigma Aldrich and were used without further purification.

#### 3.2.1 Fluorescence Measurements

Samples were prepared by diluting Cc in 2 mL of 50 mM HEPES buffer (pH ~7.5) to a final concentration of ~15 – 20 μM in a 1-cm quartz cuvette that was sealed with a septum cap and deaerated with argon (Ar) for ~15 min. Fe$^{3+}$ samples were reduced to Fe$^{2+}$ by adding freshly prepared sodium hydrosulfide from a concentrated stock solution (~100 mg of sodium hydrosulfide per mL in 50 mM HEPES buffer, pH~7.5) via a Hamilton syringe. Carbon monoxide (Airgas UltraHigh purity) was added by purging the Fe$^{2+}$ species under 1 atm constant pressure for ~1 min.

A 40 mM SDS stock solution was prepared in 50 mM HEPES buffer at pH ~7.5 and placed in a separate container (glassware-tube), sealed with a septum cap, and also deaerated with Ar for ~15 min. SDS titrations were carried out by sequentially adding small aliquots (5 – 20 μL increments) of the 40 mM SDS stock solution to the Fe$^{2+}$ Cc samples. Changes in emission
were observed in the spectral region of 295 nm – 525 nm (excitation at 280 nm) using an ISS PC1 (ISS. Inc., Champaign, IL) single-photon counting spectrofluorimeter.

### 3.2.2 Circular Dichroism Measurements

Fe$^{2+}$Cc samples were prepared by reducing Cc with excess sodium hydrosulfide. Removal of unreacted sodium hydrosulfide products was accomplished by application to a G-25 column eluted with 50 mM HEPES buffer pH 7.5. The reduced Cc sample was then diluted into ~0.74 mM SDS (50 mM HEPES buffer at pH ~7.5). CD spectra were recorded on Aviv Quick Start 215 CD Spectrometer® at 25 ºC using 1 mm quartz cuvette. Spectra were averaged over three scans (210 – 300 nm, 1 nm intervals, 1 nm bandwidth, 1 s response time) and background corrected.

### 3.2.3 PAC and TA Measurements

Carbon monoxide bound samples for TA and PAC measurements were prepared by dissolving Cc into ~0.74 mM SDS (50 mM HEPES buffer at pH ~7.5) to give a final concentration of ~15-20 μM into a 1-cm quartz cuvette, sealed with a septum cap and deaerated with Ar for ~15 min. Approximately 30 μL of the freshly prepared stock solution of sodium dithionite was added to reduce the samples followed by purging the sample with CO for ~1 min. The set up and analysis for PAC and TA is as described in Chapter 2.

### 3.3 Results and Discussion

Fluorescence measurements of Fe$^{2+}$Cc-SDS samples were conducted by taking advantage of a single Trp residue at position 59, which presents a sensitive probe for determining Cc
structural compactness.\(^{[1,4-6]}\) In the Cc native state, the observed emission of Trp59 is significantly quenched due to energy transfer with the heme. Unfolding of the complex results in an increase of the fluorescence quantum yield due to an increase in the distance between Trp59 and the heme.\(^{[1, 5-8]}\) As a result, the emission of Trp59 increase as the concentration of SDS was increased, indicative of denaturant-induced protein unfolding (data not shown). In addition, the fraction of unfolded protein versus SDS concentration (Figure 3.1, top) indicates that in the presence of CO, the equilibrium is shifted toward the unfolded state at lower concentrations of SDS. This result is consistent with previous studies that have shown that destabilization of Fe\(^{2+}\)-Met80 coordination occurs in the presence of denaturant (i.e. GdnHCl) and upon addition of CO binding to the heme.\(^{[1,7]}\) The data indicates that at ~0.74 mM SDS CO-Fe\(^{2+}\)Cc is approximately 20% unfolded. To confirm difference in the secondary structure of CO-bound and CO-free complexes, far-UV CD measurements (Figure 3.1, bottom) were conducted under the experimental conditions of 0.74 mM SDS. The data indicates that CO-Fe\(^{2+}\)Cc does not have the same 2° structure as the CO-free form. However, the CO-bound form still exhibit 2° characteristics thus confirming a partially folded complex. Based off the results from the fluorescence measurements, samples prepared for TA and PAC measurements were done in the presence of 0.74 mM SDS (50 mM HEPES buffer at pH ~7.5).
Figure 3.1: (Top) SDS-Induced equilibrium unfolding of Fe$^{2+}$Cc in the presence (red) and absence (black) of CO (monitored by Trp Fluorescence at ΔI 308 nm). The dotted line indicates the SDS concentration at which the presence of CO results in 20% of the protein being unfolded while in the CO-free form remains relatively folded. (Bottom) Far-UV CD spectra of Fe$^{2+}$Cc (red) and CO-Fe$^{2+}$Cc in 0.74 mM SDS (blue).

Figure 3.2 displays a kinetic difference spectrum of Fe$^{2+}$Cc following CO photolysis overlaid with the equilibrium difference spectra of Microperoxidase-11 (MP-11, a five-coordinate high-spin model system) and CO-Fe$^{2+}$Cc. The equilibrium difference spectra were constructed by subtracting deoxy Fe$^{2+}$MP-11 from CO-Fe$^{2+}$MP-11 and Fe$^{2+}$Cc minus CO-Fe$^{2+}$Cc. The equilibrium difference spectrum of CO-Fe$^{2+}$Cc exhibits a band centered at ~427 nm,
a minimum at ~413 nm and an isosbestic point at ~417 nm. The kinetic difference spectrum of Fe$^{2+}$Cc is distinct from the equilibrium difference spectrum and displays a maxima at ~433 nm, two minima ~407 nm and ~413 nm, and an isosbestic point ~417 nm (inset).

Examination of the single wavelength decays obtained at the absorbance maximum (427 nm), minimum (413 nm), and isosbestic point (417 nm) from the kinetic different spectra of CO-Fe$^{2+}$Cc (figure 3.3) reveal biphasic exponential kinetics: a fast lifetime of 537 µs and a slower lifetime of 1.5 ms. Thus, indicative of a fast and slow CO recombination to the Fe$^{2+}$ heme as similarly observed by Kumar et al.$^{[7]}$

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**Figure 3.2:** Transient and equilibrium difference spectra of different Fe$^{2+}$Cc species. (Red) Circles: equilibrium difference spectra of the steady-state spectra of CO-Fe$^{2+}$Cc subtracted from Fe$^{2+}$Cc. (Black) Squares: equilibrium difference spectra of CO-Fe$^{2+}$MP-11 in 0.74 mM SDS (50 mM HEPES buffer at pH ~7.5) subtracted from the steady-state spectra of deoxy MP-11 steady-state spectrum in 0.74 mM SDS (50 mM HEPES buffer, pH ~7.5) to mimic the five-coordinate (high-spin) model equilibrium difference spectra. Remaining spectra are kinetic difference spectra constructed from various single wavelengths transient absorption traces over time. Inset: shows the transient and steady-state spectra normalized to the same absorbance at 407 nm to better show the differences in shape between the transient and the steady-state difference spectrum.
Figure 3.3: (Left) Single wavelength transient absorption traces of CO-Fe$^{2+}$Cc-SDS obtained at 413 nm (red) (minimum in the kinetic difference spectrum) 417 nm (blue) (isosbestic point in the kinetic difference spectrum) both decreasing in absorbance with biphasic decays. (Right) Single wavelength transient absorption trace obtained at 427 nm (maximum in the kinetic difference spectrum).

Molar volume and enthalpy changes associated with the refolding processes subsequent to CO photo-dissociation from CO-Fe$^{2+}$Cc-SDS were measured using PAC. The frequency shift of the sample acoustic wave in comparison to the reference acoustic wave indicates that there are multiple kinetic processes occurring between ~20 ns to ~20 μs (Figure 3.4, left). Deconvolution of the acoustic waves was best fit to three kinetic processes associated with Fe$^{2+}$Cc folding (Figure 3.4, right). The prompt phase (<20 ns) has an observed Q of ~56 kcal mol$^{-1}$ with a β = 1, a second phase with a lifetime of 588 ns and an observed Q of 7 kcal mol$^{-1}$ with a β = 1, and, finally, a slow third phase that experienced heterogeneity with a β = 0.5, a lifetime of 3.9 μs, and an observed Q of ~35 kcal mol$^{-1}$.

In the presence of 0.74 mM SDS, the fraction of Fe$^{2+}$Cc unfolded was found to be ~20% versus the percentage (~80%) that is not CO bound in solution. Therefore the total heat released to the solvent (Q$_{\text{total}}$) for the prompt phase has two components that have to be taken into consideration. The first corresponds to the amount of heat released by the ~80% of Cc that remained intact (not CO-bound) and did not undergo any photolysis (Q$_{\text{intact}}$) and the second
associated with the amount of heat absorbed during the reaction of CO photolysis from the Cc complex ($Q_{CO}$).

$$Q_{total} = Q_{intact} + Q_{CO} \quad (3.0)$$

Because $Q_{intact}$ undergoes no net reaction and therefore deposits all of the absorbed energy as heat within the response time of the detector (<20 ns), $Q_{intact}$ can be expressed as the pulse energy ($E_{hu}$) scaled to the fraction of the incident light absorbed by the intact portion of Fe$^{2+}$Cc so that Equation 3.0 becomes

$$Q_{CO} = Q_{total} - f^{Abs} E_{hu} \quad (3.1).$$

Thus allowing for the calculation of $\Delta H$ for the prompt phase (as described in Chapter 2) where the observed Q is replaced with $Q_{CO}$ for the system. This yields the prompt phase (<20 ns) with an endothermic $\Delta H = 89 \pm 4$ kcal mol$^{-1}$ and is characterized with a negligible $\Delta V$ of $0.2 \pm 0.3$ mL mol$^{-1}$ after scaled to the quantum yield of CO photolysis ($\Phi_{CO-Cc} = 0.46$) and $\Delta V$ was calculated using Equation 2.9 (in Chapter 2). Following the prompt phase is a second and third kinetic phase (588 ns and 3.9 $\mu$s) with $\Delta H$ of $-7 \pm 3$ kcal mol$^{-1}$ and $35 \pm 8$ kcal mol$^{-1}$ that are also accompanied by volume changes of $2 \pm 5$ mL mol$^{-1}$ and $-5 \pm 1$ mL mol$^{-1}$, respectively.
**Figure 3.4:** (Left) Overlay of PAC traces for CO photo-dissociation of COFe$^{2+}$Cc frequency shifted (red) Calorimetric reference (black), three component fit of sample acoustic wave (blue) and the residuals (green). Protein concentration is ~ in 0.74 mM SDS at pH ~7.5. (Right) Plot of $\phi E_{hv}$ vs. $C_p/\beta$ for the three components associated with COFe$^{2+}$Cc photolysis.

From the temperature dependence study of PAC, the rate constants ($k_{obs}$) were obtained for temperatures in the range of 6 – 20 °C for the second and third kinetic phases. The calculated activation parameters for the ~600 ns and ~4 μs kinetic phases are $\Delta H^\ddagger = 1 \pm 4$ kcal mol$^{-1}$ / $13 \pm 8$ kcal mol$^{-1}$ and $\Delta S^\ddagger = -46 \pm 7$ cal mol$^{-1}$ K$^{-1}$ / $-18 \pm 14$ cal mol$^{-1}$ K$^{-1}$, respectively.

**Figure 3.5:** Erying plot of the (black) ~600 ns (Fe-Met) and (red) ~4 μs (Fe-His) ligation phases to Fe$^{2+}$Cc.
3.3.1 Prompt Phase

The prompt phase, $U \rightarrow U^* \rightarrow U'$ (See Figure 1.9, Chapter 1), is all of the integrated processes occurring faster than the instrument response of the PAC detector (<20 ns). Specifically, the PAC data was best fit to a single exponential decay ($\beta = 1$) function that gave rise to a negligible $\Delta V_{\text{obs}}$ of 0.2 mL mol$^{-1}$ (0.3 Å$^3$ molecule$^{-1}$) and a $\Delta H_{\text{obs}}$ of 89 kcal mol$^{-1}$. The observed $\Delta H$ and $\Delta V$ can be divided into contributions due to Fe-CO dissociation, heme spin state transition (resulting from a 6-coordinate low spin to 5-coordinate high spin heme), solvation of CO due to the ligand going into the bulk solvent, and any concomitant conformational changes occurring upon the protein refolding. Thus, the total changes in enthalpy and molar volume can be expressed as:

$$\Delta H_{\text{obs}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{Solv}} + \Delta H_{\text{Conf}}$$

(3.3)

$$\Delta V_{\text{obs}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{Solv}} + \Delta V_{\text{Conf}}$$

(3.4)

To account for the energetics associated with Fe-CO, LS-HS, and CO-Solv the Fe$^{2+}$ heme model system, MP-11, was utilized assuming the complex undergoes similar processes upon CO photolysis as the unfolded Fe$^{2+}$Cc system in the prompt phase. Measurements of CO-Fe$^{2+}$MP-11-SDS obtained from PAC under identical experimental conditions as the CO-Fe$^{2+}$Cc-SDS in this study (data not shown) yielded a $\Delta H_{\text{MP-11}} = -17$ kcal mol$^{-1}$ and $\Delta V_{\text{MP-11}} = 2.1$ ml mol$^{-1}$. By subtracting $\Delta V_{\text{obs}} - \Delta V_{\text{MP-11}}$ and $\Delta H_{\text{obs}} - \Delta H_{\text{MP-11}}$ results in the conformational component ($\Delta V_{\text{Conf}}/\Delta H_{\text{Conf}}$) that contributes to the earliest steps in the protein folding process.

The $\Delta H_{\text{Conf}}$ indicates that an additional endothermic process(es) of 106 kcal mol$^{-1}$ is taking place in <20 ns. The exact origin of $\Delta H_{\text{Conf}}$ is unclear. Interestingly, this process is uphill.
in energy by about ~100 kcal mol⁻¹, enthalpically that is an unfavorable process ($\Delta G = \Delta H - T\Delta S, \Delta G < 0$) hence $\Delta H_{\text{Conf}}$ is an entropically driven process. We propose that $\Delta H_{\text{Conf}}$ mainly results from reorganization of the protein (does not include desolvation of the system). One of these events may include the collapse phase of the partially folded complex. Previous studies have calculated the formation of compact conformational states in Cc (under various conditions) ranges ~5 – 40 kcal mol⁻¹[9-11] which may contribute to $\Delta H_{\text{Conf}}$. In addition, $\Delta H_{\text{Conf}}$ may arise from two possibilities. (1) The formation of one or more salt-bridge interactions subsequent to CO photo-dissociation (contribute energies of ~5 – 10 kcal mol⁻¹). (2) The SDS-induced partially folded state of Fe²⁺Cc may expose several hydrophobic residues around the heme cavity to the solvent, and in turn, the protonated residues causes electrostatic interactions (contributing energies of ~10 kcal mol⁻¹) between the distribution of negative head groups in SDS and the solvent. In addition, Lys residues around the exterior of the protein are likely interacting with the SDS molecules as well, also contributing to the electrostatic interaction. Interestingly,

For $\Delta V_{\text{Conf}}$ the remaining process occurring within the excitation is found to be ~1.9 mL mol⁻¹ (~3.2 Å³ molecule⁻¹). The small $\Delta V_{\text{Conf}}$ indicates that there is no large-scale protein collapse following CO photo-dissociation, which is consistent with the initial denatured state being a partially folded conformation. In addition, the CD spectra obtained (see Figure 3.1, bottom) indicates that the system exhibits 2º structures which can also be interpreted as a small $\Delta V_{\text{Conf}}$.

3.3.2 Slow Phases

The two additional phases in Fe²⁺Cc-SDS folding occurs after photo-dissociation of CO opens up the heme distal coordination site allowing different ligation states (native and non-
native contacts) with lifetimes of 588 ns and 3.9 μs. Kumar et al.[7] detected a 300 ns phase that was attributed to Met(80/65) binding as well as a 2.5 μs phase attributed to His (26/33) binding. A different group, also observed a 710 ns process using Transient Grating (TG) and TA (pH ~13), suggesting the lifetime occurred from an induced Fe-Met coordination.[3] In the current work, Fe\textsuperscript{2+}Cc in 0.74 mM SDS intermolecular binding is kinetically similar to these previous reported studies and most likely arises from also U’→ U’Met and U’→ U’His (See Figure 1.9, Chapter 1). Thus, the thermodynamics and volume changes obtained from PAC are associated with the two slower phases with ΔH\textsubscript{obs} of −7 kcal mol\textsuperscript{-1}/35 kcal mol\textsuperscript{-1} and ΔV\textsubscript{obs} of 2 mL mol\textsuperscript{-1}/−5 mL mol\textsuperscript{-1}, respectively.

The bond formation energies of Fe-Met and Fe-His have been reported as ΔH\textsubscript{Fe-Met} = 8 kcal mol\textsuperscript{-1}[12] and ΔH\textsubscript{Fe-His} = 23 kcal mol\textsuperscript{-1}[13]. Analysis of these ΔH\textsubscript{bond} values are taken into consideration with ΔH\textsubscript{obs} values from the current study (ΔH\textsubscript{obs} = ΔH\textsubscript{bond} + ΔH\textsubscript{struc}) resulting in an endothermic ΔH\textsubscript{struc} reaction of 15 kcal mol\textsuperscript{-1} for Met(80/65) and 12 kcal mol\textsuperscript{-1} for His(26/33). Interestingly, the ΔH\textsubscript{obs} = −7 kcal mol\textsuperscript{-1} for the U’→ U’Met ligation process was best fit to a single exponential decay (β = 1). This shows that the system undergoes a homogeneous process when Met is bound to the heme. However, the PAC kinetics associated with U’→ U’His ligation was best fit to a stretched exponential decay function (β = 0.5). This indicates that Fe\textsuperscript{2+}Cc-SDS folding also experiences a frustrated pathway in the folding funnel due to the non-native contact of His(26/33) binding to the heme iron, thus yielding a second population exhibiting heterogeneous relaxation upon folding. Some other sources that may contribute to the endothermic ΔH\textsubscript{struc} in both U’→ U’Met and U’→ U’His binding includes: (1) perturbations involving structural reorganization of the protein in order to accommodate the environment around the heme, (2) hydrophobic/electrostatic interaction due to the denaturant, (3) high-spin to
low-spin transitions to the heme and/or (4) redistribution of backbone dihedral angles in the protein.\textsuperscript{[14-19]}

Since the Fe\textsuperscript{2+}Cc-SDS complex is starting from a partially folded state that is compact in conformation, the protein is essentially reorganizing ligands around the heme. Therefore, it is likely that there are no large chain diffusions required to bind the ligands. Hence, the volume changes corresponding to the 588 ns and 3.9 μs phases are relatively small, 2 and –5 mL mol\textsuperscript{-1} (3.3 and –8.3 Å\textsuperscript{3} molecule\textsuperscript{-1}) resulting in the \( \Delta V_{\text{obs}} \) for both ligation processes (Met80/65 and His26/33) are mainly due to small scale reorganization of the complex upon refolding.

From temperature dependence measurements, activation parameters for the transition states associated with ligand binding of Fe-Met and Fe-His was found to be \( \Delta H^i = 1 \text{ kcal mol}^{-1} \)/\( \Delta S^i \) = \(-46 \text{ cal mol}^{-1} \text{ K}^{-1}\) and \( \Delta H^f = 13 \text{ kcal mol}^{-1} / \Delta S^i = -18 \text{ cal mol}^{-1} \text{ K}^{-1}\), respectively. The data shows that the Fe-Met coordination is barrierless and enthalpically downhill in energy. As a result, the folding process of Fe\textsuperscript{2+}Cc favors Met binding to the heme iron and occurs on a faster timescale. In the case of Fe-His coordination, the slower rate is likely due to the ligand binding having a higher activation barrier with a conformation that ends enthalpically uphill (Figure 3.6). This is potentially causing the heterogeneous kinetics displayed in PAC. Thus, results from the temperature dependence measurements indicate that Fe\textsuperscript{2+}Cc ligand binding process favors Met binding which is enthalpically driven to go the native process, however, His binding is entropically driven to a frustrated pathway.
Figure 3.6: Enthalpy profiles of Fe$^{2+}$Cc Fe-Met (Black) and Fe-His (Red) ligand binding process.

3.3.3 Transient Absorption Data

In the case of TA, photo-dissociation of CO leads to a rapid transient formation of a 5-coordinate high spin heme followed by an immediate reform to a 6-coordinate low spin heme. This is due to different ligands binding to the open distal coordination site. However, photolysis did not detect the three phases observed in PAC, but exhibited two additional slower phases with lifetimes of 537 $\mu$s and 1.5 ms. Both phases are associated with kinetic competition of CO rebinding to the heme.

The TA data provides a kinetic model for CO recombination in which the native-like conformation competes with CO. Specifically, if the native-like formation out competes CO recombination (U′ → N), thus rate-limiting, it is likely to occur on the slowest observed lifetime of 1.5 ms ($\tau_4$). However, if CO immediately rebinds to the heme and avoids the native-like formation (U′ → U), it is assigned to 537 $\mu$s ($\tau_3$) (See Figure 1.9, Chapter 1).
In summary, the results obtained in this study are kinetically similar to previous studies reported on Fe$^{2+}$ Cc in high pH and low concentrations of GdnHCl, suggesting a uniform mechanism for the early folding events regardless of the denaturant environment. Furthermore, the PAC data revealed three kinetic processes with the third slowest phase (~4 μs) yielding heterogeneous kinetics. This was assumed to only occur when a non-native His(26/33) bound to the Fe$^{2+}$ heme. The results also suggest that protein folding of Fe$^{2+}$ Cc-SDS is kinetically driven from a compact partially folded conformation. Overall, the PAC data obtain have allowed for a thermodynamic profile for the folding pathway summarized by figure 3.7.

![Figure 3.7](image-url)  
**Figure 3.7:** Thermodynamic profile for the enthalpy (red) and corresponding molar volume (blue) changes for CO-photolysis of Fe$^{2+}$Cc fast folding events in the presence of ~0.74 mM SDS with 50 mM HEPES buffer, pH ~7.5.

### 3.4 References


CHAPTER 4: FERROUS SYSTEM II
CO Photo-dissociation from Chloramine-T Modified Horse Heart Cytochrome-c

4.1 Background

The distal ligand, Met80, plays an essential role in modulating the proteins reduction potential as well as to provide stability to the overall tertiary structure. Previous studies have demonstrated that the Fe-Met80 can be replaced by intrinsic or extrinsic ligands under appropriate conditions.\cite{1} One approach to Met80 modification is via oxidation to a sulfoxide using N-chloro-4-toluosulfonamide (Chloramine-T, CT) which retains ligation to the heme iron via the sulfoxide oxygen.\cite{2} It has now been demonstrated that the ferrous form of CT-Cc binds carbon monoxide (CO) by displacing the weaker Fe(II)-O bond of the sulfoxide.\cite{2,3} Photorelease of CO allows for structural dynamics associated with the local distal pocket of the heme to be probed on fast timescales. Previous photolysis studies of CO-CT-Cc have led to the development of an overall mechanism for ligand recombination in which both the Met80-sulfoxide and CO ligand compete to rebind to the vacant 6th coordination site of the heme iron with lifetimes of 50 μs and 500 μs, respectively.\cite{3} The slow recombination rate of Met80-sulfoxide was attributed to electrostatic repulsion between the sulfoxide group and the high spin heme iron as well the breaking of a hydrogen bond formed between Tyr69 and the Met80-sulfoxide oxygen in the CO ligated form of the protein.\cite{3} The rate of Met80-sulfoxide recombination is noteworthy as results from Zhong\cite{4}, Kruglik\cite{5}, and Champion et al.\cite{6} indicate native Met80 rebinding to the heme iron occurs on ~ps timescales. The fast recombination rate
was suggested to be due to the Met80 ligand being part of the rigid protein backbone and, thus, not able to move far from the heme iron subsequent to photolysis.\cite{6}

In this study, PAC is used to probe the enthalpy and molar volume changes associated with distal pocket reorganization in Cc subsequent to CO photo-release from CO-CT-Cc. In addition, molecular dynamics (MD) simulations were performed in order to provide atomistic resolution to the events occurring subsequent to CO photo-release. The results from PAC reveal two dynamic events occurring prior to Met80-sulfoxide/CO ligand recombination, which MD indicates are related to ligand migration into hydrophobic cavities and heme pocket structural relaxations.

### 4.2 Materials and Methods

Horse heart Cc and CT were both purchased from Sigma and used without further purification. The changes in absorption were observed in the spectral region of 300 – 750 nm using a Shimadzu UV-2401 spectrophotometer.

#### 4.2.1 Preparation of CT-Cc

CT-Cc samples were prepared according to Bosshard et al.\cite{7} by reacting 1 mM of Fe$^{3+}$Cc with 5 mM CT in 100 mM Tris buffer, pH 8.45. Samples were incubated for ~5 hrs followed by application to a Bio-Rex 70 column equilibrated with 10 mM Tris buffer pH 8.45. The column was eluted with 10 mM NaCl in 10 mM Tris buffer (pH ~8.45), and then by 250 mM NaCl in 10 mM Tris buffer (pH 8.45). Two bands emerged from the column with the first being CT-Cc.
4.2.2 PAC Measurements

Samples for PAC studies were prepared by diluting CT-Cc into 1.5 mL of 10 mM Tris buffer (pH ~7.5) to a final concentration of ~10 – 15 μM in a 1 cm optical quartz cuvette, sealed with a septum cap, and deaerated with Ar for ~15 min. The Fe$^{3+}$ sample was reduced to Fe$^{2+}$ by adding freshly prepared sodium hydrosulfite from a concentrated stock solution (~100 mg of sodium hydrosulfite per mL in 10 mM Tris buffer, pH 7.45) via a Hamilton syringe. Carbon monoxide (Airgas UltraHigh purity) was added by purging the Fe$^{2+}$ sample under constant pressure (1 atm) for ~1 min. The set-up, theory and analysis for PAC have been described in Chapter 2.

4.2.3 Molecular Dynamics Simulations

The structure of Cc was exported from the protein data bank[8] ID code 1HRC.[9] Visual Molecular Dynamics (VMD) was used to prepare and visualize the protein.[10] Molecular dynamics simulations were performed (see Preface) using NAnoscale Molecular Dynamics (NAMD) Software.[11] The 1HRC structure was modified to add distal CO to the heme (CO-Cc). CT-Cc was prepared by modifying Met80 to Met80-sulfoxide CT-CC by removing the two methyl groups from Met80 and adding oxygen. Distal CO was added to the heme, yielding CO-CT-Cc. The protein, heme and CO parameters were obtained from the standard CHARMM22 force field[12] and DMSO parameters from the CHARMM36 force field[13]. The charges that were adjusted from the Met residue parameters were as follows: O (-0.55), S (0.22), CG (-0.02). An explicit solvent TIP3P water box with 10 Å padding was used to solvate the charge-neutral domain of the protein.[14] The water box was then neutralized with explicit KCl. The water molecules were treated as rigid using the SETTLE algorithm.[15] Six different 300 ns MD
simulations were computed at 298K. These simulations were setup by first minimizing the potential energy of the aqueous protein by conjugate gradient. This was followed by heating using velocity reassignment from 0K to 298K with a step size of 0.001K and equilibration by velocity rescaling at constant temperature and pressure (NPT) for 400 ps.

The results for analysis were computed using Langevin dynamics at a constant temperature of 298K and a pressure of 1 atm (NPT) for 300 ns with a 2 fs time-step.\[16\] Periodic boundary conditions were used, with a cutoff of 10.0 Å for the short-range interactions, approximated as Lennard-Jones interactions.\[17\] The particle mesh Ewald sum method was used for the computation of the long-range electrostatic interactions, with 64 grid points in each direction, giving a grid spacing of 0.8 Å.\[18, 19\] Structures were stored during the productions runs at intervals of 5 ps for analysis. After equilibration of CO-CT-Cc, the bond between CO and Fe was broken and allowed to relax during production runs.

4.3 Results and Discussion

Figure 4.1 displays the optical absorption spectrum of the Fe\(^{2+}\) forms of Cc, CT-Cc, and CO bound CT-Cc in 10 mM Tris buffer solution (pH 7.45). The Q-bands of CT-Cc are similar to native Cc and are observed in the visible region centered at 548 nm (\(\alpha\)-band) and 518 nm (\(\beta\)-band). However, the Soret band for the CT-Cc is distinct from that of Cc exhibiting a ~4 nm bathochromic shift with a Soret maximum at 418 nm. The positions of the CT-Cc absorption bands are characteristic of a 6-coordinate low spin heme. Addition of CO to the ferrous CT-Cc results in a ~5 nm hypsochromic shift of the Soret band to 413 nm and a single broad Q-band characteristic of CO binding to Fe\(^{2+}\) heme.\[2\]
Figure 4.1: Optical absorption spectra of Fe$^{2+}$Cc (black), Fe$^{2+}$CT-Cc (red), and CO bound Fe$^{2+}$CT-Cc complex (blue). Protein concentration is ~10 µM in 10 mM Tris buffer, pH ~7.45.

Figure 4.2 displays an overlay of PAC traces for the photolysis of CO-CT-Cc and the calorimetric reference. The frequency shift of the sample acoustic trace relative to the reference acoustic trace indicates multiple kinetic processes occurring between ~20 ns to ~20 µs. Deconvolution of the PAC traces resulted into two kinetic processes being resolved: a prompt phase occurring with a lifetime faster than the time resolution of the PAC instrument (<20 ns) and a second phase with a lifetime of 534 ns.

Figure 4.2: (Left) Overlay of PAC traces for the CO photolysis of CO-CT-Cc frequency shifted (red), calorimetric reference (black), two component fit of the sample acoustic wave (blue) and the residuals (green). Protein concentration is ~10 µM in 10 mM Tris buffer, pH ~7.45. (Right) Plot of $\phi E_{hv}$ vs. $\text{Cp} / \beta$ for the two components associated with CO-CT-Cc photolysis.
4.3.1 Prompt Phase

In the case of the prompt phase, photo-dissociation of CO from CO-CT-Cc is associated with a $\Delta H$ of $53 \pm 2$ kcal mol$^{-1}$ and a $\Delta V$ of $-4.7 \pm 0.4$ mL mol$^{-1}$ occurring in <20 ns. The thermodynamic parameters for the prompt phase were scaled to $\Phi_{\text{CO-CT-Cc}} = 0.17$. The observed $\Delta H$ and $\Delta V$ can be divided into contributions due to Fe-CO bond dissociation, heme spin-state transition (resulting from a 6-coordinate low spin to 5-coordinated high spin heme), and concomitant protein conformational changes occurring within the heme distal pocket. Thus, the total changes in enthalpy and molar volume for this process can be expressed as:

$$\Delta H_{\text{obs}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{Conf}}$$

(4.0)

$$\Delta V_{\text{obs}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{Conf}}$$

(4.1).

From previous studies of Fe$^{2+}$ model systems,$^{[20-22]}$ photo-dissociation of the Fe-CO bond is associated with a $\Delta H_{\text{Fe-CO}} = \sim 17$ kcal mol$^{-1}$ and $\Delta H_{\text{LS-HS}} \sim 0$ kcal mol$^{-1}$, giving a $\Delta H_{\text{Conf}}$ of $\sim 36$ kcal mol$^{-1}$.

The $\Delta H_{\text{Conf}}$ likely arises from a conformational response of the protein due to CO release from the heme consistent with the MD simulations that demonstrate a reorganization of the distal pocket subsequent to release of the CO ligand. Initially, CO migrates $\sim 5.3$ Å from the heme into a hydrophobic pocket (Figure 4.3, left) formed by residues Tyr67, Leu68, Pro71, and Phe82 (Figure 4.3, right). The pocket is formed by the movement of Phe82 $\sim 4$ Å allowing for the formation of a $\pi$-stacking interaction with the heme and additional hydrophobic interactions between Leu68 and Pro71 (Figure 4.4). Furthermore, Leu64 shifts $\sim 2.5$ Å and both Leu98 and
Ile95 shift ~1.3 Å, all towards the back of the pocket, allowing CO to migrate further into the interior of the protein (Figure 4.4).

In the case of $\Delta V_{\text{obs}}$, $\Delta V_{\text{LS- HS}}$ and $\Delta V_{\text{Fe-CO}}$ are likely to be negligible for a heme constrained within a protein pocket. Therefore, $\Delta V_{\text{Conf}} \sim -5$ mL mol$^{-1}$ ($-8$ Å$^3$ molecule$^{-1}$) is due to local conformational changes surrounding the distal pocket and possibly electrostriction involving the heme propionates and peripheral amino acids at the surface of the protein. The structures highlighted in Figure 4.4 suggest movement of the propionates leading to greater solvent exposure which would increase the electrostriction resulting in the observed volume decrease.

Figure 4.3: MD structure of CO-CT-Cc illustrating (Left) the first hydrophobic pocket surrounding the CO ligand following dissociation from the heme and (Right) the residues composing the hydrophobic site.
Figure 4.4: MD structures of CO-CT-Cc highlighting the distal residues surrounding the CO ligand before (Red) and after (Blue) dissociation from the heme and subsequent migration into the first hydrophobic pocket.

4.3.2 Slow Phase

The slow phase has a lifetime of 534 ns (at 20 °C) with a corresponding $\Delta H$ of 8 kcal mol$^{-1}$ and a $\Delta V$ of 1.3 mL mol$^{-1}$. The activation enthalpy is also found to be 4 kcal mol$^{-1}$ (obtained from temperature dependent rate constants extracted from the PAC fits, Figure 4.6). In this case the $\Delta H$ and $\Delta V$ are associated with a conformational transition that occurs subsequent to the transfer of CO into the initial hydrophobic binding pocket. The MD simulations on longer timescales indicate CO migrates further into the distal pocket (~8 Å from the heme) and becomes trapped in a second hydrophobic site located within the interior of the protein. The residues involved are Leu64, Tyr67, Leu68, Ile95, Leu98 and the Met80-sulfoxide/Tyr$^{67}$ hydrogen bond (~2 Å) remained intact (Figure 4.5). The simulations further indicate that the residues involved in the formation of the initial docking site begin to transition back to the front of the pocket (Figure
4.7). Interestingly, one of the residues involved in the secondary hydrophobic pocket, Ile$^{95}$, is positioned in a different orientation in which it is rotated ~90°, potentially blocking CO exit from the protein (Figure 4.7). The relatively small value of $\Delta V_{\text{obs}}$ (1.3 mL mol$^{-1}$ or 2.2 Å$^3$ molecule$^{-1}$) is consistent with the migration of CO between internal cavities within the protein matrix.

![Figure 4.5: MD structure of CO-CT-Cc illustrating (Left) the secondary hydrophobic pocket surrounding the CO ligand following dissociation from the heme and (Right) the residues making up the hydrophobic site.](image)

**Figure 4.5:** MD structure of CO-CT-Cc illustrating (Left) the secondary hydrophobic pocket surrounding the CO ligand following dissociation from the heme and (Right) the residues making up the hydrophobic site.

![Figure 4.6: Erying plot of the ~530 ns CO migration phase occurring in Fe$^{2+}$ CT-Cc.](image)

**Figure 4.6:** Erying plot of the ~530 ns CO migration phase occurring in Fe$^{2+}$ CT-Cc.
Figure 4.7: MD structures of CO-CT-Cc highlighting the distal residues surrounding the CO ligand from the first hydrophobic pocket (blue) into a secondary cavity (gray).

4.4 Comparison with CO-Fe$^{2+}$Mb, CO-Fe$^{2+}$HR, CO-Fe$^{2+}$Cm-Cc

Previous studies of CO photo-dissociation from ferrous Myoglobin (Mb) using PAC demonstrated multiple conformational relaxations depending upon the origin of the protein.$^{[23-27]}$ In the case of horse heart Mb, photo-cleavage of the Fe-CO bond leads to the prompt ($\tau < 20$ ns) formation of a transient deoxyMb intermediate with the CO molecule trapped near the distal pocket with a negligible volume change and a $\Delta H$ $10 - 15$ kcal mol$^{-1}$ close to that of the Fe-CO bond energy. This prompt phase is followed by a relaxation with a lifetime of $\approx 80$ ns with a $\Delta V = \approx - 3$ mL mol$^{-1}$ and $\Delta H = - 3$ kcal mol$^{-1}$ that has been attributed to the migration of CO from the primary docking site to the internal Xe cavities or ligand exchange between individual Xe
cavities. Ligand exchange between the protein matrix and the surrounding solvent occurs with the lifetime 700 ns (at 20 °C) with a ΔV of −14 mL mol⁻¹ and ΔH of −9 kcal mol⁻¹. High resolution structures of deoxyMb indicate a water molecule is hydrogen bounded to the Ne atom of His with an occupancy of ~0.8 and the rate of water entry into the Mb distal pocket was proposed to be limited by the escape of the photo-dissociated CO molecule. Therefore, the 700 ns phase includes CO escape from the protein matrix and concomitant binding of a water molecule. The analysis of Mb crystal structures reveal that His64 is part of a hydrogen bond network of His64 –H₂O – Lys45 – 6-propionate whereas His93 participates in the proximal hydrogen bond network of 7-propionate-Ser92 – His93.

In the case of HRP, previous PAC studies reported a single kinetic event occurring <50 ns following CO dissociation with a ΔH of 16 kcal mol⁻¹ and ΔV of 8 mL mol⁻¹. The data supported a mechanism through which CO migrates out of the distal pocket into the bulk solvent and is consistent with a water molecule entering the distal cavity subsequent to CO dissociation. The authors proposed that the rapid escape of CO (occurring <50 ns) was consistent with HRP having a direct channel linking the heme group to the bulk solvent.

Interestingly, ligand photolysis studies by Silkstone et al. on carboxymethylated Cc (Cm-Cc) reported that Cm-Cc did not possess any sequestering cavities through which to facilitate ligand escape to the bulk solvent. This was evident by the high rate of geminate CO recombination and little to no recombination on longer timescales suggesting that heme pocket reorganization is quite distinct between CT-Cc and Cm-Cc.

In summary, the PAC data associated CO photo-release from CO-CT-Cc revealed two kinetic processes (<20 ns and 534 ns) occurring prior to Met80-sulfoxide/CO rebinding to the heme iron. The prompt phase (<20 ns) thermodynamics are consistent with Fe-CO dissociation,
heme spin transition, and conformational changes, which in this case included the formation of a hydrophobic CO docking site. It is proposed that the second kinetic phase (~530 ns) involves CO migration ~8 Å from the heme and becoming trapped in a secondary hydrophobic site. The MD simulations also revealed that the Ile95 90° likely assists in preventing CO from leaving the pocket. In addition, the simulations also confirmed that the Met80-sulfoxide oxygen has formed a hydrogen bond between Tyr69 thus preventing immediate recombination to the heme iron. The mechanism based upon the results from PAC and the previous TA studies for ligand recombination[3] are summarized in Figure 4.8.

\[
\begin{align*}
\text{Prompt phase (} & \tau_1 < 20 \text{ ns)} \\
\Delta H &= 52 \pm 2 \text{ kcal mol}^{-1} \\
\Delta V &= -4.7 \pm 0.4 \text{ mL mol}^{-1}
\end{align*}
\]

\[
\begin{align*}
\text{hv} & \\
\text{His-Fe}^{3+}.\text{CO} & \rightarrow [\text{His-Fe}^{2+}.\text{U}] & \rightarrow [\text{His-Fe}^{3+}]^* \\
\tau_2 &= 534 \text{ ns} \\
\Delta H &= 8 \pm 3 \text{ kcal mol}^{-1} \\
\Delta V &= 1.3 \pm 0.5 \text{ mL mol}^{-1}
\end{align*}
\]

\[
\begin{align*}
\text{(} & \tau \sim 50 \text{ ns)} \\
\text{His-Fe}^{3+}.\text{MetS}=\text{O} \\
\text{(} & \tau \sim 500 \text{ ns)} \\
\text{His-Fe}^{3+}.\text{CO} \quad \text{(} & \tau \sim 17 \text{ ms)}
\end{align*}
\]

**Figure 4.8:** Overall mechanism for ligand rebinding occurring in CO-Fe\(^{2+}\)CT-Cc with data obtained from PAC highlighted and the relatively slow kinetic phases obtained from previous TA studies found in Reference 3.

### 4.5 References


CHAPTER 5: FERROUS SYSTEM III
Surfactant-Induced Protein Folding in Ferrous Chloramine-T Modified Horse Heart
Cytochrome-c

5.1 Background

Thus far, ferrous Cc has been the system chosen to elucidate the mechanism of folding. Particularly, the kinetics, enthalpy, and molar volume changes associated with the fast folding of horse heart Fe$^{2+}$Cc have been obtained (in Chapter 3) via PAC under two different conditions. These studies revealed that fast coordination of native and non-native residues resulted in two folding populations. One included a ‘frustrated’ folding pathway due to non-native binding of His to the heme (exhibiting heterogeneous kinetics) and the second involved Met80 (or Met65) allowing for the native-like conformation to ensue. These early events occurred on timescales of less than 5 μs.

Here Fe$^{2+}$CT-Cc folding process is also evaluated, thus providing more details on the energetic barriers involved with early folding events. Chloramine-T-Cytochrome-c (in which the native Met80 sulfur atom has been oxidized to a sulfoxide) folding mechanism has not been examined. However, previous studies on CO-Fe$^{2+}$CT-Cc ligand recombination upon CO photolysis$^{[1]}$ (discussed in Chapter 4), have demonstrated that in the absence of denaturant the Met80–sulfoxide recombination to the heme iron occurs on relatively slow timescales (~50 μs) due to the sulfoxide, electrostatic repulsion, and the breakage of a hydrogen bond formed between Tyr69/Met80-sulfoxide oxygen.$^{[1]}$ In the folding mechanism of native Cc, the Fe$^{2+}$-Met80 coordination is obviously an important obligatory step in the early stages of folding that
leads to the native $3^{\circ}$ structure. Hence, an important question is: does modification of Cc Met80 ligand via oxidation alter this folding mechanism as well as the thermodynamics?

To investigate the effects of the folding lifetimes as well as the magnitudes of enthalpy and molar volume changes associated with CO-Fe$^{2+}$CT-Cc, PAC along with TA has been employed to probe the folding process. The experiments described in this work utilize SDS as a denaturant under conditions allowing for refolding of the native-like conformation from a surfactant-induced partially unfolded state. Specifically, the experiments investigated here were conducted in the presence of ~0.2 mM SDS in 50 mM HEPES (pH ~7.5) buffer solution. The data obtained from PAC have been used to construct a detailed thermodynamic profile for the early folding events occurring in the reaction pathway and, to our knowledge, is the first time the kinetics, energetics and molar volume changes associated with Fe$^{2+}$CT-Cc folding have been measured.

5.2 Materials and Methods

Horse heart Cc and CT were both purchased from Sigma and used without further purification. The changes in absorption were observed in the spectral region of 300 – 750 nm using a Shimadzu UV-2401 spectrophotometer.

5.2.1 Preparation of CT-Cc

CT-Cc samples were prepared according to Bosshard et al.[2] and as previously discussed in Chapter 4.

5.2.2 Fluorescence Measurements

Samples were prepared by diluting CT-Cc in 2 mL of 50 mM HEPES buffer (pH ~7.5) to a final concentration of ~15 μM in a 1-cm quartz cuvette, sealed with a septum cap, and
deaerated with Ar for ~15 min. Fe$^{3+}$ samples were reduced to Fe$^{2+}$ by adding freshly prepared sodium hydrosulfide from a concentrated stock solution (~100 mg of sodium hydrosulfide per mL in 50 mM HEPES buffer, pH~7.5) via a Hamilton syringe. Carbon monoxide (Airgas UltraHigh purity) was added by purging the Fe$^{2+}$ species under 1 atm constant pressure for ~1 min.

A 40 mM SDS stock solution was prepared in 50 mM HEPES buffer at pH ~7.5 and placed in a separate container (glassware-tube), sealed with a septum cap, and also deaerated with Ar for ~15 min. SDS titrations were carried out by sequentially adding small aliquots (5 – 20 µL increments) of the 40 mM SDS stock solution to the Fe$^{2+}$CT-Cc samples. Changes in emission were observed in the spectral region of 295 nm – 525 nm (excitation at 280 nm) using an ISS PC1 (ISS. Inc., Champaign, IL) single-photon counting spectrofluorimeter.

5.2.3 Circular Dichroism Measurements

Fe$^{2+}$CT-Cc samples were prepared by reducing the modified sample with excess sodium hydrosulfide. Removal of unreacted sodium hydrosulfide and oxidation products was accomplished by application to a G-25 column eluted with 50 mM HEPES buffer pH 7.5. The reduced CT-Cc sample was then dissolved into ~0.2 mM SDS (50 mM HEPES buffer at pH ~7.5). CD spectra were recorded on Aviv Quick Start 215 CD Spectrometer® at 25 ºC using 1 mm quartz cuvette. Spectra were averaged over three scans (210 – 300 nm, 1 nm intervals, 1 nm bandwidth, 1 s response time) and background corrected.
5.2.4 PAC and TA Measurements

Carbon monoxide bound samples for TA and PAC studies were prepared by dissolving CT-Cc into ~0.2 mM SDS (50 mM HEPES buffer, pH ~7.5) to give a final concentration of ~15 µM into a 1-cm quartz cuvette, sealed with a septum cap, and deaerated with Ar for ~15 min. Approximately 30 µL of freshly prepared stock solution of sodium dithionite was added to reduce the samples followed by purging the sample with CO for ~1 min. The set up and analysis for PAC and TA is as described in Chapter 2.

5.3 Results and Discussion

Figure 5.1 displays an optical absorption spectra of Fe\(^{2+}\)CT-Cc and CO-Fe\(^{2+}\)CT-Cc in the presence of ~0.2 mM SDS, 50 mM HEPES buffer solution (pH ~7.5). The Soret band of Fe\(^{2+}\)CT-Cc exhibits a maximum at 417 nm and the Q-bands are observed in the visible region centered at 548 nm (α-band) and 518 nm (β-band). The positions of the Fe\(^{2+}\)CT-Cc absorption bands are characteristic of a 6-coordinate low spin heme. Addition of CO to the Fe\(^{2+}\)CT-Cc results in a ~3 nm hypsochromic shift of the Soret band to 414 nm and a single broad Q-band, characteristic of CO binding to Fe\(^{2+}\) heme.[3]

Fluorescence measurements of Fe\(^{2+}\)CT-Cc-SDS were conducted by taking advantage of Trp59 energy transfer with the heme. The distance between the residue and heme presents a sensitive probe for determining Cc structural compactness.[4-7] The data indicates that at ~0.2 mM SDS, CO-Fe\(^{2+}\)CT-Cc is approximately 25% unfolded while the CO free form is relatively folded (Figure 5.2, left). Details of the unfolded structure or the unfolding sequence are not known for the CT-Cc derivative. However, it is likely that SDS binds to the ferrous system and results in a partially folded complex. To confirm differences in the secondary structure of CO-
bound versus CO-free complex, far-UV CD measurements (Figure 5.1, right) were conducted on CO-Fe$^{2+}$CT-Cc and Fe$^{2+}$CT-Cc in the presence of 0.2 mM SDS. The data shows that there are differences in the conformation. Specifically, CO-Fe$^{2+}$CT-Cc which is in the initial equilibrium state exhibits 2° structure characteristics, thus in a compact partially folded state. Based on the results obtained from fluorescence measurements, samples for both TA and PAC measurements were carried out in the presence of ~0.2 mM SDS (50 mM HEPES buffer, pH ~7.5).

**Figure 5.1:** Optical absorption spectra of CO-Fe$^{2+}$CT-Cc (red) and Fe$^{2+}$CT-Cc (black) in 0.2 mM SDS (pH~7). Inset displays the visible region.

**Figure 5.2:** (Left) SDS-Induced equilibrium unfolding of Fe$^{2+}$CT-Cc in the presence (red) and absence (black) of CO (monitored by Trp Fluorescence at ΔI ~300 nm). The dotted line indicates the SDS concentration at which the presence of CO results in 25% of the protein being unfolded while in the CO-free form remains relatively folded. (Right) Far-UV CD spectra of Fe$^{2+}$CT-Cc (red) and CO-Fe$^{2+}$CT-Cc in 0.2 mM SDS (blue).
The PAC data displays a frequency shift in the sample acoustic wave relative to the reference acoustic wave (see Figure 5.3). Deconvolution of the PAC traces resulted in three kinetic processes being resolved. Specifically, a prompt phase occurring with a lifetime faster than the resolution of the PAC detector (<20 ns), a second phase with a lifetime of 826 ns and a third phase with a lifetime of 7.4 µs have been extracted.

![Figure 5.3](image)

**Figure 5.3:** (Left) Overlay of PAC traces for the CO photolysis of CO-Fe^{2+}CT-Cc frequency shifted (red), calorimetric reference (black), three fit component of the sample acoustic wave (blue), and the residuals (green). Protein concentration is ~15 µM in 0.2 mM SDS in 50 mM HEPES buffer, pH ~7.5. (Right) Plot of \( \phi_{E_{hv}} \) vs. Cp/\( \beta \) for the three fit components associated with CO-Fe^{2+}CT-Cc photolysis.

### 5.3.1 Prompt Phase:

In the presence of 0.2 mM SDS, the fraction of unfolded Fe^{2+}CT-Cc was found to be ~25% and, therefore, the total heat released \( (Q_{\text{total}}) \) to the solvent associated with the prompt phase has two components. The first corresponds to the amount of heat released by ~75% of CT-Cc that remained folded (not CO-bound), and thus does not undergo any folding processes. The second component is associated with the amount of heat that initiates the reaction, CO photolysis from unfolded CT-Cc. In this case, \( \Delta H \) can be calculated as per equation 3.1 in Chapter 3. The prompt phase (<20 ns) was best fit to a single exponential decay (\( \beta = 1 \)) function with an...
endothermic $\Delta H = 91 \pm 4$ kcal mol$^{-1}$ and is accompanied by a $\Delta V$ of $5 \pm 1$ mL mol$^{-1}$ after scaling to the quantum yield of CO photolysis ($\Phi_{\text{CO-CT-Cc}} = 0.21$).

The observed $\Delta H$ and $\Delta V$ can be divided into contributions as discussed in Chapter 3 (see Equations 3.3 and 3.4) and gives rise to a $\Delta H_{\text{Conf}}/\Delta V_{\text{Conf}}$ of 108 kcal mol$^{-1}$ and 3 mL mol$^{-1}$ respectively. The exact origin of $\Delta H_{\text{Conf}}$ (108 kcal mol$^{-1}$) is unclear. In the case of native Fe$^{2+}$Cc folding, Kumar et al.$^8$ indicated that following CO photo-release a hydrophobic collapse (burst phase) occurs prior to coordination of Met/His ligands to the Fe$^{2+}$ heme. In Chapter 3, a model was proposed for native Cc-SDS fast folding in which the prompt phase included this burst phase from a partially folded conformation. In addition, $\Delta H_{\text{Conf}}$ for native Cc-SDS fast phase (106 kcal mol$^{-1}$) was also suggested to result from the exposure of hydrophobic residues around the heme cavity to the solvent, Lys residues patch interacting with SDS molecules, as well as the formation of one or more salt-bridge interactions subsequent to CO photo-dissociation. In the current study, this is likely the case for Fe$^{2+}$CT-Cc-SDS fast phase in which the estimated $\Delta H_{\text{Conf}}$ exhibits a similar thermodynamic value (106 kcal mol$^{-1}$ versus 108 kcal mol$^{-1}$, respectively). The similarities in enthalpy between Cc-SDS and CT-Cc-SDS suggest that the SDS molecules binding to the protein has the greatest contribution to $\Delta H_{\text{Conf}}$. Studies have indicated that submicellar concentrations (surfactant is less than the critical micelle concentration (CMC)) predominately consist of monomers in solution and interactions mainly consist of electrostatic contributions between the negatively charged head groups of SDS and the solvent expose hydrophobic sites.$^9$ For $\Delta V_{\text{Conf}}$, the remaining process occurring within the excitation is found to be 3 mL mol$^{-1}$ ($\sim$5 Å$^3$ molecule$^{-1}$). The small $\Delta V_{\text{Conf}}$ indicates that there is no large-scale protein collapse following CO photo-dissociation, consistent with the initial denatured state being a compact MG conformation.
5.3.2 Slow Phases:

The PAC data has revealed two additional kinetic phases in Fe$^{2+}$CT-Cc-SDS folding that occur subsequent to photo-dissociation of CO. Both processes were best fit to a single exponential decay ($\beta = 1$) with an $\Delta H_{\text{obs}}$ of $-3 \pm 2$ kcal mol$^{-1}$/\Delta V_{\text{obs}}$ of $1 \pm 0.5$ mL mol$^{-1}$ (~830 ns phase) followed by a $\Delta H_{\text{obs}}$ of $-17 \pm 11$ kcal mol$^{-1}$/\Delta V_{\text{obs}}$ of $7 \pm 1$ mL mol$^{-1}$ (~7 $\mu$s phase). In native Fe$^{2+}$Cc-SDS studies (Chapter 3), the two slower rates were the result of ligand competition between phases $U' \rightarrow U'\text{Met}$ and $U' \rightarrow U'\text{His}$ (See Figure 1.9, Chapter 1) as previously reported.[4,8] This appears to be the case for Fe$^{2+}$CT-Cc-SDS with the exception of Met-sulfoxide (Met-S=O) ligands being in competition with His residues for coordination to the Fe$^{2+}$ heme. In the absence of denaturant, the rate of Met-S=O rebinding was controlled by hydrogen binding within a cavity of the distal pocket. Most likely this cavity does not exist when the system is unfolded but the recombination rate of the S=O ligand to the heme is affected regardless. This was shown in a previous study conducted by Larsen et al.[10] In an effort to understand ligand binding dynamics of Fe$^{2+}$-S=O, the authors conducted a study on dimethylsulfoxide (DMSO), which ligates to the Fe$^{2+}$ heme iron via the sulfoxide oxygen.[3,10] The rebinding of DMSO to the Fe$^{2+}$ porphyrin model system, Fe$^{2+}$PPIX, exhibited a recombination rate of ~500 ns.[10] The authors attributed the relatively slow rate to strong dipole interactions and steric affects between the S=O ligand and high-spin heme.[10] Hence, in the case of Fe$^{2+}$CT-Cc-SDS refolding, the ~830 ns phase is likely due to Met-S=O binding to the heme while the ~7 $\mu$s phase is assigned to His binding. The difference in kinetics between the two systems (Fe$^{2+}$PPIX-DMSO vs. Fe$^{2+}$CT-Cc) is likely due to CT-Cc having a protein covalently attached to the heme. However in the case of the ~7 $\mu$s phase assigned to His binding, previous studies have found that non-native His ligation impedes the folding process as a result of an
accumulation of kinetic barriers (intermediates), which frustrates folding.\[^{6,11}\] Ultimately, these kinetic barriers causes traps that slow down the folding process and likely the case for His binding to Fe\(^{2+}\)CT-Cc-SDS.

The bond formation energies of Fe-Met-S=O and Fe-His have been reported as $\Delta H_{FeS=O} = -6$ kcal mol\(^{-1}\)\[^{10}\] and $\Delta H_{Fe-His} = 23$ kcal mol\(^{-1}\)\[^{12}\], respectively. Analysis of these $\Delta H_{bond}$ values are taken into consideration with $\Delta H_{obs}$ values from the current study resulting in an exothermic $\Delta H_{struc}$ reaction of $-9$ kcal mol\(^{-1}\) for Met-S=O(80/65) and $-40$ kcal mol\(^{-1}\) for His(26/33). Interestingly, unlike the native form (discussed in Chapter 3) both ligation processes were best fit to a single exponential decay ($\beta = 1$). Other sources that may contribute to the exothermic $\Delta H_{struc}$ in both $U'\to U'Met-S=O$ and $U'\to U'His$ binding include: (1) perturbations involving structural reorganization of the protein, (2) hydrophobic/electrostatic interaction due to the denaturant, (3) high-spin to low-spin transitions of the heme and/or (4) redistribution of backbone dihedral angles in the protein.\[^{11-17}\]

Since the Fe\(^{2+}\)Cc-SDS complex is likely starting from a compact MG state, the protein is essentially reorganizing ligands around, known as the "ligand exchange phase\[^{18}\]". Therefore, it is likely that there is no large scale chain diffusions required between binding the ligands. Hence, the volume changes corresponding to the 826 ns and 7.4 $\mu$s ligation processes with corresponding $\Delta V_{obs}$ of 1 and 7 mL mol\(^{-1}\) (1.7 and 12 Å\(^3\) molecule\(^{-1}\)), respectively, are relatively small compared to Cc (~144000 Å\(^3\) molecule\(^{-1}\)) overall volume dimensions. Thus $\Delta V_{obs}$ is mainly due to small scale reorganization of the complex upon ligand binding.

Temperature dependence measurements from PAC allowed for the activation parameters associated with the ligand binding of Fe-Met-S=O (~830 ns phase) and Fe-His (~7 $\mu$s phase) to be calculated (Figure 5.4, left). The activation energies for ligand association were found to be
ΔH‡ = 7 ± 1 kcal mol⁻¹ / ΔS‡ = -56 ± 2 cal mol⁻¹ K⁻¹ and ΔH‡ = -3 ± 2 kcal mol⁻¹ / ΔS‡ = -36 ± 2 cal mol⁻¹ K⁻¹, respectively. The data shows that the Fe²⁺-His coordination is barrierless. As a result, the folding process of Fe²⁺ CT-Cc seems to favor His binding to the heme iron. Coordination of non-native His is assumed to create a kinetic barrier during protein folding in which facilitates a native-like intermediate.⁶,¹⁴ In the case of Fe²⁺-Met-S=O coordination, the data shows that Met-S=O binding to the heme has a higher activation barrier with a conformation that ends enthalpically downhill (Figure 5.4, right). The activation energy reported for recombination of Fe²⁺-DMSO largest contribution (the activation barrier for Fe²⁺-S=O binding) was from solvent interactions, constituting ~90% to the total activation energy. In addition, electrostatic contributions came from the large dipole moment (4.11 D) of the system but only contributed ~25% to the total activation energy.¹⁰

Figure 5.4: (Left) Erying plot of the (black) ~830 ns (Fe-Met-S=O) and (red) ~7 μs (Fe-His) ligation phases to Fe²⁺ CT-Cc. (Right) Enthalpy profile of Fe²⁺ CT-Cc, Fe-Met-S=O (Black) and Fe-His (Red) ligand binding process.

Figure 5.5 displays kinetic difference spectrum of CO-Fe²⁺ CT-Cc subsequent to CO photolysis and the equilibrium difference spectra of CO-Fe²⁺ CT-Cc and CO-Fe²⁺ MP-11. CT-Cc transient difference spectra displays a minimum at ~417 nm, a maximum centered at ~435 nm,
and two isosbestic points centered around 415 nm and 430 nm. In order to simulate the equilibrium 6-coordinate low-spin complex, an equilibrium difference spectra was constructed by subtracting CO-Fe\textsuperscript{2+}MP-11 in 50% DMSO/H\textsubscript{2}O from Fe\textsuperscript{2+}MP-11 in the presence of ~0.2 mM SDS and then compared with the equilibrium difference spectra of Fe\textsuperscript{2+}CT-Cc minus CO-Fe\textsuperscript{2+}CT-Cc. The two equilibrium difference spectra are similar, exhibiting a maximum at ~420 nm and a minimum at ~413 nm. However, there are clear differences in the transient spectrum of CT-Cc relative to the two equilibrium difference spectra. This is indicative of the transient species being 5-coordinate high-spin following CO photolysis.

![Figure 5.5](image)

**Figure 5.5**: (Red circles): Kinetic difference spectrum of Fe\textsuperscript{2+}CT-Cc constructed from various single wavelength transient absorption traces over time. (Black squares): Equilibrium difference spectrum of CO Fe\textsuperscript{2+}MP-11 in 50% DMSO:H\textsubscript{2}O subtracted from the steady-state spectrum of deoxy MP-11 in 0.2 mM SDS (50 mM HEPES buffer at pH ~7.5). (Blue triangles): Equilibrium difference spectra of CO-Fe\textsuperscript{2+}CT-Cc subtracted from Fe\textsuperscript{2+}CT-Cc in 0.2 mM SDS (50 mM HEPES buffer, pH ~7.5).

Examination of the single wavelength transient absorption decay at 435 nm (maximum) reveals a biphasic exponential process with rate constants of $4 \times 10^4$ s\textsuperscript{-1} and $2.9 \times 10^3$ s\textsuperscript{-1} (Figure 5.6). Transient absorption data obtained at the isosbestic points (415 nm and 430 nm) in the
kinetic difference spectrum can also be fit to a biphasic exponential process. Specifically, at 415 nm the decay has a decrease in absorbance with rate constants of \(3.3 \times 10^2\) s\(^{-1}\) and \(4.4 \times 10^3\) s\(^{-1}\). At 430 nm, the decay shows an increase in absorbance with rate constants of \(3.3 \times 10^4\) s\(^{-1}\) and \(7.7 \times 10^3\) s\(^{-1}\) (Figure 5.6).

![Graphs of absorption changes over time at 415 nm and 430 nm](image)

**Figure 5.6**: Single wavelength transient absorption traces of CO-Fe\(^{2+}\)-CT-Cc in the presence of \(~0.2\) mM SDS (50 mM HEPES buffer, pH~7.5) obtained at 415 nm, 430 nm (isosbestic points in the kinetic difference spectra) and 435 nm (maximum in the kinetic difference spectra).

Concentration dependence study of CO (data not shown) on Fe\(^{2+}\)-CT-Cc revealed that the \(~300\) \(\mu\)s (\(~3 \times 10^3\) s\(^{-1}\)) phase is second order with respect to CO. Thus the fast phase (\(~25\) \(\mu\)s) is likely the His(26 or 33) residue bound to the heme as previously revealed through PAC, with a relatively similar kinetic phase (\(~7 – 10\) \(\mu\)s). Hence, the slowest phase (\(~3\) ms) may be the result of a native-like conformation ensuing. It appears that Fe\(^{2+}\)-CT-Cc has the same general paradigm for folding as native Fe\(^{2+}\)-Cc that Kumar\[^8\] and Jones\[^4\] previously proposed as well as Fe\(^{2+}\)-Cc-SDS fast folding discussed in Chapter 3. As a result, the data in the current study suggests that upon refolding of CT-Cc from a MG state, the denatured chain undergoes a hydrophobic collapse into a more compact conformation (U\(\rightarrow\)U\(^*\)\(\rightarrow\)U’) occurring in <20 ns. This phase is then mediated by intramolecular binding between ligands (U’\(\rightarrow\)U’Met-S=O and U’\(\rightarrow\)U’His).
occurring in ~830 ns (τ₁) and 7 μs (τ₂), respectively. 1) CO rebinds directly back to the Fe²⁺ heme (U’→U) assigned to ~300 μs (τ₃), therefore avoiding the native-like conformation, or 2) the system reaches a stable rate-limiting transition state that crosses the activation barrier into a native-like conformation that is a late event occurring at ~3 ms (τ₄), (See Figure 1.9, Chapter 1). Overall, the PAC data obtain have allowed for a thermodynamic profile for the fast folding events occurring in the reaction pathway summarized by figure 5.7.

![Figure 5.7: Thermodynamic profile for the enthalpy (red) and corresponding molar volume (blue) changes for CO-photolysis of native Fe²⁺Cc fast folding events in the presence of 0.74 mM SDS vs. Fe²⁺CT-Cc in the presence of 0.2 mM SDS.](image)

5.4 References

CHAPTER 6: FERRIC SYSTEMS I & II
Ferric Cytochrome-c Protein Unfolding in Different Denaturant Environments, Using Nitrogen Monoxide as a Photo-trigger

6.1 Background

As discussed in Chapter 1, Cc exist in both Fe$^{2+}$ and Fe$^{3+}$ oxidation state without any major structural differences.\cite{1} However, the folding mechanism between the two states differ considerably. In the pioneering work of Jones et al., the authors demonstrated that reduced Cc Met80 coordination to the heme iron is destabilized in the presence of denaturant and can be replaced by CO. This also applies to Fe$^{2+}$Cc in the presence of SDS as previously discussed in Chapter 3. Carbon monoxide photolysis from both reduced Cc systems revealed 5 kinetic processes assigned as: a burst phase (<20 ns), Met ligand (~600 ns) competing with non-native His (~4 $\mu$s) coordination to the heme, and relatively slower kinetic phases attributing to CO recombination (~540 $\mu$s) competing with the native-like conformation (~1.5 ms). However, in nearly all of the studies on oxidized Cc folding, it has been suggested that a near-native conformation forms on the order of seconds, with bis-His coordination being rate-limiting. In fact, the Fe$^{3+}$Cc folding studies determined that reorganization of the ferric complex did not result in binding with either Met ligands (80 or 65) during the initial folding stages.\cite{2-6} The unfolding sequence of Cc is known to occur from 4 discrete folding units known as foldons with Fe$^{2+}$Cc being more stable than Fe$^{3+}$Cc.\cite{7} Though there are clear differences in the mechanistic details of folding/unfolding between the reduced and oxidized forms of Cc, information regarding earlier events (<$\mu$s) occurring in Fe$^{3+}$Cc remains less well characterized. One main
reason is due to methods not yet available to initiate Fe\textsuperscript{3+}Cc folding/unfolding on sufficiently fast timescales.

Met80 ligand of oxidized Cc can be displaced by the addition of extrinsic ligands such as: imidazole, azide, cyanide, pyridine, and/or nitrogen monoxide (NO).\textsuperscript{[8-10]} However, the Fe\textsuperscript{3+}-NO coordination is sufficiently labile such that NO can be photo-dissociated from the heme in \( \sim 50 \) fs\textsuperscript{[11]} with reasonable quantum yield.\textsuperscript{[12]} Hence, NO serves as an ideal photo-trigger for probing early events occurring in oxidized Cc folding on ns – ms timescales. Of particular interest in this study is the use of NO as a photo-trigger for probing the kinetics and energetics associated with Fe\textsuperscript{3+}Cc folding from a partially folded state via photo-thermal methods.

In a previous study conducted by Miksovska et al.\textsuperscript{[13]} suggested that NO rebinds to two populations of the unfolded Fe\textsuperscript{3+}Cc (in \( \sim 2.8 \) M GdnHCl).\textsuperscript{[13]} One population contained a 5-coordinate heme that can drive the conformation towards the native pathway, while the second population involved a 6-coordinate heme with potentially a bis-His mis-ligation rapidly formed during the refolding process.\textsuperscript{[13]} However, this study did not take into consideration that the GdnHCl induced equilibrium in the absence of NO is shifted to a more unfolded conformation (versus the initial partially folded state). In addition, information regarding the thermodynamics associated these unfolding events occurring in Fe\textsuperscript{3+}Cc are lacking. Therefore, in this study, NO photo-release has been developed to initiate the unfolding process, allowing for the enthalpy and volume changes to be probed through PAC. Specifically, unfolding of the protein was examined under denaturing conditions consisting of \( \sim 2.8 \) M GdnHCl and \( \sim 0.4 \) mM SDS (both in 50 mM HEPES buffer, pH \( \sim 7.5 \)). Under both denaturant conditions, NO triggered the partial unfold of the complex and photo-dissociation leaves the protein in a conformational state that favors an
even more unfolded complex. The data obtained from PAC has been used to construct a detailed thermodynamic profile for the early unfolding events occurring in the reaction pathway.

### 6.2 Materials and Methods

Horse heart Cc and SDS were all purchased from Sigma Aldrich and were used without further purification.

#### 6.2.1 Fluorescence Measurements

Samples were prepared by diluting Cc in 2 mL of 50 mM HEPES buffer (pH ~7.5) to a final concentration of ~15 – 20 μM in a 1-cm optically transparent quartz cuvette that was sealed with a septum cap and deaerated with argon (Ar) for ~15 min. Nitrogen monoxide was prepared by saturating Fe(II)SO₄ in HCl and reacting the solution with NaNO₂. The resulting NO gas generated was released and purged through the Fe³⁺ Cc samples for ~1 min.

A 40 mM SDS stock solution was prepared in 50 mM HEPES buffer (pH ~7.5) and placed in a separate container (glassware-tube), sealed with a septum cap, and also deaerated with Ar for ~15 min. Titrations were carried out by sequentially adding small aliquots (5 – 20 μL increments) of the 40 mM SDS stock solution to the Fe³⁺ Cc samples. Recordings of the changes in emission were observed in the spectral region of 295 nm – 525 nm (excitation at 280 nm) using an ISS PC1 (ISS. Inc., Champaign, IL) single-photon counting spectrofluorimeter.

#### 6.2.2 PAC and TA Measurements

Nitrogen monoxide bound samples for TA and PAC measurements were prepared by dissolving Fe³⁺ Cc into ~0.4 mM SDS or ~2.8 M GdnHCl in 50 mM HEPES buffer solution (pH
~7.5) to give a final concentration of ~15 – 20 μM into a 1-cm quartz cuvette. The sample is then sealed with a septum cap and deaerated with Ar for ~15 min, followed by purging the sample with NO for ~1 min. The set up and analysis for PAC and TA is as described in Chapter 2.

6.3 Results and Discussion

The steady-state absorption spectra of Fe$^{3+}$Cc in ~0.4 mM SDS (pH ~7.5) in the presence and absence of NO is displayed in Figure 6.1 (top). In the absence of NO, the sample exhibits a Soret maximum at ~408 nm and a broad band in the visible region centered around ~543 nm. NO addition to the Fe$^{3+}$Cc results in an increase in the absorbance as well as a ~8 nm bathochromic shift of the Soret band to ~416 nm with two Q-bands that form in the visible region at ~528 nm and 562 nm.

Fe$^{3+}$Cc in ~2.8 M GdnHCl (pH ~7.5) in the absence and presence of NO absorption spectrum is displayed in Figure 6.1 (bottom). In the absence of NO, the spectrum exhibits a Soret band at ~406 nm and a broad band in the visible region centered around ~544 nm. NO addition to Fe$^{3+}$Cc also results in a bathochromic shift of the Soret band to ~415 nm with two visible Q-bands that form around ~528 nm and ~561 nm, characteristic of NO binding to the Fe$^{3+}$ complex.$^{[13]}$

GdnHCl-induced equilibrium unfolding of Fe$^{3+}$Cc in the presence and absence of NO was previously conducted by Miksovska et al.$^{[13]}$, monitoring Trp59 fluorescence. These authors found that the equilibrium $m_G$ value (the slope of the linear extrapolation of unfolding free energy changes versus denaturant concentration) and $C_m$ (transition midpoint) value were ~2.8 M GdnHCl (Figure 6.2, bottom).$^{[13]}$ At this concentration, the Fe$^{3+}$Cc-GdnHCl is relatively ~40% unfolded protein. In the current study, it was determined that the SDS-induced denaturant
concentration for Fe\textsuperscript{3+}Cc is \textasciitilde0.4 mM (Figure 6.2, top). This concentration of SDS results in a \textasciitilde25\% unfolded protein. Addition of surfactants to proteins are known to either increase or decrease the helical conformation\textsuperscript{[14]} This arises primarily from strong attractive forces between the SDS and water molecules and (at even low concentrations) alters the protein conformation\textsuperscript{[14]}

\[\text{Fe}^{3+}\text{Cc} (~0.4\text{mM SDS, pH~7})\]

\[\text{NOFe}^{3+}\text{Cc} (~0.4\text{mM SDS, pH~7})\]

\[\text{Fe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5})\]

\[\text{NOFe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5})\]

\[\begin{align*}
\text{Absorbance (O.D.)} \\
\text{Wavelength (nm)}
\end{align*}\]

\[\begin{align*}
\text{Fe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5}) \\
\text{NOFe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5})
\end{align*}\]

\[\begin{align*}
\text{Absorbance (O.D.)} \\
\text{Wavelength (nm)}
\end{align*}\]

\[\begin{align*}
\text{Fe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5}) \\
\text{NOFe}^{3+}\text{Cc} (~2.8\text{M GdnHCl, pH~7.5})
\end{align*}\]

**Figure 6.1:** Absorption spectra of Soret and Q-bands of (Top) NO-bound Fe\textsuperscript{3+}Cc (red) and Fe\textsuperscript{3+}Cc (black) in \textasciitilde0.4 mM SDS (50 mM HEPES pH \textasciitilde7.5) and (Bottom) NO-bound Fe\textsuperscript{3+}Cc (green) and Fe\textsuperscript{3+}Cc (blue) in \textasciitilde2.8 M GdnHCl (50 mM HEPES buffer, pH \textasciitilde7.5).
Based on the results obtained from the previous and current fluorescence studies, Fe$^{3+}$Cc samples for both TA and PAC measurements were conducted in the presence of ~2.8 M GdnHCl and ~0.4 mM SDS (50 mM HEPES buffer, pH ~7.5), respectively.

**Figure 6.2:** (Top) SDS-Induced equilibrium unfolding of Fe$^{3+}$Cc in the presence (black) and absence (red) of NO (monitored by Trp Fluorescence at ∆I ~300 nm). The dotted line indicates the SDS concentration at which the presence of NO results in 25% of the protein being relatively unfolded while the NO-free form is 75% relatively unfolded. (Bottom) GdnHCl-induced equilibrium unfolding data of Fe$^{3+}$Cc in the presence (circle) and absence of (squares) of NO (*Similar figure can be found in Reference 13).
6.3.1 Fe$^{3+}$Cc in the Presence of ~2.8 M GdnHCl

In the case of NO-Fe$^{3+}$Cc in the presence of ~2.8 M GdnHCl, the PAC data displays a frequency shift in the sample acoustic wave relative to the reference acoustic wave (see Figure 6.3). Specifically, a prompt phase with a lifetime faster than the resolution of the PAC detector (<20 ns), a second phase with a lifetime of 863 ns and the third phase with a lifetime of 6.3 μs have been extracted.

![Figure 6.3](image.png)

**Figure 6.3:** (Left) Overlay of PAC traces for NO photo-dissociation of NOFe$^{3+}$Cc frequency shifted (red), calorimetric reference (black), three component fit of the sample acoustic wave (blue), and the residuals (green). Protein concentration is ~15 μM in ~2.8 M GdnHCl (50 mM HEPES, pH ~7.5). (Right) Plot of $\Phi_{\text{hv}}$ vs. $C_p/\beta$ for the three components associated with NO-Fe$^{3+}$Cc photo-dissociation.

### 6.3.1.1 Prompt phase:

In the presence of ~2.8 M GdnHCl, the fraction of Fe$^{3+}$Cc unfolded and NO bound protein was found to be ~40%. Therefore, the heat released ($Q_{\text{NO}}$) to the solvent for the prompt phase can be calculated based on Equation 3.1 allowing for the determination of $\Delta H (E_{\text{hv}} - Q_{\text{NO}}$, discussed in chapter 2). The prompt phase (<20 ns) was best fit to a single exponential decay ($\beta = 1$) function giving a $\Delta H = 182 \pm 11$ kcal mol$^{-1}$ and a $\Delta V$ of $-24 \pm 4$ mL mol$^{-1}$. The fast phase $\Delta H$ and $\Delta V$ was scaled to the quantum yield for NO photolysis ($\Phi_{\text{NO}} = 0.16$) under the current experimental conditions.
The observed $\Delta H$ and $\Delta V$ can be divided into contributions due to Fe-NO dissociation, heme spin state transition, solvation of the NO ligand, and any concomitant conformational changes occurring upon the protein refolding. Thus, the total changes in enthalpy and molar volume can be expressed as:

$$\Delta H_{\text{obs}} = \Delta H_{\text{Fe-NO}} + \Delta H_{\text{LS-HS}^+} + \Delta H_{\text{Solv}} + \Delta H_{\text{Conf}}$$  \hspace{1cm} (6.0)

$$\Delta V_{\text{obs}} = \Delta V_{\text{Fe-NO}} + \Delta V_{\text{LS-HS}^+} + \Delta V_{\text{Solv}} + \Delta V_{\text{Conf}}$$  \hspace{1cm} (6.1).

To account for energetics associated with Fe-NO, LS-HS, and NO-Solv, Fe$^{3+}$MP-11 was utilized assuming the complex undergoes similar processes following NO photolysis. PAC measurements of NO-Fe$^{3+}$MP-11 under identical experimental conditions (data not shown) as NO-Fe$^{3+}$Cc-GdnHCl yield a $\Delta H_{\text{MP-11}} = 59$ kcal mol$^{-1}$ and $\Delta V_{\text{MP-11}} = 8$ ml mol$^{-1}$. By subtracting $\Delta V_{\text{obs}} - \Delta V_{\text{MP-11}}$ and $\Delta H_{\text{obs}} - \Delta H_{\text{MP-11}}$ results in the conformational component ($\Delta V_{\text{Conf}}/\Delta H_{\text{Conf}}$) that contributes to the earliest steps involved with Fe$^{3+}$Cc folding process.

$\Delta H_{\text{Conf}}$ indicates that an additional endothermic process(es) of 123 kcal mol$^{-1}$ is taking place in <20 ns. The first contributing factor to take into consideration is Fe$^{3+}$Cc-GdnHCl initial conformation, partially folded state. An event to consider results from further inspection of the GdnHCl-induced equilibrium unfolding of Fe$^{3+}$Cc, found in the work of Miksovska et al.$^{[13]}$. Aside from the ~40% relatively unfolded complex (~2.8 M GdnHCl) in the presence of NO, a closer observation shows that in the absence of NO (at this same concentration) the system equilibrium is actually driven to a more unfolded conformation (~40% $\rightarrow$ ~80% relatively unfolded). Hence, upon NO photo-release, the system is progressing towards a more denatured conformation. Bai et al. identified the unfolding sequence of Cc as 4 discrete folding units
known as foldons, color-coded: red, yellow, green, and blue (see Figure 6.4).\[7\] If the unfolding sequence is taken into consideration, the initial \(~40\%\) unfolded complex (\(~42\) residues) includes the red and yellow foldon \(\Omega\) loops potentially resulting in the initial partially unfolded state. However at \(~80\\%\) (\(~83\) residues) unfolding of the green foldon 60’s helix is likely occurring following NO photo-release, while the blue foldon which includes the N-/C-terminal helices remains intact. Numerous studies have determined the unfolding reaction enthalpy of Cc from a range of partially folded states to the denature state is \(~30 – 45\) kcal mol\(^{-1}\).\[15-17\] Particularly, Kuroda et al.\[15\] demonstrated that Fe\(^{3+}\)Cc has four distinctive thermodynamic states that could be observed during the thermal unfolding process: native, MG1, MG2, and the denatured state. The authors reported thermodynamic parameters for the transition between each state (except between the N \(\rightarrow\) MG1 state) as: \(\Delta H\) of 58.1 kcal mol\(^{-1}\)/\(\Delta C_p\) of 1.4 Cal K\(^{-1}\) mol\(^{-1}\) for the N \(\rightarrow\) D transition; \(\Delta H\) of 43.7 kcal mol\(^{-1}\)/\(\Delta C_p\) of 1.1 Cal K\(^{-1}\) mol\(^{-1}\) for the MG1 \(\rightarrow\) D transition; and \(\Delta H\) of 30.8 kcal mol\(^{-1}\)/\(\Delta C_p\) of 0.4 Cal K\(^{-1}\) mol\(^{-1}\) for the MG2 \(\rightarrow\) D transition. These same authors predicted the N \(\rightarrow\) MG1 transition (through DSC measurement) had parameters of: \(\Delta H\) of 70 – 80 kcal mol\(^{-1}\) and \(\Delta C_p\) of 0.9 – 1.05 Cal K\(^{-1}\) mol\(^{-1}\)\[15\], which were later confirmed experimentally by Quershi et al.\[18\] Thus in the case of Fe\(^{3+}\)Cc-GdnHCl for the current study, the remainder \(\Delta H_{Conf}\) (which includes the unfolding enthalpy) is estimated to be about \(~20 – 50\) kcal mol\(^{-1}\) is potentially due to conformational changes associated with reorganization of the pocket and hydrogen bonds breaking in the protein upon unfolding.

For \(\Delta V_{Conf}\), the remaining process occurring within the excitation is found to be \(~36\) mL mol\(^{-1}\) (\(~60\) Å\(^3\) molecule\(^{-1}\)). The volume contraction is likely representing the decrease in helical conformation following NO photo-dissociation. Previous studies have indicated that decrease in \(\Delta V\) unfolding is the result of internal cavities and voids eliminated upon the disruption of a
folded structure as well as exposure of polar and charged groups due to electrostriction.\textsuperscript{[19]}

![Figure 6.4: Structure of Horse heart Cc (1hrc) highlighting the color-coded foldons and sequence as identified by Bai et al.\textsuperscript{[7]} (*Similar structure can be found in Reference 20*)](image)

**6.3.1.2 Slow Phases:**

The two additional kinetic phases occurring after photo-dissociation of NO the NO-Fe\textsuperscript{3+}Cc results in the conversion of a partially folded to a more denatured-like conformation. Both processes were best fit to a single exponential decay (β \(= 1\)) with a \(\Delta H_{\text{obs}}\) of 13 ± 5 kcal mol\(^{-1}\)/\(\Delta V_{\text{obs}}\) of 18 ± 2 mL mol\(^{-1}\) (863 ns phase) followed by a \(\Delta H_{\text{obs}}\) of 12 ± 20 kcal mol\(^{-1}\)/\(\Delta V_{\text{obs}}\) of –49 ± 7 mL mol\(^{-1}\) (6.3 \(\mu\)s phase).

Due to the system being in a more denatured conformation, the ~860 ns phase is likely water beginning to solvate the pocket. Specifically, the ~860 ns phase of Fe\textsuperscript{3+}Cc can be envisioned as the progressive breakage of hydrogen bonds/hydrophobic interactions in the protein interior gradually exposing amino acid residues normally buried in the protein interior, to solvent and GdnHCl molecules. Thus, the total changes in \(\Delta H\) and \(\Delta V\) for this process can be expressed as:
\[ \Delta V_{\text{obs}} = \Delta V_{\text{Solv}} + \Delta V_{\text{Struc}} \]  \hspace{1cm} (6.2)

\[ \Delta H_{\text{obs}} = \Delta H_{\text{Solv}} + \Delta H_{\text{Struc}} \]  \hspace{1cm} (6.3)

Rodriguez-Larrea et al.\cite{21} determined the energies associated with solvation for proteins with ~60 – 130 total residues is ~24 kcal mol\(^{-1}\). The enthalpic contributions included breaking of internal interactions and hydration of the residues that became exposed to the solvent.\cite{21} The corresponding \( \Delta V_{\text{obs}} \), which exhibits a volume expansion of 18 mL mol\(^{-1}\) (30 Å\(^3\) molecule\(^{-1}\)), is consistent with the proposed events. Hence, the expansion in volume likely includes the protein interior and amino acid residues becoming solvent/denaturant exposed.

The slower phase occurring in ~6 μs is less clear. Miksovska et al. proposed that an intrinsic His residue preferentially binds to the transient 5-coordinate heme of Fe\(^{3+}\)Cc-GdnHCl upon NO photo-dissociation, on similar timescales.\cite{6,13} The coordination of non-native His is assumed to create kinetic barriers during protein folding, hence facilitating the trapping of a native-like intermediate.\cite{6,13} To determine if His binding is indeed the ~6 μs phase observed in PAC, a pH-dependent study was conducted on Fe\(^{3+}\)Cc in 2.8 M GdnHCl under acidic (pH ~4) and alkaline (pH ~10) conditions upon NO photo-release and probed through PAC (see Table 6.1). At pH \( \leq 5 \) both His (26 and 33) imidazole rings are protonated, thus misligation to the heme is prevented and differences in the kinetics relative to pH ~7 should be observed. On the other hand, in pH \( \geq 7 \) the His residues are deprotonated, hence if ligation of the His residues occurs there should be similar lifetimes observed between pH ~7 and pH ~10. The PAC measurements indicate that this is not the case for Fe\(^{3+}\)Cc-GdnHCl under the various pH conditions. Although, there are no differences in lifetimes amongst each pH the trends of the observed enthalpies and volume changes in each kinetic phase resolved as a function of pH. In the prompt and second
kinetic phase there is ~100 kcal mol\(^{-1}\)/~40 kcal mol\(^{-1}\) difference as the system is deprotonated (going from pH 4 → 10), respectively. This is likely due to the pH affecting the overall charge of the protein, NH\(^3+\) (pH ~8 – 10) and COOH (pH ~2 – 4). In addition, the ~6 µs phase has a \(\Delta V\) that varies from a large contraction (pH ~4) to essentially no volume change (pH ~10), potentially due to electrostriction. Overall, the pH study indicates His may not be binding to the heme in Fe\(^{3+}\)Cc-GdnHCl. Instead, due to the protein progression to a denature-like conformation, some other ligand (L) is potentially binding to the exposed heme.

Table 6.1: Summary of PAC data for NO-Fe\(^{3+}\)Cc in the presence of 2.8 M GdnHCl (varying pH) with lifetimes highlighted in red

<table>
<thead>
<tr>
<th></th>
<th>(\tau_p)</th>
<th>(\Delta H_p) kcal/mol</th>
<th>(\Delta V_p) mL/mol</th>
<th>(\tau_2)</th>
<th>(\Delta H_2) kcal/mol</th>
<th>(\Delta V_2) mL/mol</th>
<th>(\tau_3)</th>
<th>(\Delta H_3) kcal/mol</th>
<th>(\Delta V_3) mL/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOFe(^{3+})Cc</td>
<td>&lt;20 ns</td>
<td>331 (\pm) 4</td>
<td>-52 (\pm) 2</td>
<td>942 ns</td>
<td>46 (\pm) 28</td>
<td>40 (\pm) 11</td>
<td>7.8 (\mu)s</td>
<td>-65 (\pm) 29</td>
<td>-53 (\pm) 11</td>
</tr>
<tr>
<td>(2.8 M GdnHCl, pH=4)</td>
<td></td>
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</tr>
<tr>
<td>NOFe(^{3+})Cc</td>
<td>&lt;20 ns</td>
<td>182 (\pm) 11</td>
<td>-28 (\pm) 4</td>
<td>863 ns</td>
<td>13 (\pm) 5</td>
<td>18 (\pm) 2</td>
<td>6.3 (\mu)s</td>
<td>12 (\pm) 20</td>
<td>-49 (\pm) 7</td>
</tr>
<tr>
<td>(2.8 M GdnHCl, pH=7)</td>
<td></td>
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</tr>
<tr>
<td>NOFe(^{3+})Cc</td>
<td>&lt;20 ns</td>
<td>226 (\pm) 6</td>
<td>-19 (\pm) 2</td>
<td>1 (\mu)s</td>
<td>6 (\pm) 29</td>
<td>13 (\pm) 8</td>
<td>7.2 (\mu)s</td>
<td>7 (\pm) 37</td>
<td>5 (\pm) 10</td>
</tr>
<tr>
<td>(2.8 M GdnHCl, pH=10)</td>
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The total changes in \(\Delta H\) and \(\Delta V\) for ~6 µs phase can be expressed as:

\[
\Delta H_{\text{obs}} = \Delta H_{\text{Fe-L}} + \Delta H_{\text{Struc}} \tag{6.4}
\]

\[
\Delta V_{\text{obs}} = \Delta V_{\text{Fe-L}} + \Delta V_{\text{Struc}} \tag{6.5}
\]

where \(\text{Fe-L}\) is an unknown ligand (L) binding to the heme. \(\Delta H_{\text{Struc}}\) is estimated to be an endothermic process of ~42 kcal mol\(^{-1}\), potentially structural changes accommodating the open conformation and the N-/C- terminal helix interactions. The corresponding \(\Delta V_{\text{obs}}\) of ~49 mL mol\(^{-1}\) (~81 Å\(^3\) molecule\(^{-1}\)) where \(\Delta V_{\text{Fe-L}}\) must account for solvation of the intrinsic ligand in an
already solvated environment due to the more unfolded conformation of Fe$^{3+}$Cc-GdnHCl. Thus, the origin of the volume contraction likely arises primarily from structural rearrangement of the complex.

Temperature dependence measurements from PAC allowed for the activation parameters associated with the two slower kinetic phases, 863 ns and 6.3 $\mu$s, to be determined (Figure 6.5). The activation energies were found to be $\Delta H^\ddagger = 1 \pm 2$ kcal mol$^{-1}$/K and $\Delta S^\ddagger = -46 \pm 1$ cal mol$^{-1}$/K, and $\Delta H^\ddagger = -0.2 \pm 0.8$ kcal mol$^{-1}$/K and $\Delta S^\ddagger = -41 \pm 1$ cal mol$^{-1}$/K, respectively. The data shows that both kinetic events are essentially barrierless.

![Eyring plot of the (black) ~860 ns and (red) ~6 $\mu$s phases of Fe$^{3+}$Cc in 2.8 M GdnHCl (50 mM HEPES, pH~7.5).](image)

**Figure 6.5:** Eyring plot of the (black) ~860 ns and (red) ~6 $\mu$s phases of Fe$^{3+}$Cc in 2.8 M GdnHCl (50 mM HEPES, pH~7.5).

The mechanism based upon the results from PAC presented here as well as previous TA studies$^{[13]}$ are summarized in Figure 6.6. Overall, the proposed mechanism for early events occurring in Fe$^{3+}$Cc-GdnHCl unfolding subsequent to NO photo-dissociation leads to a rapid (< 20 ns) expansion into a secondary unfolded state that is more denatured than the initial conformation. As a consequence, the 863 ns phase is due to the interior of the protein becoming
solv-exposed (i.e. solvating amino acids, breakage of hydrophobic interactions, etc.). This pathway can lead to: 1) bind an intrinsic ligand (L) directly to the heme (~6 µs) followed by the slowest step which represents NO replacement of the extrinsic ligand (~5 ms) or 2) the NO ligand rebinding directly to the high-spin complex (~250 µs).

**Figure 6.6:** Overall mechanism for early unfolding events occurring in Fe$$^{3+}$$Cc-GdnHCl with data obtained from PAC highlighted and the two slowest kinetic phases obtained from previous TA studies conducted by Miksovska et al.\textsuperscript{[13]} (Similar schematic found in Reference 14)

### 6.3.2 Fe$$^{3+}$$Cc in the presence of ~0.4 mM SDS

In the case of NO-Fe$$^{3+}$$Cc in the presence of ~0.4 mM SDS, the PAC data also revealed three kinetic events taking place subsequent to NO photolysis (Figure 6.7). Specifically, photolysis revealed observed lifetimes of <20 ns, ~640 ns, and ~8 µs. Under the denaturant condition for the prompt phase, the fraction of Fe$$^{3+}$$Cc unfolded and NO bound was found to be ~25% (see figure 6.2). In addition, this fast phase (<20 ns) was best fit to a single exponential decay ($β = 1$) function with an endothermic $ΔH = 197 ± 11$ kcal mol$^{-1}$ and a $ΔV$ of $–7 ± 1$ mL mol$^{-1}$. The prompt phase was also scaled to the quantum yield for NO photolysis ($Φ_{NO} = 0.23$). PAC measurements of NO-Fe$$^{3+}$$MP-11 under identical experimental conditions (data not shown)
yield a $\Delta H_{MP-11} = 17 \pm 6$ kcal mol$^{-1}$ and $\Delta V_{MP-11} = 4 \pm 1$ ml mol$^{-1}$, resulting in the conformational component being calculated with $\Delta V_{Conf} = -11$ mL mol$^{-1}$ and $\Delta H_{Conf} = 180$ kcal mol$^{-1}$.

**Figure 6.7:** (Right) Overlay of PAC traces for the NO photo-dissociation of NO-Fe$^{3+}$-Cc frequency shifted, (red) calorimetric reference (black), three component fit of the sample acoustic wave (blue), and the residuals (green). Protein concentration is ~15 µM in ~0.4 mM SDS, 50 mM HEPES pH~7.5. (Left) Plot of $\phi E_{hv}$ vs. Cpp/$\beta$ for the three components associated with NO-Fe$^{3+}$Cc photolysis.

The $\Delta H_{Conf}$ of 180 kcal mol$^{-1}$ indicates additional endothermic processes are occurring in <20 ns. Just as discussed in the previous system (Fe$^{3+}$Cc-GdnHCl), the first contributing factor to take into consideration results from the system equilibrium being driven to a more unfolded conformation (~25% → ~75% relatively unfolded) upon NO photo-release. Hence, conformational changes and hydrogen bonds breaking due to the protein unfolding are the main contributors for $\Delta H_{Conf}$. For $\Delta V_{Conf}$, the remaining process occurring is found to be ~11 mL mol$^{-1}$ (~ ~18 Å$^3$ molecule$^{-1}$) and, again, likely represents the decrease in helical conformation following NO photo-dissociation (as previously discussed). Hence, the data suggests a uniform mechanism for the early events occurring in Fe$^{3+}$Cc unfolding regardless of the denaturant-induced state (~0.4 mM SDS vs. ~2.8 GdnHCl). However, the distinct denaturant environment may account for the differences in the kinetics and magnitude of the $\Delta H_{obs}/\Delta V_{obs}$ in the PAC measurements. Therefore, the two slow phases (~640 ns and ~8 ßs) kinetic phases in Fe$^{3+}$Cc-
SDS occurs after photo-dissociation of NO results in the conversion of a partially unfolded conformation to a secondary unfolded state. Both processes were also best fit to a single exponential decay ($\beta = 1$) with a $\Delta H_{\text{obs}}$ of $5 \pm 2$ kcal mol$^{-1}$/$\Delta V_{\text{obs}}$ of $5 \pm 1$ mL mol$^{-1}$ (642 ns phase) followed by a $\Delta H_{\text{obs}}$ of $30 \pm 5$ kcal mol$^{-1}$/$\Delta V_{\text{obs}}$ of $-11 \pm 2$ mL mol$^{-1}$ (7.9 $\mu$s phase). Specifically, the ~640 ns phase of $\text{Fe}^{3+}\text{Cc}$-SDS is the breakage of hydrophobic/hydrogen bond interactions and the protein interior/amino acid residues becoming solvent/SDS exposed with corresponding $\Delta V_{\text{obs}}$ exhibiting a volume expansion of 5 mL mol$^{-1}$ (~8 Å$^3$ molecule$^{-1}$). The slower phase occurring in ~8 $\mu$s is binding of a ligand with structural changes accommodating the more unfolded conformation and a corresponding $\Delta V_{\text{obs}}$ that has a volume contraction of $-11$ mL mol$^{-1}$ (~18 Å$^3$ molecule$^{-1}$). The activation energies (Figure 6.8) of the two slower phases were found to be $\Delta H^\ddagger = -1 \pm 3$ kcal mol$^{-1}$/$\Delta S^\ddagger = -42 \pm 5$ cal mol$^{-1}$ K$^{-1}$ (642 ns) and $\Delta H^\ddagger = -4 \pm 9$ kcal mol$^{-1}$/$\Delta S^\ddagger = -34 \pm 12$ cal mol$^{-1}$ K$^{-1}$ (7.9 $\mu$s). Just as $\text{Fe}^{3+}\text{Cc}$-GdnHCl system previously discussed, the activation parameters for $\text{Fe}^{3+}\text{Cc}$-SDS show that both kinetic events are barrierless and consistent with the proposed mechanism.

**Figure 6.8:** Erying plot of the (black) ~640 ns and (red) ~8 $\mu$s phases of $\text{Fe}^{3+}\text{Cc}$ in 0.4 mM SDS, (50 mM HEPES buffer, pH~7.5).
Overall, the data suggests a uniform mechanism for the early events occurring in Fe$^{3+}$Cc regardless of the denaturant environments. The mechanism based upon the results of NO-Fe$^{3+}$Cc-SDS from PAC presented here as well TA studies (not discussed) are shown in Figure 6.9. In addition, the PAC data obtain has allowed for a thermodynamic profile for the fast events occurring in Fe$^{3+}$Cc (in the presence of ~2.8 M GdnHCl vs. ~0.4 mM SDS) reaction pathway and is summarized in Figure 6.10.

Figure 6.9: Overall mechanism for early events occurring in Fe$^{3+}$Cc-SDS with data obtained from PAC highlighted and the two slowest kinetic phases obtained from TA studies.

Figure 6.10: Thermodynamic profile for the enthalpy (orange) and corresponding molar volume (teal) changes for NO-photolysis of Fe$^{3+}$Cc fast folding events in the presence of ~2.8 M GdnHCl vs. in the presence of ~0.4 mM SDS (both in 50 mM HEPES buffer, pH~7.5).
6.4 References

7.1 Background

Previous studies have shown that the ferric derivative of CT-Cc displays significant changes relative to the native form that are a consequence of observed perturbations to the distal heme pocket. One being the heme crevice of Fe$^{3+}$CT-Cc is destabilized relative to the native form, binding of sulfoxide to the ferric heme occurs through coordination of the sulfoxide oxygen instead of the Met80 sulfur atom,$^{[1,2]}$ and the redox potential decreases from 260 mV to 175 mV.$^{[3]}$ Although these perturbations reduce coupling between the electronic transition of the polypeptide backbone and the heme, there is no loss of the overall protein stability upon oxidizing the Met sulfur atoms.$^{[2]}$ Thus, oxidation of Met80 provides a unique opportunity to probe structural dynamics occurring in a relatively rigid distal pocket that may be important to the ferric Cc heme function. In this study the goal is to investigate the thermodynamics involved with the reorganization of Fe$^{3+}$CT-Cc distal heme pocket, which has not been examined via PAC.

In Chapter 4, photo-release of CO from CO-Fe$^{2+}$CT-Cc allowed for the local distal pocket environment of the ferrous form to be probed on fast timescales. Through PAC, it was revealed that two kinetic processes (<20 ns and 534 ns) occurred prior to Met80-sulfoxide/CO rebinding in Fe$^{2+}$CT-Cc. Though CO only interacts with the ferrous form, NO can bind to the Fe$^{3+}$ heme of the modified system by replacing the relatively weak Met80-sulfoxide (Met80-S=O) oxygen coordinated to the heme. The Fe$^{3+}$-NO coordination is sufficiently liable and
photo-dissociation of NO can be used as a photo-trigger for probing the energetics with photothermal methods. Specifically in this study, PAC is employed to probe the kinetic, enthalpy, and molar volume changes subsequent to NO photo-release from NO-Fe$^{3+}$CT-Cc. In addition, MD simulations were also performed in order to provide atomistic resolution to the events occurring subsequent to NO photo-release.

7.2 Materials and Methods

Horse heart Cc and CT were both purchased from Sigma and used without further purification. The changes in absorption were observed in the spectral region of 300 – 750 nm using a Shimadzu UV-2401 spectrophotometer.

7.2.1 Preparation of CT-Cc

CT-Cc samples were prepared according to Bosshard et al.\textsuperscript{[4]} and as described in Chapter 4.

7.2.2 PAC Measurements

Samples for PAC studies were prepared by diluting CT-Cc into 1.5 mL of 10 mM Tris buffer (pH ~7.5) to a final concentration of ~10 – 15 μM in a 1 cm optical quartz cuvette, sealed with a septum cap, and deaerated with Ar for ~15 min. Nitrogen monoxide was prepared by saturating Fe(II)SO$_4$ in HCl and reacting the solution with NaNO$_2$. The resulting NO gas generated was released and purged through the Fe$^{3+}$ heme samples under constant pressure for ~1 min. The set-up, theory and analysis for PAC have been described in Chapter 2.
7.2.3 Molecular Dynamics Simulations

Molecular dynamic simulations (see Preface) of Cc were performed, with Met80 in wild and Met80-S=O forms. Initial coordinates for the system were obtained from Protein Data Bank (PDB)\textsuperscript{[5]}, entry 1HRC\textsuperscript{[6]}. Sulfoxide form of Met80 was modeled by addition of oxygen to the sulfur atom. Distal NO ligand was added to the Fe\textsuperscript{3+} heme group. The parameters of S=O group were adjusted based on CHARMM36 force field\textsuperscript{[7,8]}. The complex was solvated in a cubic box of 150 mM KCl solution of TIP3 water\textsuperscript{[9]}, with a minimum distance of 10 Å between the protein and the edge of the box. After minimization, the system was gradually heated to 298 K over a period of 400 ps. During system preparation, harmonic restraints were added to keep NO in a fixed position to Fe\textsuperscript{3+}, these restraints were released during 100 ns long production run. All simulations were performed with NAMD software\textsuperscript{[10]} and CHARMM 36 force field. The simulations used a time step of 2 fs with SETTLE\textsuperscript{[11]} algorithm to constrain bonds with hydrogen atoms\textsuperscript{[12]}, Nosé-Hoover thermostat for temperature control\textsuperscript{[13]}, and the particle mesh Ewald method\textsuperscript{[14]} for long-range electrostatic interactions. Trajectories were analyzed with VMD\textsuperscript{[15]} and PyMol\textsuperscript{[16]}.

7.3 Results and Discussion

Figure 7.1 displays the optical absorption spectra of Fe\textsuperscript{3+}CT-Cc and NO-Fe\textsuperscript{3+}CT-Cc in 10 mM Tris buffer solution (pH ~7.5). The Soret band exhibits a maximum at 403 nm with a board visible band centered near 530 nm. Addition of NO to the ferri CT-Cc results in a ~13 nm red shift of the Soret band to 416 nm and Q-bands are observed in the visible region centered at 528 nm and 562 nm, characteristic of NO binding to Fe\textsuperscript{3+} heme.\textsuperscript{[17]} Previous studies have shown that Fe\textsuperscript{3+}CT-Cc absorption spectra in neutral pH is similar to the native form with the exception of an
increase in the extinction coefficient of the Soret band and the elimination of the 695 nm charge transfer band, attributed to the Met-sulfur coordination to the heme iron.\textsuperscript{[2,3]}

\textbf{Figure 7.1:} Optical absorption spectra of Fe\textsuperscript{3+}CT-Cc (black) and NO-bound Fe\textsuperscript{3+}CT-Cc complex (red) in 10 mM Tris buffer solution (pH \~7.5).

An overlay of the acoustic traces for NO photolysis from NO-Fe\textsuperscript{3+}CT-Cc versus the corresponding reference acoustic traces is displayed in Figure 7.2 (left). The absence of a phase shift between the sample and reference acoustic signal indicates that volume and enthalpy changes associated with NO photolysis occurs on a timescale faster than the response time of the acoustic detector (<20 ns). The fit of the data in Figure 7.2 (right) to equation $E_{hv} - Q$ (as discussed in Chapter 2) and scaled to $\Phi_{NO} = 0.21$ gave rise to an endothermic $\Delta H$ of 50 ± 1 kcal mol\textsuperscript{-1} and a corresponding $\Delta V$ of 2.6 ± 0.1 mL mol\textsuperscript{-1}.

The observed $\Delta H$ and $\Delta V$ can be divided into contributions due to Fe-NO bond dissociation, heme spin-state transition, and concomitant protein conformational changes.
occurring within the heme distal pocket. Thus, the total changes in enthalpy and molar volume for this process can be expressed as:

\[ \Delta H_{\text{obs}} = \Delta H_{\text{Fe-NO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{Conf}} \]  
(7.0)

\[ \Delta V_{\text{obs}} = \Delta V_{\text{Fe-NO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{Conf}} \]  
(7.1)

**Figure 7.2:** (Left) PAC traces for NO photo-dissociation from NOFe\(^{3+}\)CT-Cc (red) versus Reference (black) in 10 mM HEPES buffer, pH ~7.5. (Right) Plot of \( \Phi E_{\text{hv}} \) vs. \( \text{Cp/}^{\beta} \) for NO photo-dissociation from NO-Fe\(^{3+}\)CT-Cc.

From previous studies of Fe\(^{3+}\) model systems,\(^{[17]}\) photo-dissociation of Fe\(^{3+}\)-NO bond is associated with a \( \Delta H_{\text{Fe-NO}} \sim 24 \text{ kcal mol}^{-1} \) and \( \Delta H_{\text{LS-HS}} \sim 0 \text{ kcal mol}^{-1} \), giving a \( \Delta H_{\text{Conf}} \) of \( \sim 26 \text{ kcal mol}^{-1} \). The MD simulations show that reorganization of the distal pocket occurs subsequent to release of the NO ligand. Specifically, NO migrates \( \sim 5 \text{ Å} \) from the heme into a hydrophobic pocket formed by residues Tyr67, Leu68, Pro71, and Phe82 (Figure 7.3), that harbors the dissociated NO. In addition to the hydrophobic docking site, the Met80-S=O group is hydrogen bonded with Tyr67 (\( \sim 1.9 \text{ Å} \)) thus preventing immediate recombination of both NO and Met80-S=O to the heme. It is also noteworthy to point out from the simulations that following NO migration from the heme, Tyr67 benzene ring forms an aromatic – aromatic interaction with a
pyrrole ring of the heme group known as an “edge to face” type stacking.\cite{18-22} The two aromatic rings are \(~4.4\) Å apart center-to-center, hence the interactions between the rings are also likely preventing immediate NO recombination to the heme potentially due to electrostatic repulsion between the ligand and induced dipole between the rings.

In the case of $\Delta V_{\text{obs}}$, $\Delta V_{\text{LS-HS}}$ and $\Delta V_{\text{Fe-NO}}$ are likely to be negligible for a heme constrained within a protein pocket. Therefore, the estimated changes arise from $\Delta V_{\text{Conf}} \sim -3$ mL mol$^{-1}$ ($-5$ Å$^3$ molecule$^{-1}$) is due to local conformational changes surrounding the distal pocket and possibly electrostriction involving the heme propionates and peripheral amino acids at the surface of the protein (as seen in the ferrous form). The structures highlighted in Figure 7.3 (right) suggest movement of the propionates leading to greater solvent exposure which would increase the electrostriction resulting in the observed volume decrease.

![Figure 7.3](image)

**Figure 7.3:** (Left) MD structure of NO-Fe$^{3+}$CT-Cc illustrating the residues making up the hydrophobic site surrounding the NO ligand following dissociation from the heme. (Right) MD structures of NO-Fe$^{3+}$CT-Cc highlighting the distal residues surrounding the NO ligand before (Red) and following (Gray) dissociation from the heme into the hydrophobic docking site pocket.

Photolysis of NO from Fe$^{3+}$CT-Cc results in the formation of a 5-coordinate high-spin complex as judged from the transient difference spectra (Figure 7.3). The transient difference
spectra (transient minus NO-Fe$^{3+}$CT-Cc ground state) obtained 500 ns and 10 μs subsequent to photolysis exhibit a maximum at ~400 nm, a minimum at ~418 nm, and an isosbestic point near ~410 nm. The fact that there is no change over time occurring from 500 ns – 10 μs following NO photolysis is consistent with the MD simulation in which shows no other ligand binds to the Fe$^{3+}$ heme on fast timescales. Hence, the Fe$^{3+}$ heme remains a 5-coordinate high-spin complex. The corresponding equilibrium difference spectrum of deoxy Fe$^{3+}$MP-11 (5-coordinate high spin model) minus NO-Fe$^{3+}$MP-11 is similar to the transient different spectra, again indicating no Met-S=O binding on fast timescales.

**Figure 7.4:** (Red line): Equilibrium difference spectra of NO-Fe$^{3+}$CT-Cc subtracted from Fe$^{3+}$CT-Cc. (Blue triangles): Kinetic difference spectrum of Fe$^{3+}$CT-Cc constructed from various single wavelength transient absorption traces over time, plotted 500 ns subsequent to photolysis. (Green triangles): Kinetic difference spectrum of Fe$^{3+}$CT-Cc plotted 10 μs subsequent to photolysis. (Black squares): Equilibrium difference spectrum of NO Fe$^{3+}$MP-11 subtracted from the steady-state spectrum of deoxy MP-11.

Examination of the single wavelength transient absorption spectra (Figure 7.5) at 400 nm reveals biphasic decay kinetics with rate constants of $(6.6 \pm 0.5) \times 10^2$ s$^{-1}$ and $18 \pm 0.01$ s$^{-1}$. Transient absorption data obtained at the isosbestic point of 411 nm also revealed biphasic decay.
with an absorbance decrease and corresponding rate constants of $(1.2 \pm 0.3) \times 10^2$ s$^{-1}$ and $17 \pm 0.02$ s$^{-1}$. The fast and slow phase corresponds to $\sim$28% and $\sim$72% of the relative amplitude, respectively.

**Figure 7.5:** Single wavelength TA traces of NO-Fe$^{3+}$CT-Cc obtained at 400 nm (black) (maximum in the kinetic difference spectra for the 5-coordinate high-spin species) and 411 nm (red) (isosbestic point in the kinetic difference spectra).

Ferric MP-11 was used as a model system in order to mimic both the 5-coordinate high-spin Cc and 6-coordinate low-spin (in the presence of DMSO) His/Met-S=O Cc species. Nitrogen monoxide photolysis and recombination to Fe$^{3+}$MP-11 in DMSO:buffer mixtures are shown in Figure 7.6 (left). In the absence of DMSO the rate constant of NO recombination to Fe$^{3+}$MP-11 is $7.5 \times 10^2$ s$^{-1}$ at 1.4 mM [NO]$^{17}$ giving a second order rate constant of $1.05 \times 10^6$ M$^{-1}$ s$^{-1}$. Photolysis of NO-Fe$^{3+}$MP-11 complex in aqueous solutions containing increasing amounts of DMSO results in biphasic decay kinetics for recombination to the transient 5-coordinate complex. One kinetic phase (fast phase) rate constant increases as a function of DMSO concentration thus attributed to DMSO binding. The second (slow phase) can be assigned
as subsequent NO displacement of the bound DMSO molecule. From the dependence of the fast rate constant on DMSO concentration (Figure 7.6, right), a second order rate constant for diffusional DMSO binding is found to be $1.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$.

![Figure 7.6: (Left) Single wavelength transient absorption traces of NO-Fe$^{3+}$MP-11 (concentration of MP-11 is $\approx$15 µM) in buffer solution in the presence of various concentration of DMSO (0, 130 and 190 mM). (Right) Plot of fast phase rate constant vs. DMSO concentration.](image)

From the TA data, results obtained at the maximum (400 nm) resulted in two recombination rates: the fast phase due to Met-S=O binding to the heme with a recombination rate ($k_1$) of $6.6 \times 10^2 \text{ s}^{-1}$ ($\approx$1.5 ms) and the slowest phase with a recombination rate of $18 \text{ s}^{-1}$ ($\approx$55 ms) that is assigned as NO replacement of the Met-S=O ligand, reflective of Met-S=O off rate. The rate constant for Met-S=O dissociation ($k_{-1}$, from $(1/R) = (1/k_{-1})(k_1/ k_2)$, where $R = 18 \text{ s}^{-1}$ for the observed rate of NO exchange) is found to be $\approx$0.6 s$^{-1}$. Since the slowest phase ($\approx$1.5 ms) is not observed in the single wavelength decay at the isosbestic point (411 nm), the rate constant ($k_2$) of $1.2 \times 10^2 \text{ s}^{-1}$ (8 ms) is likely diffusional NO binding. Therefore, it can be concluded from the results that NO recombination competes with Met-S=O ligation to the vacant 6$^{\text{th}}$ coordination site of the Fe$^{3+}$ heme.
7.4 Comparison of Modified Cc (Fe\textsuperscript{2+} vs. Fe\textsuperscript{3+})

Obviously the difference in the two modified systems (aside from the oxidation state of the heme group) are the ligands (CO vs. NO) used for photolysis to probe the structural reorganization occurring in the local distal environment of the heme. Specifically, the results from PAC and MD simulation revealed some similarities and differences in the ligands pathway. In both instances the ligand first migrate ~5 Å from the heme into a hydrophobic pocket (formed by Tyr67, Leu68, Pro71, and Phe82), however, NO remains in this pocket while CO continues to travel further away from the heme (~8 Å) into a second docking site that is formed with distinct residues (see Chapter 4). One would first suspect that the differences in mechanisms of the modified Cc systems result in the overall charge of the Fe\textsuperscript{2+}-(0) versus Fe\textsuperscript{3+}-(+1) heme (at pH~7), as the charge influences which external ligand is able to bind to the heme. However, this assumption is ruled out due to the first docking site being identical in both heme oxidation states. Therefore, the differences in the ligand pathway may be the result of the Cc derivative favoring NO over CO. This is likely due to NO generally having a greater affinity in heme proteins and being more physiologically relevant (i.e. NO sensor/storage),\textsuperscript{[26]} hence in the ferric form, NO remains in the first hydrophobic pocket closest to the heme. In the ferrous form the surrounding residues facilitates the escape of CO from the pocket. However, due to the 90° of Ile95 in CT-Cc, CO escaping into the bulk solvent did not occur (see Chapter 4). the data presented here demonstrates that Fe\textsuperscript{3+}CT-Cc Met80-S=O group dissociates from the heme, rebinds to the heme, and is modulated by residues within the distal heme pocket (much like the modified ferrous form). Transient absorption revealed that Met-S=O exhibits a relatively slow recombination rate (~1.5 ms). Hence, the activation barrier for Met-S=O recombination in Fe\textsuperscript{3+}CT-Cc is similar to the modified ferrous form in which involves electrostatic repulsion between the S=O large
dipole moment and the high-spin $d_{x^2}$ metal center$^{[1,2]}$ as well as the energy required to break the hydrogen bond formed between Tyr67 and Met-S=O (as seen in the MD simulations).

In summary, the results presented in this study have provided a detailed mechanism on the thermodynamics and structural changes associated with a relatively rigid distal pocket that may be important to ferric Cc function. Transient absorption revealed that Met-S=O compete with NO recombination to the heme on relatively slow timescales. The PAC data revealed a kinetic phase that was faster than the resolution time of the detector (< 20 ns) in which occurred prior to Met-S=O/NO rebinding to the heme group. Molecular dynamic simulations show that the < 20 ns was due to the formation of a hydrophobic docking site that harbors the NO ligand as well as the Met80-sulfoxide oxygen forms a hydrogen bond between Tyr67, thus preventing immediate recombination to the heme. It can be concluded that Met-S=O binding to the heme in Fe$^{3+}$CT-Cc is modulated by residues within the distal heme pocket. The overall mechanism for ligand rebinding to NO-Fe$^{3+}$CT-Cc is summarized in Figure 7.7.

**Figure 7.7:** Overall mechanism for ligand rebinding occurring in NO-Fe$^{3+}$CT-Cc with data obtained from PAC highlighted and the relatively slow kinetic phases obtained from TA studies explained in the text.
7.5 References

[16] Delano, W.L., PyMol v.13, Schrödinger, LLC.
CHAPTER 8: FERRIC SYSTEM IV
Surfactant-Induced Protein Folding in Ferric Chloramine-T Modified Horse Heart Cytochrome-c

8.1 Background

To gain further insight into the folding/unfolding mechanism of Fe\(^{3+}\)Cc, the modified form (Fe\(^{3+}\)CT-Cc) was also investigated. Previous studies on native ferric Cc have suggested a near-native conformation formed on the order of seconds, with bis-His coordination being rate-limiting.\(^1,2\) Coordination of the non-native His in Fe\(^{3+}\)Cc is assumed to create a kinetic barrier during protein folding trapping a native-like intermediate.\(^1,2\) On the other hand, fast folding studies also on native Fe\(^{3+}\)Cc that used NO as a photo-initiator to trigger the unfolding reaction (presented in Chapter 6) led to a different proposed folding mechanism. The mechanism involved (under the denaturant conditions of ~2.8 M GdnHCl and ~0.4 mM SDS) a rapid expansion of the protein into a partially folded state that was more denatured than the initial unfolded conformation and, instead of a bis-His coordination, led to a ligand (L) other than His binding directly to the heme. Hence, an important question is: does modification of Cc (via oxidation of the sulfur atoms of the Met ligands) alter the folding mechanism as well as the thermodynamics?

To investigate the folding lifetimes as well as the magnitude of enthalpy and molar volume changes associated with NO-Fe\(^{3+}\)CT-Cc folding mechanism, PAC along with TA has been employed to probe the folding process. The experiments described in this work utilize SDS as a denaturant under conditions that allows for refolding of the native-like conformation from a
surfactant-induced partially folded state. Specifically, the experiments examined here were conducted in the presence of ~0.2 mM SDS in 50 mM HEPES (pH ~7.5) buffer solution. The data obtained from PAC have been used to construct a detailed thermodynamic profile for the early folding events occurring in the reaction pathway and, to our knowledge, is the first time the kinetics, energetics and molar volume changes associated with Fe\(^{3+}\)CT-Cc folding have been measured.

**8.2 Materials and Methods**

Horse heart Cc and CT were both purchased from Sigma and used without further purification. The changes in absorption were observed in the spectral region of 300 – 750 nm using a Shimadzu UV-2401 spectrophotometer.

**8.2.1 Preparation of CT-Cc**

CT-Cc samples were prepared according to Bosshard et al.[3] and as previously discussed in Chapter 4.

**8.2.2 Fluorescence Measurements**

Samples were prepared by diluting CT-Cc in 2 mL of 50 mM HEPES buffer (pH ~7.5) to a final concentration of ~15 μM in a 1-cm quartz cuvette, sealed with a septum cap, and deaerated with Ar for ~15 min. Nitrogen monoxide was prepared by saturating Fe(II)SO\(_4\) in HCl and reacting the solution with NaNO\(_2\). The resulting NO gas generated was released and purged through the Fe\(^{3+}\) heme samples under constant pressure for ~1 min.
A 40 mM SDS stock solution was prepared in 50 mM HEPES buffer (pH ~7.5) and placed in a separate container (glassware-tube), sealed with a septum cap, and also deaerated with Ar for ~15 min. SDS titrations were carried out by sequentially adding small aliquots (5 – 20 μL increments) of the 40 mM SDS stock solution to the Fe³⁺CT-Cc samples. Changes in emission were observed in the spectral region of 295 nm – 525 nm (excitation at 280 nm) using an ISS PC1 (ISS. Inc., Champaign, IL) single-photon counting spectrofluorimeter.

### 8.2.3 PAC and TA Measurements

Nitrogen monoxide bound samples for TA and PAC studies were prepared by dissolving CT-Cc into ~0.2 mM SDS (50 mM HEPES buffer, pH ~7.5) to give a final concentration of ~15 μM into a 1-cm quartz cuvette. The Fe³⁺ sample is then sealed with a septum cap, deaerated with Ar for ~15 min, and then followed by purging the sample with NO for ~1 min. The set up and analysis for PAC and TA is as described in Chapter 2.

### 8.3 Results and Discussion

Figure 8.1 displays the optical absorption spectra of Fe³⁺CT-Cc and NO-Fe³⁺CT-Cc in the presence of ~0.2 mM SDS, 50 mM HEPES buffer solution (pH ~7.5). The Soret band is centered at 403 nm with a broad visible band centered at ~540 nm. Addition of NO to the ferric CT-Cc results in a ~13 nm red shift of the Soret band to 416 nm and Q-bands are observed in the visible region centered at 528 nm and 562 nm, characteristic of NO binding to Fe³⁺ heme.⁴
Fluorescence measurements of Fe$^{3+}$CT-Cc-SDS were conducted by taking advantage of Trp59 energy transfer with the heme. The data show that at ~0.2 mM SDS, NO-Fe$^{3+}$CT-Cc is approximately 20% unfolded while the NO free form is relatively folded (Figure 8.2). Details of the unfolded structure or the unfolding sequence is not known for the CT-Cc derivative. However, it is likely that SDS binds to the ferric system and results in a partially folded intermediate. Based on the results obtained from fluorescence measurements, samples for both TA and PAC measurements were done in the presence of ~0.2 mM SDS (50 mM HEPES buffer, pH ~7.5).
**Figure 8.2:** SDS-Induced equilibrium unfolding of Fe$^{3+}$CT-Cc in the presence (red) and absence (black) of NO (monitored by Trp Fluorescence at ΔI ~300 nm). The dotted line indicates the SDS concentration at which the presence of NO results in ~20% of the protein being relatively unfolded while the NO-free form remains relatively folded.

The PAC data displays a frequency shift in the sample acoustic wave in comparison to the reference acoustic wave (see Figure 8.3). Deconvolution of the PAC traces revealed three kinetic processes. Specifically, a prompt phase occurring with a lifetime faster than the resolution of the PAC detector (<20 ns), a second phase with a lifetime of 987 ns and a third phase with a lifetime of 10 μs was extracted.

**8.3.1 Prompt Phase:**

In the presence of ~0.2 mM SDS, the fraction of Fe$^{3+}$CT-Cc was found to be ~20% unfolded and, therefore, the total heat released ($Q_{\text{total}}$) to the solvent for the prompt phase has two components that have to be taken into consideration (see Chapter 3). Hence, equation 3.1 in Chapter 3 allows for ΔH to be calculated (as described in Chapter 2) for the prompt phase. The prompt phase (<20 ns) was best fit to a single exponential decay (β = 1) function with an
endothermic $\Delta H = 66 \pm 5$ kcal mol$^{-1}$ and a $\Delta V$ of $-30 \pm 1$ mL mol$^{-1}$ after scaled to the quantum yield of NO photolysis ($\Phi_{NO-CT-Cc} = 0.23$).

**Figure 8.3:** (Left) Overlay of PAC traces for the NO photo-dissociation of NO-Fe$^{3+}$CT-Cc frequency shifted (red) calorimetric reference (black), three component fit of the sample acoustic wave (blue) and the residuals (green). Protein concentration is $\sim$15 µM in 0.2 mM SDS (50 mM HEPES buffer, pH $\sim$7.5. (Right) Plot of $\phi E_{hv}$ vs. $C_p/\beta$ for the three components associated with NO-Fe$^{3+}$CT-Cc photolysis.

The observed $\Delta H$ and $\Delta V$ are divided into contributions are as discussed in Chapter 6 (see Equations 6.0 and 6.1) and gives rise to a $\Delta H_{Conf}/\Delta V_{Conf}$ of 49 kcal mol$^{-1}$ and $-34$ mL mol$^{-1}$ respectively. The exact origin of $\Delta H_{Conf}$ (49 kcal mol$^{-1}$) is unclear. In Chapter 6, we proposed a mechanism for native Fe$^{3+}$Cc-SDS fast folding in which the prompt phase included the system equilibrium being driven to a more unfolded conformation ($\sim$25% $\rightarrow$ $\sim$75% relatively unfolded) upon NO photo-release. However, in the current study, this appears to not be the case for Fe$^{3+}$CT-Cc-SDS. Aside from the initial $\sim$20% unfolded complex ($\sim$0.2 mM SDS) in the presence of NO, a closer observation of Figure 8.2 shows that in the absence of NO (at this same concentration) the system equilibrium is driven to a near-native conformation that is at least $\sim$95% folded. Hence, it appears that Fe$^{3+}$CT-Cc-SDS has the same general paradigm for the initial folding events occurring as native ferrous Cc. If this is indeed the case, the prompt phase of Fe$^{3+}$CT-Cc refolding occurs from a partially folded state that likely undergoes a hydrophobic
collapse into a more compact conformation \((U \rightarrow U^* \rightarrow U', \text{ see Figure 1.9})\). It is noteworthy to point out that this burst phase occurs before coordination of Met-S=O/His to the Fe\(^{3+}\) heme. The MG stabilization is characterized as having hydrophobic interaction and secondary structure contributing to the thermodynamics\(^{[5]}\). Therefore, it seems reasonable that \(\Delta H_{\text{Conf}}\) of 49 kcal mol\(^{-1}\) primarily arises from interactions present in the MG conformation (contributing energies of \(\sim-5-40\) kcal mol\(^{-1}\)\(^{[6-8]}\)) Any additional \(\Delta H_{\text{Conf}}\) is also likely to include exposure of Lys residues around the heme cavity (electrostatic interactions contributing energies of \(\sim-3-8\) kcal mol\(^{-1}\)), which is consistent with \(\sim1-3\) Lys potentially exposed to the SDS molecules. However, it cannot not be excluded that \(\Delta H_{\text{Conf}}\) could also result from electrostatic interaction between the negatively charged head groups of the SDS molecule and the solvent.

For \(\Delta V_{\text{Conf}}\), the remaining process occurring within the excitation is found to be \(-34\) mL mol\(^{-1}\) \((-56.5\) Å\(^3\) molecule\(^{-1}\)). The same arguments for \(\Delta H_{\text{Conf}}\) can be applied to \(\Delta V_{\text{Conf}}\). Particularly, the magnitude of \(\Delta V_{\text{Conf}}\) \((-34\) mL mol\(^{-1}\)) is likely the result of the potential electrostriction event occurring. As the molar volume change associated with solvation of a single charge is \(-20\) mL mol\(^{-1}\), for anion solvation.

### 8.3.2 Slow Phases:

The two additional phases occurring in Fe\(^{3+}\)CT-Cc-SDS are less clear. However, photodissociation of NO opens up the Fe\(^{3+}\) heme distal coordination site potentially allowing for intermolecular ligands to compete for the vacant site upon the protein refolding. Both processes were best fit to a single exponential decay \((\beta = 1)\) with a \(\Delta H_{\text{obs}}\) of \(41 \pm 4\) kcal mol\(^{-1}\)/\(\Delta V_{\text{obs}}\) of \(14 \pm 1\) mL mol\(^{-1}\) (987 ns phase) followed by a \(\Delta H_{\text{obs}}\) of \(132 \pm 32\) kcal mol\(^{-1}\)/\(\Delta V_{\text{obs}}\) of \(-3 \pm 5\) mL mol\(^{-1}\) (10 \(\mu\)s phase). In modified ferrous Cc (Fe\(^{2+}\)CT-Cc-SDS) folding studies (discussed in Chapter 5),
the two slower rates were the result of ligand competition between U’→ U’Met-S=O and U’→ U’His (see Figure 1.9). Though this is likely the case for Fe$^{3+}$CT-Cc-SDS (since it appears to follow a similar folding mechanism), no assignment of the ligands (Met-S=O or His) can justifiably be made for the observed kinetics. Unfortunately PAC measurements do not provide structural assignments for which ligands are bound to the heme. Hence, it is not obvious which ligand is associated with the observed PAC kinetic phases ($\sim$1 µs and $\sim$10 µs). In addition, the foldons of ferric CT-Cc have not been characterized nor are there studies conducted on S=O binding to Fe$^{3+}$ porphyrin model systems (as there are for the ferrous form) to aid in elucidating Met-S=O binding energetics in Fe$^{3+}$CT-Cc folding. However, we propose two folding models that can occur in Fe$^{3+}$CT-Cc initial folding process. The first proposed mechanism is similar to the ferrous form, discussed in Chapter 5. Subsequent to a hydrophobic collapse of the protein is then mediated by ligand competition for binding between U’→ U’Met-S=O and U’→ U’His occurs in $\sim$1 µs ($\tau_1$) and 10 µs ($\tau_2$), respectively. The second mechanism takes into consideration the proposed idea that native ferric Cc is known to bypass coordination of the Met80 ligand and instead forms bis-His coordination that slows down the folding the kinetics. Hence $\tau_1$ is potentially due to hydrogen bond forming between Tyr67 and Met-S=O (as seen in the absence of denaturant, discussed in Chapter 7) upon the protein refolding thus preventing Fe$^{3+}$-S=O coordination and $\tau_2$ results in the formation of bis-his ligation of the heme. Nevertheless, Fe$^{3+}$CT-Cc-SDS folding studies seem to be in agreement with Fe$^{3+}$CT-Cc studies in the absence of denaturant (see Chapter 7) as well as Fe$^{2+}$CT-Cc-SDS folding studies (see Chapter 5) in which oxidation of the Met sulfur atoms impedes the kinetics and affect the thermodynamics associated with Cc fast folding.
Temperature dependence measurements from PAC allowed for the activation parameters associated with the indistinguishable ligand binding of the 987 ns and 10 μs phases to be calculated (Figure 8.4). The activation energies for ligand association was found to be $\Delta H^\ddagger = 27 \pm 14 \text{ kcal mol}^{-1} / \Delta S^\ddagger = 5 \pm 24 \text{ cal mol}^{-1} \text{ K}^{-1}$ and $\Delta H^\ddagger = 7 \pm 5 \text{ kcal mol}^{-1} / \Delta S^\ddagger = -28 \pm 9 \text{ cal mol}^{-1} \text{ K}^{-1}$, respectively. The folding process of Fe$^{3+}$CT-Cc seems to favor the 10 μs phase binding to the heme iron whereas the 987 ns coordination phase has an activation barrier that is almost 4x higher. Hence, the fast folding mechanism of Fe$^{3+}$CT-Cc is a thermodynamically driven process.

![Erying plot of the phases of Fe$^{3+}$CT-Cc](image)

**Figure 8.4:** Erying plot of the (black) ~1 μs and (red) ~10 μs phases of Fe$^{3+}$CT-Cc in 0.2 mM SDS, (50 mM HEPES buffer, pH~7.5).

Figure 8.5 displays kinetic difference spectrum of NO-Fe$^{3+}$CT-Cc subsequent to NO photolysis and the equilibrium difference spectra of NO-Fe$^{3+}$CT-Cc. CT-Cc transient difference spectra displays a minimum at ~417 nm, a maximum centered at ~395 nm, and an isosbestic point centered around ~400 nm. The equilibrium difference spectrum exhibits a maximum at ~402 nm and a minimum at ~417 nm. There are clear differences in the transient spectrum of
CT-Cc relative to the two equilibrium difference spectra. This is indicative of the transient species being 5-coordinate high-spin following NO photolysis, on relatively fast timescales.

![Graph](image)

**Figure 8.5:** (Black line): Equilibrium difference spectra of NO-Fe³⁺CT-Cc subtracted from Fe⁵⁺CT-Cc. (Red triangles): Kinetic difference spectrum of Fe³⁺CT-Cc constructed from various single wavelength transient absorption traces over time, plotted 500 ns subsequent to photolysis.

Examination of the single wavelength transient absorption decay at the minimum (417 nm) reveals a biphasic exponential process with rate constants of $3 \times 10^2$ s⁻¹ and $2.3 \times 10^3$ s⁻¹ (Figure 8.6, right). Transient absorption data obtained at the isosbestic point (407 nm) in the kinetic difference spectrum can also be fit to a biphasic exponential process. Specifically, the decay has an increase in absorbance with rate constants of $2.5 \times 10^2$ s⁻¹ and $1.4 \times 10^3$ s⁻¹ (Figure 8.6, left). In addition, the maximum (407 nm) in the kinetic difference spectrum was also a biphasic exponential process with an increase in absorbance that has rate constants of 90 s⁻¹ and $1.4 \times 10^3$ s⁻¹ (Figure 8.6, left).
Figure 8.6: (Left) Single wavelength transient absorption traces of NO-Fe$^{3+}$CT-Cc-SDS obtained at 395 nm (blue) (maximum in the kinetic difference spectrum) and 400 nm (red) (isosbestic point in the kinetic difference spectrum) both increasing in absorbance with biphasic decays. (Right) Single wavelength transient absorption trace obtained at 417 nm (minimum in the kinetic difference spectrum).

Concentration dependence study of NO (data not shown) on Fe$^{3+}$CT-Cc revealed that the fast phase, $\sim 700 \mu$s ($\sim 1.4 \times 10^3$ s$^{-1}$), is second order with respect to NO. Hence, the slowest phase, $\sim 11$ ms ($\sim 90$ s$^{-1}$), is attributed to the native-like conformation. Overall, it appears that Fe$^{3+}$CT-Cc has the same general paradigm for folding as native Fe$^{2+}$Cc that Kumar$^{9}$ and Jones$^{10}$ previously proposed as well as Fe$^{2+}$Cc-SDS fast folding discussed in Chapter 3. The PAC data obtain have allowed for a thermodynamic profile for the fast folding events occurring in the reaction pathway is summarized by figure 8.7.

Figure 8.7: Thermodynamic profile for the enthalpy (orange) and corresponding molar volume (teal) changes for NO-photolysis of Fe$^{3+}$CT-Cc fast folding events in the presence of $\sim 0.2$ mM SDS.
8.4 References

CHAPTER 9: SUMMARY AND FUTURE PERSPECTIVE

9.1 Summary of Cytochrome-c Fast Folding

The earliest events occurring in protein folding is key to understanding how an unfolded protein can form into its native conformation on physiological timescales. For this reason, horse heart Cc has become an ideal systems for understanding the mechanism of protein folding.\textsuperscript{[1-12]} Cytochrome-c heme group is covalently bonded to the polypeptide chain (providing a spectroscopic probe) and has the unique ability to unfold and refold reversibly without degradation of the protein. In addition, the single Trp residue (at position 59) serves as a built-in fluorescent probe that provides a good indicator of conformational changes that can be observed in low to highly concentrated solutions of denaturants (i.e.: SDS, GdnHCl, urea, etc.).\textsuperscript{[13]} Jones et al.\textsuperscript{[14]} took advantage of this in addition to demonstrating that in the presence of a denaturant, the axial Fe\textsuperscript{2+}-Met80 bond is destabilized and can be displaced by CO. The displacement of the Met80 ligand with CO triggered the unfolding of Fe\textsuperscript{2+}Cc and photolysis of CO initiates refolding. In this dissertation the progression of Fe\textsuperscript{2+}, Fe\textsuperscript{3+}, and modified Cc fast refolding and/or unfolding were monitored through PAC utilizing CO or NO photo-initiation methods. Overall, the PAC data provided information regarding direct kinetics, enthalpy, and molar volume changes associated with early folding events in an effort to better understand the mechanism through which protein folding occurs (on fast timescales).

In the case of ferrous Cc folding, native Cc-SDS versus Kumar\textsuperscript{[15]} and Jones\textsuperscript{[14]} folding studies on native Cc-GdnHCl revealed that the rate constants varied between each system depending upon the denaturant used. However, regardless of denaturant the general paradigm for
folding is a uniform mechanism for the early events (see Figure 1.9, Chapter 1). Specifically, the mechanism starts with a partially unfolded CO-bound Cc (UFeco), following CO photolysis a transient phase (high spin, U → U*Fe → U’) that is described as a hydrophobic collapse happens faster than the resolution time of the PAC detector (<20 ns), followed by competition ligand rebinding with fast coordination of Met (U’ → U’Met, τ = ~600 ns) and non-native His (U’ → U’His, τ = ~4 μs) to the Fe2+ heme that are kinetically driven. The PAC measurements revealed one population that included a ‘frustrated’ folding pathway due to non-native coordination of His to the heme (exhibiting heterogeneous kinetics) and the second population involved Met binding allowing for the native-like conformation to ensue (exhibiting a homogeneous kinetics). Destabilizing the Met ligands (via oxidation of the sulfur atom) affects both the thermodynamics and rates relative to the native form, but not the general paradigm folding mechanism. Specifically, PAC revealed three kinetics phases that also included a partially folded conformation undergoing a hydrophobic collapse, occurring in <20 ns. This phase is then followed by intramolecular binding between ligands (U’ → U’Met-S=O and U’ → U’His) occurring in ~830 ns and ~7 μs, respectively. While there are clear differences in the enthalpy and molar volume changes of the modified vs. native form, the most significant distinction arose from Fe2+CT-Cc preferential His binding. In addition, the non-native His coordination exhibited homogenous kinetics relative to the native form. We propose that this is likely due to an accumulation of kinetic barriers that traps the protein proceeds in a frustrated pathway into a near native-like conformation.

In the case of ferric Cc unfolding, PAC studies conducted on native Cc-SDS and Cc-GdnHCl shows that the energetics definitely depends upon the way in which the system is denatured (GdnHCl vs. SDS molecules). Thus, the denaturant affect the rate in which events are
happening and the thermodynamics associated with those steps involved. However, the unfolding process remains a uniform mechanism between the two ferric systems examined. In addition, it is clear that the overall charge of the heme matters (Fe$^{2+}$- (0) versus Fe$^{3+}$- (+1) heme, at pH~7). Not only does the charge influence which external ligand (CO vs. NO) is able to bind to the heme, as it also appears that the charge of the heme affects the folding mechanism (in comparison to the ferrous form). In our hands, ferric Cc in the presence of denaturant and subsequent to NO photo-release drove the system equilibrium to an unfolded conformation (versus a native-like conformation in the ferrous form, following CO photo-release). The PAC data revealed three kinetic phases, all in which exhibited homogenous kinetics. Specifically, the proposed mechanism for early events occurring in Fe$^{3+}$Cc following NO photo-dissociation includes a rapid (< 20 ns) expansion into a secondary partially folded intermediate that is more denatured than the initial partially folded conformation. This phase is followed by a ~860 ns phase that is likely due to the interior of the protein becoming solvent-exposed including solvation of amino acids in the interior of the protein being exposed to the solvent, breakage of hydrophobic interactions, etc. and finally leads to a 5-coordinate (Cc*) high-spin complex that binds some unknown ligand (L) directly to the heme (~6 μs). Destabilization of the Met ligands to ferric Cc mechanism is less understood. PAC also revealed three kinetic phases all displaying homogenous kinetics and the sulfoxides impedes the kinetics and affect the thermodynamics associated with Cc fast folding. But more interesting is that the systems equilibrium was shifted towards a native-like conformation upon NO photo-release. Hence, it appears that Fe$^{3+}$CT-Cc has a similar paradigm for the initial folding events occurring as native ferrous Cc (and modified ferrous Cc). If this is indeed the case, the prompt phase of Fe$^{3+}$CT-Cc refolding occurs from a partially folded state that likely undergoes a hydrophobic collapse into a more compact
conformation and occurs < 20 ns. Although kinetics, enthalpy, and molar volume changes were measured for each resolvable phase via PAC, it was not possible to identify the specific events and/or which ligands are associated with the two slower kinetic phases (~990 ns and ~10 μs). However, we propose two folding models that can occur following the hydrophobic collapse phase. The first is to consider the protein is mediated by ligand competition for binding between U’ → U’Met-S=O and U’ → U’His, occurring in ~1 μs (τ1) and 10 μs (τ2), respectively. A second event to consider is that τ1 potentially forms a hydrogen bond between Tyr67 and Met-S=O (preventing Fe^{3+}-S=O coordination) and τ2 results in the formation of bis-his ligation of the heme as seen in previous Fe^{3+}Cc folding studies.[16-20]

9.2 Future Direction: Photo-thermal Studies on Cc Fast Folding in a “Crowded” Environment

Proteins folding inside cells consist of an environment that is very different from functioning in buffer solutions.[21-23] One main reason is that the intracellular environment of a cell is known to be highly crowded due to the presence of large amounts of macromolecules (i.e.: proteins, nucleic acids, ribosomes, carbohydrates, etc.). Macromolecules, often referred to as crowding agents, occupy large portions of volume in the cell thus excluding intercellular space to other biomolecules. Crowding has been shown to affect a series of processes, such as protein stability and folding.[21-23] Specifically, studies have suggested that denatured populations of protein occupy larger conformational space and, therefore, are likely to be most affected by what authors referred to as “excluded volume” space.[21-23] Hence, it is necessary to understand the mechanism of protein folding in a crowded environment, particularly Cc folding via PAC. Since Cc fast folding thermodynamics and kinetics are well understood in solution, one can now
analyze if a crowded environment affects the folding mechanism, rates, and energetics. More importantly, molecular crowding can be mimicked experimentally by the use of crowding agents; such as Dextran 40, Dextran 70, and Ficoll 70 to name a few. These crowding agents are known to be highly branched polysaccharide unit that are neutral, inert, and occupy space without interacting with proteins.[21-23]

9.3 References