DESIGN, CHARACTERIZATION, AND ELECTRON TRANSFER PROPERTIES OF SYNTHETIC METALLOPROTEINS

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A Dissertation

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ABSTRACT

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The binding of Cu(I) to the random coil peptide C16C19-GGY produces a self-organized metal-peptide assembly which displays an intense room-temperature luminescence at 600 nm. It was shown that this synthetic metalloprotein exists as a 4-helix bundle which contains a cyclic Cu₄S₄ cofactor in which each Cu(I) atom is bridged by two cysteine residues and has a terminal N/O ligand. The strong luminescence of the Cu(I) protein suggests that it might function as a photoinduced electron-transfer agent. The emission follows biexponential decay kinetics with \( \tau_1 = 1.0 \, \mu\text{sec} \) and \( \tau_2 = 7.5 \, \mu\text{sec} \). These components have approximately equal amplitudes and the results indicate that the Cu₄S₄ cofactor contains two independent lumophores. Both lifetime components are quenched by a series of [Ru(NH₃)₅L]³⁺ (L = chloro, amine, lutidine, pyridine, nicotinamide, and 3,5-dimethyl pyridine dicarboxylate). The quenching mechanism is assigned to a photoinduced electron-transfer event by transient spectroscopy. The results show the occurrence of bimolecular forward electron-transfer in the inverted Marcus regime.

Electron-transfer (ET) reactions occur between a negatively charged cyclic metallopeptide \([\text{Ru(bpy)}_2(\text{phen-am})-\text{cyclo(Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu})]^{3-} = \text{Rucyclic}\), and ferricytochrome \(c = \text{cyt} \, c\), in which an acetamido linker was used to attach the ruthenium polypyrindyl complex to the cysteine side chain of a head to tail cyclic peptide. In the presence of cyt \(c\), the triplet state of ruthenium metallopeptide decays via parallel pathways that involve two different encounter peptide-protein complexes. That the electron transfer rate constants of both encounter complexes decrease with increasing viscosity demonstrates that the kinetics are gated by rate-limiting configurational changes occurring within the complexes. NMR experiments
confirm that two separate conformations exist for Rucyclic. The two conformations of Rucyclic might be arising from the cis/trans isomerization of proline and result in the formation of two encounter complexes when interact with cyt c.

Metallopeptides, 5-Chloro-PhenRuCE₅G with a different redox potential but a similar conformation with those of RuCE₅G give different driving forces of the excited-state ET reaction. The reorganization energy (λ) and donor-acceptor separation (r) of the preformed complex between metallopeptides and cyt c are determined to be 1.25 eV and 16.4 Å by measuring the actual electron transfer rates of these four metallopeptides at their most stable configurations. These values are comparable with those of some protein-protein systems reported previously.
To my parents,

Fangzheng Hong and Guilan Liu,

and to my sister Lin Hong for their love and support.
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CHAPTER I
ELECTRON TRANSFER PROPERTIES OF A DESIGNED MINIATURE COPPER (I) PROTEIN

I.1 Introduction and Background

Metalloproteins comprise approximately one-third of all structurally-characterized proteins and are vital to biological energy conversion in photosynthesis, respiration, and a growing number of signaling processes governing gene regulation and expression. Long-range electron transfer has often been studied in different types of protein models due to its fundamental role in mechanisms of photosynthesis, the process of chemical and biological energy conversion. Thus, incorporating electron-transfer functionality into synthetic metalloproteins has been an attractive target in the field of protein design and engineering.

The ultimate goals of protein design are the engineering of synthetic metalloproteins whose chemical functions and physical/chemical properties can be both tailorable and distinct from those found in the naturally occurring systems. Much work in this field has focused on either re-engineering native proteins to enable them to perform new chemical functions or de novo design of artificial proteins whose chemical reactivity can be incorporated through rational synthesis. Notable examples of metalloprotein design include the well-studied four-helix bundle heme proteins, which will be discussed in detail in this chapter, the designed metalloprotein in which two self-associated helix-loop-helix units are used to bind two separate iron atoms,\(^1\)-\(^4\) the bridged Ni(II)-(\(\mu_2\)-SCys)-[Fe\(_4\)S\(_4\)] protein in which the helix-loop-helix motif creates a bridged metal binding site,\(^5\) and several series of cysteine-containing helical bundle proteins.\(^6\)-\(^15\)
1.1.1 Designed electron-transfer active metalloproteins

There are two fundamentally different approaches to achieving the construction of novel electron transfer and redox catalysts. The “bottom up” approach refers to the de novo design of polypeptide scaffolds, designed to accommodate metallic cofactors; and “top down” approach refers to the re-engineering of evolved metalloproteins to perform new redox chemistry. Recent progress in electron-transfer active protein design is expanding both the types of protein scaffold available to the designer and the range of metals and redox factors that can be incorporated.

Four-α-helix bundles have proven to be a particularly versatile structural motif since many cofactors that may serve as active sites can be implemented. Several research groups have described the synthesis of heme protein models which usually consist of four-helix bundle that can accommodate a histidine-ligated heme in the hydrophobic interior. Pioneering work by Dutton and co-workers incorporated the hydrophobic di-heme subunits of the membrane redox protein cyt $bc_1$ into a prototype hydrophilic four-helix heme-binding maquette. However, a further challenge is to obtain structural information when an amphiphilic maquette is organized at an interface, either in a membrane, an air/aqueous interface, self-assembled onto a solid semiconductor or electrode substrate as part of a device. In the past five years, some progress has been made in this area. Firstly, de novo design of a cyt $b$ maquette for electron transfer and coupled reactions on electrodes were achieved. A four-α-helix bundle redox protein, which contains charged surface patches, like native cyt $c$, and bis-histidine ligated protoporphyrin IX (heme), like native cyt $b$, was designed and synthesized. The positively charged residues facilitate adsorption to negatively charge modified gold electrodes surfaces and aid Cyclic Voltammetry (CV) measurements. CV demonstrates the reversible electrochemistry typical for
cyt b and pH dependence of redox midpoint potentials reveals the coupling of the b-heme oxidation and reduction to proton exchange.

Furthermore, a family of four-α-helix bundle peptides, AP0, AP1, AP2 and AP3 (for amphiphilic protein n) were designed to possess both hydrophilic and hydrophobic domains along the bundle’s exterior. In addition to possessing bis-histidyl metalloporphyrin binding sites at one (AP3) or two (AP2) positions within the hydrophilic domain, AP0 and AP1 also possess a bis-histidyl metalloporphyrin binding site at one position within their hydrophobic domain. This is a key development toward creating an artificial electron transfer chain across the interface between polar and nonpolar media since binding appropriate electron donor and acceptor cofactors at these sites would permit electron transfer along the long axis of the bundle. In addition, their vectorial insertion across the interface between polar and nonpolar media indicates that the electron-transfer properties of the individual molecules can be translated into a macroscopic property of the interface.\textsuperscript{21}

In related works, Haehnel and co-workers\textsuperscript{22-26} have been conducting elegant studies on the functional metalloprotein design using a template-assisted synthetic four α-helix bundle as the scaffold. A family of four-α-helix bundles was constructed based on a cyclic peptide as template-assisted synthetic protein (TASP) to ensure prealignment of the individual helices. Stable copper binding sites (two histidines and one cysteine) were incorporated into four-helix-bundle TASP constructed by three different helices with only sixteen amino acid residues based on the structure of Cu-Mop5.\textsuperscript{22} Alteration of the steric interactions in the vicinity of the His$_2$Cys ligand were found to be the origin for the conversion of a tetragonal to a tetrahedral copper binding site. Further, by introducing one bis-histidine binding site into the hydrophobic interior, TASP proteins were able to incorporate a single heme group or other metalloporphyrin (Fe$^{3+}$,
Co$^{3+}$, or Zn$^{2+}$). Different functional properties, including redox activity$^{26}$ and the capability of light induced electron transfer$^{23}$ were introduced into this modular protein.

There are still more examples$^{27-29}$ which use the four-helix-bundle as a building motif for the design of electron-transfer active proteins. All the above studies demonstrate that incorporation of different metals or cofactors into the same protein scaffold could enable the protein maquette with different functional properties.

I.1.2 Marcus theory of electron transfer

During the past half-century, the field of electron transfer has been greatly advanced by the detailed analytic theory developed by R. A. Marcus$^{30}$ and also by the introduction of new technology, such as photochemical initiation. In the Marcus theory, the rate constant $k$ for intramolecular electron transfer is given by $k = \kappa_{el} \nu_n \kappa_n$, where $\kappa_{el}$ is the electronic transmission coefficient, $\nu_n$ is a nuclear vibrational frequency, and $\kappa_n$ is the nuclear factor which can be expressed as

$$\kappa_n = \exp[-(\Delta G^0 + \lambda)^2 / 4\lambda k_B T]$$

If there is a substantial mixing of the reactant electronic states, the value of $\kappa_{el}$ is close to unity, and the electron transfer is termed adiabatic. When the redox sites are far apart, the electronic coupling between them will be weak, and the reaction will be nonadiabatic ($\kappa_{el} \ll 1$). Under these conditions the product $\kappa_{el} \nu_n$ is independent of $\nu_n$ and given by

$$\kappa_{el} \nu_n = \frac{2\pi}{h\sqrt{\pi\lambda k_B T}} H_{AB}^2$$
The rates of non-adiabatic electron transfer reactions are given by Fermi’s golden rule,\textsuperscript{30,31} where the quantity (FC) is the “Frank-Condon” factor which refers to the overlap of the nuclear wave functions of the reactant and product.

\[ k_{ET} = \frac{2\pi}{\hbar} H_{AB}^2 FC \]

The semiclassical formalism predicts that the Frank-Condon factor, thus the nonadiabatic electron transfer rate \( k_{ET} \) between redox active proteins depends not only on the driving force for the reaction \( -\Delta G^0 \), but also on the reorganization energy \( \lambda \), and the electronic coupling \( H_{AB} \) between reactants and products in the transient state as expressed by Equation II.1.\textsuperscript{30}

\[ k_{ET} = \frac{2\pi^2}{\hbar \sqrt{\pi \lambda k_B T}} H_{AB}^2 \exp\left[-\frac{(\Delta G^0 + \lambda)^2}{4\lambda k_B T}\right] \quad \text{(Eq. I.1)} \]

The most important prediction of the Marcus theory of electron transfer is that as the driving force of the electron transfer reaction increases, the electron transfer rate will initially increase, reach a maximum, and then decrease in the region where the driving force is larger than the reorganization energy \( -\Delta G^0 > \lambda \).\textsuperscript{30,32} This strongly exergonic regime is generally referred to as the Marcus inverted regime. The reorganization energy \( \lambda \) is the energy required to structurally reorganize the donor and acceptor as well as their solvation spheres upon electron transfer and reflects nuclear configuration displacements from the reactant to the product state. This parameter considers the donor and acceptor pair before and after electron transfer and is comprised of two components. The inner-sphere reorganization energy \( \lambda_i \) reflects redox dependent nuclear perturbations of the redox centers, such as changes in bond lengths, as a result of the electron transfer. The outer-sphere reorganization energy \( \lambda_o \) reflects changes in the
surrounding medium, such as changes in solvent orientation, due to the electron transfer. For biological systems, \( \lambda_0 \) may also include configurational changes in the protein matrix.

The electronic coupling between electron donors (D) and acceptors (A) is a function of the distance \( (r) \) between donors and acceptors. Dutton and co-workers\(^ {33} \) have found that in a wide range of biological systems \( k_{ET} \) decreases exponentially with the distance between the redox centers according to Equation II.2.

\[
k_{ET} = k_0 \exp[-\beta(r - r_0)]\exp[-\frac{(\Delta G^0 + \lambda)^2}{4\lambda RT}]
\]

(Eq. I.2)

The product \( \kappa_{el} \nu_n \) is given by

\[
\kappa_{el} \nu_n = 10^{13} \exp[-\beta(r - r_0)]
\]

where \( r_0 \) is defined so that the reaction is adiabatic (i.e., \( \kappa_{el} = 1 \)) at \( r = r_0 \). Meanwhile, the electronic coupling between two proteins is also determined by the specific arrangement of the transient protein-protein complex which is determined by local interactions such as hydrogen bonds, \textit{van der Waals} interactions as discussed by Beratan,\(^ {34-36} \) and hydrophobic and electrostatic interactions. Therefore, by determining electron transfer reaction rates under different conditions, such as different temperature and reaction driving forces, much useful information can be obtained on an electron-transfer active protein, such as the reorganization energy, electronic coupling, and distance between redox centers.

\textit{1.1.3 Bimolecular electron transfer reactions}

Marcus theory envisages three kinetic regimes for electron transfer reactions depending on the driving force range:
(1) a “normal regime” for small driving forces ($-\Delta G^0 < \lambda$)

(2) an “activationless” regime ($-\Delta G^0 = \lambda$)

(3) an “inverted regime” for strongly exergonic reactions ($-\Delta G^0 > \lambda$)

The existence of the Marcus inverted region has been well established in fixed distance electron transfer reaction,\textsuperscript{37-47} such as those which are covalently-bound, in frozen media, or in electrostatic complexes of proteins. However, observations of the inverted region in bimolecular photoinduced electron transfer reactions are extremely rare. These reactions follow the Rehm-Weller behavior,\textsuperscript{48,49} as depicted in Fig I.1, as the driving force increases, so does the bimolecular rate constant for the reaction (the normal region); at some point, even though the driving force continues to increase, the rate constant levels off because the rate-limiting step is now the diffusion of the reactants together. In all these above cases, the distance between the donor and acceptor is constant, thus circumventing diffusion, and making the electron transfer reaction a unimolecular process.

![Rehm-Weller plot](image)

**Figure I.1** Rehm-Weller plot.
Because of the Rehm-Weller behavior, bimolecular electron transfer systems exhibiting the Marcus inverted region remained unexplored for around twenty-five years until Gray and co-workers\textsuperscript{50,51} reported the first direct observations of inverted region behavior for a bimolecular reaction. In their studies, the back electron transfer between an excited iridium complex and various pyridinium quenchers clearly exhibit an inverted driving-force dependence for $-\Delta G^0 > 1.0$ eV while the forward electron transfer rates remain diffusion-limited. Four years later, Turro et al.\textsuperscript{52} reported the measurement of the photoinduced bimolecular reaction rates of a homologous series of electronically excited *Ru(II) diimines with cyt c, both in its oxidized and reduced forms. The electron transfer rate constants for *Ru(II) diimine/Fe(II) cyt c reaction increase and approach the diffusion limit of $8.8 \times 10^8$ M$^{-1}$s$^{-1}$ at $-\Delta G^0 = 0.7$ eV. At a higher driving force, the rate for electron transfer is observed to drop off. Similar results were achieved in *Ru(II) diimine/Fe(III) cyt c system when $-\Delta G^0 = 1.12$ eV. Utilizing the known electron transfer properties of cyt c and the Ru(II) diimine complexes, inverted region behavior for the forward bimolecular electron transfer of Fe(II) and Fe(III) cyt c were observed. The intramolecular reorganization energies for both cyt c and Ru(II) diimine complexes are minimal, and the modest reorganization energies associated with solvent, lead to a shift of the maximum of the Marcus curve to lower driving force.\textsuperscript{52} By displacing the Marcus curve vertically below the diffusion limit and shifting it horizontally to lower driving force, direct observation of the inverted region in these Fe(II)/Fe(III) cyt c and the Ru(II) diimine complexes systems were achieved.

From the research of Turro et al., it seems that the bimolecular Marcus inverted region should be discerned for those electron transfer reactions with an electron transfer rate sufficiently small compared to the rate of diffusion and a moderate reorganization energy to allow easy access of the inverted region. Based on this, Fukuzumi et al.\textsuperscript{53} studied the electron transfer
between fullerenes (C\textsubscript{76} and C\textsubscript{78}), which have small reorganization energies due to their highly delocalized \(\pi\)-electron systems, and a series of arene \(\pi\)-radical cations in dichloromethane. The driving force dependence of \(\log k_{et}\) shows a pronounced decrease (about 20 times) towards the highly exothermic region. Another example of electron transfer systems exhibiting the bimolecular Marcus inverted region is ruthenium (II)-polypyridyl complexes with phenolate ions in aqueous medium.\textsuperscript{54} It seems that if a suitable homogeneous reaction series is chosen with appreciable change in driving forces, but maintaining other parameters constant, such as \(\lambda\) and \(d\), it is possible to observe the Marcus inverted region in the bimolecular charge-separation reactions.

\textit{1.1.4 Background}

In this chapter, a more native-like, copper (I) binding peptide, C16C19GGY was designed not only to have the metal binding function, but also to be used as an electron transfer agent. The coiled-coil motif was chosen as a model system for protein design because of its simplicity, regularity, and diversity in oligomerization state. The synthetic coiled-coil sequence of C16C19GGY consists of four repetitions of the seven amino acid residue heptad, denoted by the letters (\textit{abcdefg}). Positions \(a\) and \(d\) of the heptad are occupied by hydrophobic amino acids, in this case, Ile and Leu, to form a hydrophobic core. Further, the Glu and Lys residues at positions \(e\) and \(g\) of the heptad, respectively, are positioned to form favorable interhelical charged interactions if they interact in the desired parallel coiled coil. Positions \(b\), \(c\), and \(f\) of the heptad repeat are occupied by hydrophilic residues, exposed to the solvent. A helical wheel diagram (Figure I.2) illustrates a parallel two-stranded \(\alpha\)-helical coiled coil.
Figure I.2 Schematic views of synthetic coiled coil. Top: primary amino acid sequence of the two helices of the coiled coil. Left: helices in parallel orientation. Right: helical wheel diagram of the coiled coil; view down helical axes of the coiled coil (adapted from reference 55).

The (IEALEGK) heptad used in C16C19GGY has been shown to self-assemble into two-stranded $\alpha$–helical coiled coils by a combination of circular dichroism (CD) spectroscopy, high performance size exclusion chromatography (HPSEC), analytical ultracentrifugation (AUC), and electron paramagnetic resonance (EPR) line broadening experiments. In C16C19GGY, the $a$ and $d$ positions of the third heptad was modified by substituting cysteine residues at position 16 and 19 to construct a potential metal-binding site within the hydrophobic core of the coiled coil. The Cu(I) adduct of C16C19GGY was shown to fold into a four-stranded coiled-coil as determined by analytical ultracentrifugation (AUC).
Figure I.3 Computer generated model of the Cu(I) adduct of C16C19-GGY. The metalloprotein exists as a four-stranded-helical bundle which contains a cyclic Cu(I)$_4$S$_4$(N/O)$_4$ cofactor.

Kharenko et al.\textsuperscript{56} further reported that the Cu(I) adduct of C16C19-GGY led to the observation of an intense ($\phi = 0.053$) ambient temperature luminescence centered at 600 nm which persists upon allowing the protein to stand overnight under ambient conditions. It is noteworthy that similar photoluminescent properties have been reported for Cu(I) derivatives of the cysteine rich metal-binding protein metallothionein,\textsuperscript{57,58} as well as those of the Cox17 copper chaperone,\textsuperscript{59} and the copper responsive transcription factors ACE1\textsuperscript{60} and CopY,\textsuperscript{61} all of which contain polynuclear copper(I) clusters that are buried within the protein to shield them from bulk solvent.\textsuperscript{62} This is consistent with earlier studies of small molecule Cu(I)-thiolate compounds in which luminescence is observed for polynuclear metal clusters where metal-metal interactions play an important role in stabilizing the emissive photoexcited-state.\textsuperscript{63} Thus, the observation of a 600 nm luminescence from Cu(I)-C16C19GGY thus suggests that it likely contains a polynuclear Cu(I) cluster.
The results of metal-ion titrations and X-ray absorption studies were consistent with the presence of a cyclic Cu(I)$_4$S$_4$(N/O)$_4$ cofactor in the protein in which each Cu(I) atom is coordinated to two bridging cysteine residues and has either a terminal nitrogen or oxygen ligand. It was found that this luminescence can be quenched by the addition of either ferricyanide, oxygen, or urea to respectively indicate that the emitting species is associated with the reduced Cu(I) state, has significant triplet character, and is quenched upon exposure to bulk solvent. The one-electron reduction potential of the Cu(I) adduct of C16C19GGY was determined by performing redox titrations in combination with UV-Vis absorption and/or steady-state emission spectra as shown in Figure I.5.
Figure I.5 plots the absorbance at 310 nm vs. the measured potential and shows the results of a fit to a single ($n = 1$) Nernst equation which yields a single midpoint reduction potential of 343 mV vs. NHE. Similar results were obtained when the loss of the 600 nm emission was used to monitor the metalloprotein oxidation (not shown). The strong room temperature luminescence of Cu(I)-C16C19GGY suggests that it might function as a photoinduced electron-transfer agent which can be monitored by emission lifetime experiments. In this chapter, the electron transfer properties of this Cu(I)-C16C19GGY metalloprotein is examined.

I.2 Experimental

I.2.1 Materials
The Fmoc-protected L-amino acid derivatives, 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), piperidine, diisopropylcarbodiimide, and anhydrous hydroxybenzotiazole (HOBt) were purchased from Peptides International Inc. (Louisville, KY). The reagents chloropentaammine ruthenium (III) chloride, 3,5-lutidine, nicotinamide, pyridine, 3,5-pyridine dicarboxylic acid, and tetrakis(acetonitrile) copper(I) hexafluorophosphate were purchased from the Sigma-Aldrich Company (St. Louis, MO). All reagents were used as received.

1.2.2 Synthesis of the C16C19-GGY Peptide

The 32-residue peptide, C16C19-GGY, with the sequence of Ac-K(IEALEGK)$_2$(CEACEGK)(IEALEGK)-GGY-amide, was synthesized using the solid-phase methods, on an Applied Biosystems Model 433 A peptide Synthesizer by standard Fmoc chemistry; the 0.25 mmol scale protocol with followed by N-terminal capping protection strategy by acetic anhydride. Activation was achieved by 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT) in DMF. The GGY tag was attached to the peptide to allow determination of the peptide concentration by measuring the absorption of the tyrosine residue at 275 nm ($\varepsilon_{275} = 1450 \text{ M}^{-1} \text{cm}^{-1}$). Deprotection of the amino acid side chains and cleavage from the resin was performed by a reaction with a mixture of trifluoroacetic acid (88% v/v), phenol (5% v/v), triisopropylsilane (1% v/v), 2-mercaptoethanol (1% v/v) and water (5% v/v) for 2.5 hours at room temperature. The crude peptide was then precipitated in cold anhydrous diethyl ether, collected by vacuum filtration and dried under vacuum. Final purification was achieved by preparative reversed-phase C18 HPLC, which were performed on Vydac semi preparative reversed-phase Vydac C18-
column (10 μM particle size, 10×250 mm) or preparative Vydac C-18 column (10 μM particle size, 22×250 mm). Linear gradient acetonitrile/water mixture was used as a mobile phase containing 0.1 (v/v) % trifluoroacetic acid over a course of 90 min using the flow rate of 2 (semi preparative) or 6 (preparative) ml/min. A two pump system (Waters Model 515) equipped with a Waters Model 994 diode array detector/spectrophotometer having 1 cm cell was used. For preparative separations, the monitoring wavelengths were set to 210-230 nm and purity of the collected peptide was verified by analytical HPLC runs. The collected peptide was then lyophilized with followed analysis by MALDI mass-spectroscopy.

1.2.3 Syntheses of Ru(NH$_3$)$_5$L(BF$_4$)$_3$ (L=3,5-lutidine, nicotinamide, pyridine and dimethyl 3,5-pyridine dicarboxylate)

Ru(NH$_3$)$_5$L(BF$_4$)$_3$ (L=3,5-lutidine, nicotinamide, pyridine and dimethyl 3,5-pyridine dicarboxylate) were prepared through literature method$^{65}$ with the following modification: 100 mg of [(NH$_3$)$_5$RuCl]Cl$_2$ (0.343 mmol) was added to a 2 ml of an Ag(I) solution which was made up by dissolving 0.0749 g of silver oxide (0.323 mmol) in 2 ml of hot water by dropwise addition of trifluoroacetic acid. A white precipitate was seen to form immediately. The silver chloride formed was removed by filtration. The light yellow Ru(III) complex solution was bubbled with argon for 30 minutes. Several pieces of zinc mercury amalgam were added to the solution under argon atmosphere. 0.3 g of L (L = 3,5-lutidine, nicotinamide, pyridine and dimethyl 3,5-pyridine dicarboxylate) was added to the ruthenium solution and the solution turned reddish orange immediately. The reaction was allowed to go for 2 hours under argon at ambient temperature. The zinc amalgam was then removed, and approximately 2 ml of concentrated tetrafluoroboric acid was added to the resulting solution. The solution was oxidized by adding several drops of
hydrogen peroxide (30%) and the solution turned colorless. Then 10 ml of absolute ethanol was added. The pale yellow precipitate formed was collected by filtration and washed with ether. If no precipitate upon adding of absolute ethanol, a large amount of anhydrous ether was added. The product was dried under vacuum and purified by preparative reversed-phase C18 HPLC using the linear gradient starting at 0% acetonitrile and increasing to 5% acetonitrile in 20 minutes. The purity of this product was checked by HPLC, electrochemistry, and extinction coefficient.

I.2.4 Electrochemical measurements

Electrochemical experiments were performed on a BAS 100W Electrochemical Analyzer using CV technique. All experiments were performed in aqueous solution. A 2 mL cell was equipped with a platinum working electrode, a platinum wire auxiliary electrode and an Ag/AgCl reference electrode. To achieve higher concentrations, the electrochemistry of the samples was conducted using a 200 μL micro-cell. In this case, the working electrode was contained within this micro-cell, while the auxiliary and reference electrode were placed in a separate compartment connected through the glass frit of the micro-cell. The following equation was used to recalculate the redox potentials:

\[ E \text{ (vs NHE)} = E \text{ (vs Ag/AgCl)} + 196 \text{ mV} \]

The instrument was calibrated using an external Ru(bpy)_3^{2+} standard (E = 1.26 V vs. NHE). All electrochemical measurements of [Ru(NH_3)_5L]^3+ (L= 3,5-lutidine, nicotinamide, pyridine and dimethyl 3,5-pyridine dicarboxylate) were performed in 0.2 M pH 5.4 acetate buffer.

I.2.5 Emission lifetime measurements
Emission lifetimes were measured with a nitrogen-pumped broadband dye laser (2-3nm fwhm) from PTI (GL-3300 N₂ laser, GL-301 dye laser) as described previously. The excitation wavelength was set at 337 nm for fundamental nitrogen excitation or 357 nm for BPBD (356-390nm) dye. The emission of copper/peptide complexes was monitored at 600 nm. Pulse energies were typically attenuated to ~100 µJ / pulse measured with a Molelectron Joulemeter (J4-05). The luminescence was passed through an optical filter (550 nm), a lens systems and an f/3.4 monochromator, all set a 90° angle to the excitation beam. The emission was detected with a Hamamatsu R928 PMT, which was negatively biased with a Stanford Research PS325 power supply. The PMT signal was terminated through a 50 Ω resistor to a Tektronix TDS 380 digital oscilloscope (400 MHz). Typically, 128 laser shots collected at 2-3 Hz were averaged for each data trace. Lifetime measurements were performed on argon-saturated solution of Cu(Ⅰ)(ACN)₄(PF₆), C16C19GGY and [Ru(NH₃)₅L]³⁺ (L=chloro, ammine, 3,5-lutidine, nicotinamide, pyridine and dimethyl 3,5-pyridine dicarboxylate) dissolved in 0.2 M pH 5.4 acetate buffer. The concentrations of stock solution of the C16C19GGY peptides (ca. 200 µM in 0.2 M pH 5.4 acetate buffer) was determined by UV-Vis spectroscopy using value of ε₂₇₅ = 1450 M⁻¹cm⁻¹. The stock solutions of C16C19-GGY were freshly prepared before each experiment and stored under argon. The concentrations of the stock solutions of the higher potential quenchers, [Ru(NH₃)₅L]³⁺ (L = 3,5-lutidine, nicotinamide, pyridine, and dimethyl 3,5-pyridine dicarboxylate) were obtained by reducing Ru(III) to Ru(II) using excess ascorbic acid and measuring the absorption of their MLCT bands. The concentrations of the stock solution of quenchers, [Ru(NH₃)₅L]³⁺ (L = chloro, and ammine) were measured directly by UV-Vis spectroscopy. 0.01 M [Cu(CH₃CN)₄]PF₆ stock solution were prepared by adding known volume of argon-saturated acetonitrile using an air-tight syringe to an argon-purged sealed flask, which
contained a precisely weighed sample of [Cu(CH₃CN)₄]PF₆. Buffer solutions of C16C19-GGY, [Cu(CH₃CN)₄]PF₆ with different quencher concentrations were prepared freshly before each lifetime measurement by mixing different amount of stock solutions of C16C19-GGY, [Cu(CH₃CN)₄]PF₆, quencher and 0.2 M pH 5.4 acetate buffer to a volume of 0.5 mL.

Temperature of the solution was controlled by adjusting the temperature of the cell rack with a Neslab RTE-111 circulation bath. Buffer solutions of C16C19GY and acetonitrile solution of Cu(I)(CH₃CN)₄(PF₆) were prepared freshly before each lifetime measurement by mixing different amount stock solutions of C16C19GGY, Cu(I)(CH₃CN)₄(PF₆), [Ru(NH₃)₅L]³⁺ (L=chloro, ammine, 3,5-lutidine, nicotinamide, pyridine, and dimethyl 3,5-pyridine dicarboxylate), and 0.2 M pH 5.4 acetate buffer to a final volume of 0.5 mL.

1.2.6 Steady-state luminescence measurements

Steady-state luminescence spectra were obtained with a single photon counting spectrofluorimeter from Edinburgh Analytical Instruments (FL/FS 900) as described previously ⁶⁷. The excitation was accomplished with a 450 W Xe lamp optically coupled to a monochromator (± 2 nm), and the emission was gathered at 90° and passed through a second monochromator (± 2 nm). The luminescence was measured with a Peltier-cooled (-30 °C), R995 red-sensitive photomultiplier (PMT).and was recorded over 400-750 nm range with 340 nm long-pass filter. Frozen glass emission spectra at 77K were measured by inserting a 5 mm (I.D.) NMR tube containing 5×10⁻⁵ M solution in glycerol (1:1 (v/v)), into a quartz-tipped finger Dewar filled with liquid N₂. All luminescence experiments used optically dilute solution (OD ≈ 0.1) prepared in spectroscopic grade solvents.
1.2.7 Nanosecond UV-Vis transient absorption spectroscopy

UV-visible laser flash photolysis spectrometer with frequency tripled (355 nm) pulses from a Continuum Surelite Q-switched YAG:Nd laser operating at the 5 Hz repetition rate (or a Spectra-Physics Quanta-Ray 230 GCR operating at 10 Hz) were used for excitation. The pulse energy was 5–10 mJ; the pulse duration was ca. 7 ns. The samples were placed in an open top 10 × 10 × 45 mm$^3$ rectangular quartz cuvette with four clear windows. Sample solutions were deoxygenated by purging with argon for 15-20 minutes prior to and during the photolysis. The transient absorption was monitored using a white light probe beam placed at right angles to the laser beam. The probe beam from a 150 W cw Xe arc lamp was focused through the sample and re-imaged onto the entrance slit of a SPEX 1681 (0.22m) monochromator. The monochromatic light was then detected with a modified (5- instead of the original 9-stage dynode amplifier) R928 PMT obtained from Hamamatsu (response time < 2 ns). To prevent the PMT detector from overexposure, a fast shutter (Uniblitz) was set up at the output of the Xe lamp. The combination of the output spectral irradiance from the white light source, the detector spectral response, and the optics transmittance provided the spectrometer’s spectral window between ca. 300 and ca. 800 nm with the linear dispersion of 4 nm x mm$^{-1}$.

The signals from the PMT detector were routed through a DC-coupled back-off circuit which stored and displayed digital readout for the constant component of the signal ($I_0$), and provided a compensating offset to it. The real-time current was fed into a DC-coupled vertical channel of a Tektronix TDS-380 digital oscilloscope (400 MHz bandwidth) with a 50 Ohm input impedance. The overall time response of the electronic data acquisition system was ca. 2.5 ns.

The typical sensitivity of the spectrometer was ca. $5 \times 10^{-4}$ OD with 16-shot averaging.
I.3 Results and Discussion

I.3.1 Synthesis and characterization

A 32-residue peptide, C16C19GGY, was prepared using solid-phase peptide synthesis having the following sequence

\[
\text{Ac-K(IEALEGK)(IEALEGK) (CEACEGK)(IEALEGK)-GGY-amide.}
\]

The spectroscopic tag GGY was introduced to the peptide sequence for the precise determination of the peptide concentration in aqueous solutions based on the extinction coefficient of tyrosine (\(\varepsilon_{275} = 1450 \text{ M}^{-1} \text{ cm}^{-1}\)). The apopeptide C16C19GGY was purified by preparative reverse-phase C18 HPLC. The C16C19GGY peptide was identified by MALDI mass-spectroscopy (\(m/z\) (ion), found: 3407.98, calculated: 3407.91.)

![Figure I.6 MALDI mass spectrum of synthesized peptide C16C19GGY.](image)
The strong room temperature luminescence of Cu(I)-C16C19GGY suggests that it might function as a photoinduced electron-transfer agent, and its electron transfer properties can be studied by emission lifetime experiments. As a control, Figure I.7 shows that the emission lifetime trace observed at 600 nm can be fit to a biexponential decay kinetics. (Eq I.3), in which $A_S$, $k_S$ and $A_L$, $k_L$ are the amplitudes and rate constants of the shorter and longer lifetime components, respectively.

\[ I(t) = A_S \exp(-k_S t) + A_L \exp(-k_L t) \]  

(Eq.I.3)

A non-linear least squares fit of the data yields values of $A_S = 0.038$ and $k_S = 9.5 \times 10^5$ s$^{-1}$ ($\tau_S = 1.1$ µs), and $A_L = 0.033$ and $k_L = 1.3 \times 10^5$ s$^{-1}$ ($\tau_L = 7.7$ µs), where $\tau_L$ and $\tau_S$ are the emission lifetimes of their respective components.

**Figure I.7** Triplet decay trace of ca. 25 µM Cu(I)/C16C19GGY in argon-saturated solution, in 0.2 M pH 5.4 acetate buffer at 298 K. The solid line represents fitting to a double exponential kinetics. The intensity is normalized at time zero.
The source of the two emission lifetimes in this Cu(I)-C16C19GGY adduct system is not yet known; it is speculated that the Cu₄S₄(N/O)₄ may contain two independent lumophores, corresponding to the presence of two electronically independent Cu(I)-Cu(I) dimers located within the Cu₄S₄(N/O)₄ cofactor. There is another possibility that the samples measured may contain two different metalloproteins with different emission lifetimes. However, no evidence for the presence of two different metalloproteins in these samples has been observed.

I.3.2 Effect of [Ru(NH₃)₅Pyr]³⁺ on the triplet decay kinetics and the quenching mechanism

The addition of [Ru(NH₃)₅Pyr]³⁺ to a solution of an emissive Cu(I)-C16C19GGY complex leads to quenching of the emission intensity and lifetimes. The emission decay measured from a solution containing both Cu(I)-C16C19GGY and [Ru(NH₃)₅Pyr]³⁺ can be accurately fit to a double exponential decay kinetics. Importantly, both emission lifetimes of Cu(I)-C16C19GGY shorten with the addition of [Ru(NH₃)₅Pyr]³⁺. The data shown in Figure I.8 indicate that both lifetime components of this Cu(I)-C16C19GGY protein are quenched in the presence of 100 µM electron acceptor [Ru(NH₃)₅Pyr]³⁺ and yield a lifetime of \( \tau_S = 0.86 \pm 0.01 \) µs, and \( \tau_L = 2.40 \pm 0.02 \) µs, with \( A_S/A_L \) remains approximately unity.
Figure I.8 Triplet decay traces of 100 µM C16C19GGY and 100 µM Cu(I) in 0.2 M pH 5.4 acetate buffer in the (a) absence and (b) presence of 100 µM [Ru(NH₃)₅Pyr]³⁺.

Although it is possible that the lifetime quenching is due in part or fully to energy transfer, transient absorption studies were carried out to ascertain the mechanism. As a control, the excited state absorption difference spectra of Cu(I)-C16C19GGY, obtained 0.15, 0.52, 1.2, 3.35, and 10.35 µs after a 355 nm laser pulse, show a broad positive absorption band ranging from 350 to 580 nm (Figure I.9). The absorption features decay by a biexponential kinetics. Figure I.10 shows that the time-resolved absorbance kinetics measured at 410 nm can be fit to a biexponential decay kinetics (Eq I.3). A non-linear least squares fit of the data yields values of $A_S = 0.018$ and $k_S = 6.5 \times 10^5$ s⁻¹ ($\tau_S = 1.5$ µs), and $A_L = 0.012$ and $k_L = 9.4 \times 10^4$ s⁻¹ ($\tau_L = 10.6$ µs). The kinetics is in good agreement with previously reported emission lifetimes recorded at 600 nm under similar conditions, which yields values of $k_S = 9.5 \times 10^5$ s⁻¹ ($\tau_S = 1.1$ µs), and $k_L = 1.3 \times 10^5$ s⁻¹ ($\tau_L = 7.7$ µs).
**Figure I.9** Excited state absorption difference spectra of Cu(I)-C16C19GGY, obtained from 330 µM Cu(I) and 330 µM C16C19GGY in 0.2 M pH 5.4 acetate buffer following 355 nm excitation.

**Figure I.10** Time-resolved absorbance kinetics recorded at 410 nm. Conditions: 330 µM Cu(I) and 330 µM C16C19GGY in 0.2 M pH 5.4 acetate buffer following 355 nm excitation. The solid line represents fitting to a double exponential kinetics.
When [Ru(NH₃)₅Pyr]³⁺ is present, the transient absorption difference spectra show the distinct spectral profile of an increased absorption at 400 nm (Figure I.11). This new increased absorption is consistent with the appearance of the MLCT band of the reduced [Ru(NH₃)₅Pyr]²⁺ quencher. Although excited state Cu(I)-C16C19GGY shows a similar absorption band centered at 400 nm, this new increased absorption is narrow and displays a different decay kinetics. Time-resolved absorbance kinetics recorded at 407 nm is shown in Figure I.12. The kinetics is complicated somewhat by contributions from unquenched Cu(I) excited state before 10 µs. After 10 µs, when the Cu(I) excited state has completely decayed and the interference from unquenched Cu(I) excited state can be excluded, the 407 nm absorption decays via a second-order equal-concentration kinetics. It is shown in Figure I.12 that the absorption does not completely decay even after 100 µs. This might be due to the slow second-order back electron transfer process. Based on the above observations, the new increased 400 nm absorption band can be assigned to the MLCT band of the reduced [Ru(NH₃)₅Pyr]²⁺ electron transfer product unambiguously. A 400 nm increased absorption corresponds to the MLCT band of the reduced [Ru(NH₃)₅(lutidine)]²⁺ were also observed in the transient absorption spectra of [Ru(NH₃)₅(lutidine)]³⁺ with Cu(I)-C16C19GGY (not shown), which shows a similar decay kinetic. This indicates that the quenching mechanism in this Cu(I)/Ru(III) system involves a significant contribution from a photoinduced electron-transfer event.
**Figure I.11** Transient absorption difference spectra obtained from 400 µM Cu(I) and 400 µM C16C19GGY with 800 µM [Ru(NH$_3)_5$Pyr]$^{3+}$ in 0.2 M pH 5.4 acetate buffer following 355 nm excitation.

**Figure I.12** Time-resolved absorbance kinetics recorded at 407 nm. Conditions: 400 µM Cu(I) and 400 µM C16C19GGY with 800 µM [Ru(NH$_3)_5$Pyr]$^{3+}$ in 0.2 M pH 5.4 acetate buffer following 355 nm excitation.
The bimolecular electron transfer rates for \([\text{Ru(NH}_3\text{)}_5\text{Pyr}]^{3+}\) with Cu(I)-C16C19GGY have been measured by emissive lifetime quenching method and determined from plots of the emissive decay rate constants as a function of Ru(III) quencher concentrations. It is further noted that the relative amplitudes of both the emission decay components remain approximately equal at all quencher concentrations studied. Plots of the observed emission decay constants \(k_{S}^{\text{obs}}\) and \(k_{L}^{\text{obs}}\) are linearly dependent upon the concentration of \([\text{Ru(NH}_3\text{)}_5\text{Pyr}]^{3+}\) as pseudo-first order quenching kinetics is observed (Figure I.13). Analysis of the data yields values for the bimolecular quenching constants of \(k_{S}^{\text{ET}} = (2.46 \pm 0.07) \times 10^9\ \text{M}^{-1}\text{s}^{-1}\) and \(k_{L}^{\text{ET}} = (1.36 \pm 0.05) \times 10^9\ \text{M}^{-1}\text{s}^{-1}\), respectively. Together, these results show that the luminescent polynuclear copper center in the synthetic metalloprotein Cu(I)-C16C19GGY can indeed serve as a photoinduced electron-transfer reagent by undergoing a bimolecular reaction with an exogenous acceptor in a free solution.
**Figure I.13** Observed emission decay rate constants for the long and short-lived emission components of Cu(I)-C16C19GGY (ca. 25 μM) taken as a function of the concentration of added [Ru(NH$_3$)$_5$Pyr]$^{3+}$.

I.3.3. *Stern-Volmer behavior of *Cu(I)-C16C19GGY quenching by Ru(III)*

Furthermore, as expected for a bimolecular reaction, the luminescence quenching of *Cu(I) with various Ru(III) quenchers follows the Stern-Volmer relation,

$$\frac{\tau_0}{\tau} = 1 + k_{obs}\tau_0[Ru(III)]$$

where $\tau_0$ and $\tau$ represent the lifetimes of the Cu(I) complex in the absence and presence of quenchers. The observed electron transfer rate, $k_{obs}$, can be extracted from the slope of plots of $\tau_0/\tau$ vs quencher concentration. Exemplary Stern-Volmer plots for the quenching of the charge-transfer excited state of Cu(I)/C16C19GGY by [Ru(NH$_3$)$_6$]$^{3+}$ are shown in Figure I.14. As expected, the data points follow a linear distribution and exhibit the proper intercept of unity. The Stern-Volmer plots are linear for all photoredox systems studied indicating that under the present experimental conditions dynamic quenching is the predominant process excluding the contribution of static quenching to the overall luminescence-quenching reaction.
Figure I.14 Stern-Volmer plots of the quenching of 100 µM Cu(I), 100 µM C16C19 in 0.2 M pH 5.4 acetate buffer by [Ru(NH$_3$)$_6$]$_3^{3+}$, showing the linear fit through the data points.

I.3.4 Ru(III) complexes to study the electron transfer properties of Cu(I)/C16C19GGY

Since the electron transfer reaction in this case is a bimolecular electron transfer process, the observed reaction rate constant has the form of a consecutive reaction mechanism consisting of diffusional ($k_d$) and activated ($k_{act}$) rate constants for electron transfer. The observed reaction rate constant, $k_{obs}$, depends on the rate of diffusion, $k_{diff}$, and the activated rate constant for electron transfer, $k_{act}$, which are related by

$$\frac{1}{k_{obs}} = \frac{1}{k_{diff}} + \frac{1}{k_{act}} \quad \text{(Eq. I.4)}$$

As discussed before, the kinetics of electron transfer reactions can be expressed by the Marcus theory. The reorganization energy and donor-acceptor distance can be obtained by fitting the driving force dependence of electron transfer rate according to Eq I.2:

$$k_{ET}^{obs} = k_0 \exp[-\beta(r - r_0)] \exp[-\frac{(\Delta G^0 + \lambda)^2}{4\lambda RT}] \quad \text{(Eq. I.2)}$$
$k_0$ is the nuclear frequency equal to $10^{13}$ s$^{-1}$, $\beta$ is the distance attenuation factor taken to be $\beta = 1.2$ Å$^{-1}$, $r$ is the donor-acceptor separation, $r_0$ is the *van der Waals* contact distance of the reactants, and $\lambda$ is the reorganization energy.$^{30}$ Thus, by determining electron transfer reaction rates under different reaction driving forces, structural information such as the reorganization energy, electronic coupling constant can be obtained. A series of positively-charged Ru(III) complexes were synthesized to obtain different redox potentials for the ruthenium center. Systematic variation of the substituents of the parent ligand provides a wide variation of the redox potentials, which in turn control the driving force of the *Cu(I)/Ru(III)* electron transfer reaction. A pictorial representation of the ligands utilized in this driving force dependence study is shown in Figure I.15.
**Figure 1.15** Schematic representation of the ligands utilized in the driving force dependence study of the electron transfer rate.
The assumption that the $\lambda$ value and electronic coupling constant can be obtained by measuring electron transfer rate constant at different driving forces is only feasible on the condition that the quenchers studied here are electronically homogeneous, i.e., the reorganization energy $\lambda$ and electronic coupling constant $H_{AB}$ are constant or almost invariant throughout the series. One important consideration is whether the donor-acceptor distance and the conformation are constant throughout the six ruthenium ammine complexes studied. Molecular conformations of quenchers, $[\text{Ru(NH}_3\text{)}_5\text{L}]^{3+}$ ($L = 3,5$-lutidine, nicotinamide, pyridine, and dimethyl 3,5-pyridine dicarboxylate), are almost identical since the only difference in their structure is the different substituents on pyridine. Thus, the differences in the complex conformations and donor-acceptor distances are negligible. A similar assumption was made in the investigations of bimolecular electron transfer between cyt $c$ and many positively-charged ruthenium complexes. However, in the case of $[\text{Ru(NH}_3\text{)}_5\text{Cl}]^{2+}$ and $[\text{Ru(NH}_3\text{)}_6]^{3+}$, the sixth ligands are relatively small groups of chloro or ammonia, compared to pyridine. As noted by Sutin,$^{68,69}$ the contribution to the electron exchange activation energy from the reorganization of the inner-coordination spheres in ruthenium ammine complexes is small. The inner-shell reorganization energy for $[\text{Ru(NH}_3\text{)}_5\text{(pyr)}]^{3+}$ is 1.0 kcal/mol, which is very similar to that of $[\text{Ru(NH}_3\text{)}_6]^{3+}$, 0.9 kcal/mol.$^{68}$ Based on this, it is assumed that the conformation and reorganization energy of the six quenchers studied are invariant.

Thus, different driving forces for electron transfer between $^\text{*Cu(I)-C16C19GGY}$ and Ru(III) complexes were calculated by the Rehm-Weller equation (Eq I.5).

$$-\Delta G^0 = E^{00} - E^0(\text{Cu}^\text{I}/\text{Cu}^\text{II}) + E^0(\text{Ru}^\text{III}/\text{Ru}^\text{II})$$

(Eq. I.5)
For instance, a value of $-\Delta G^0 = 2.00 \text{ eV}$ is calculated for electron transfer reactions between *Cu(I)-C16C19GGY adduct and [Ru(NH$_3$)$_5$Pyr)]$^{3+}$. $E^{00} = 2.04 \text{ eV}$ is the triplet energy of Cu(I)-C16C19GGY as determined from its emission spectrum measured at 77 K (Figure I.16).

![Emission spectrum](image)

**Figure I.16** Emission spectrum of Cu(I)-C16C19GGY recorded at 77 K. Conditions: 50 µM Cu(I) and 50 µM C16C19GGY in 0.1 M pH 5.4 acetate buffer with 50% (v/v) glycerol.

This figure shows the emission spectrum of Cu(I)-C16C19GGY adduct measured at 77K in aqueous media with 50% glycerol. Compared with the emission spectra taken at room temperature (Figure I.4), the spectrum becomes vibrationally structured, with the maximal emission band centered at 610 nm. It is known that this luminescent energy state is the lowest charge-transfer triplet state. Thus, the excited state energy can be calculated from the maximal emission band wavelength as $E^{00} = \frac{hc}{1.6 \times 10^{-19} \lambda}$, giving $E^{00} = 2.04 \text{ eV}$. $E^0(\text{Cu}^+/\text{Cu}^{II}) = 343 \text{ mV}$ was obtained by performing redox titrations in combination with UV-Vis absorption and/or
steady-state emission spectra;\textsuperscript{14} and \( E^0(\text{Ru}^{\text{III}}/\text{Ru}^{\text{II}}) = 300 \text{ mV} \) was measured by CV at room temperature in water solution with 0.2 M pH 5.4 acetate buffer as the support electrolyte.

I.3.5 Bimolecular electron transfer in Marcus inverted regime

The redox potentials and the observed bimolecular electron transfer rate constants of the series of positively charged Ru(III) complexes are listed in Table I.1. As is evident from Table I.1, the electron withdrawing or donating abilities of the substituents on the parent ligand, pyridine, provide a wide range of redox potentials. Exemplary CV diagram of [Ru(NH\textsubscript{3})\textsubscript{5}L\textsuperscript{3+} (L = dimethyl 3,5-pyridine dicarboxylate) is shown in Figure I.17.

![Figure I.17](image)

Figure I.17 CV of [Ru(NH\textsubscript{3})\textsubscript{5}(dimethyl 3,5-pyridine dicarboxylate)]\textsuperscript{3+} measured at room temperature in water with 0.2 M pH 5.4 acetate buffer as support electrolyte.
Table I.1 Redox potentials of $[\text{Ru(NH}_3\text{)}_5\text{L}]^{3+}$ and observed bimolecular electron transfer rate constants between $[\text{Ru(NH}_3\text{)}_5\text{L}]^{3+}$ ($\text{L} = \text{chloro, ammine, 3,5-lutidine, pyridine, nicotinamide, and dimethyl 3,5-pyridine dicarboxylate}$) and Cu(I)-C16C19GGY.

<table>
<thead>
<tr>
<th>Quencher</th>
<th>Reduction potential (mV)</th>
<th>$k_s$ ($10^9$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_L$ ($10^9$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuA$_5$Cl</td>
<td>-40</td>
<td>3.27 ± 0.14</td>
<td>2.53 ± 0.09</td>
</tr>
<tr>
<td>RuA$_6$</td>
<td>60</td>
<td>5.86 ± 0.21</td>
<td>3.47 ± 0.05</td>
</tr>
<tr>
<td>RuA$_5$(lutidine)</td>
<td>255</td>
<td>4.43 ± 0.06</td>
<td>2.44 ± 0.08</td>
</tr>
<tr>
<td>RuA$_6$(py)</td>
<td>300</td>
<td>2.46 ± 0.07</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>RuA$_5$(nicotinamide)</td>
<td>353</td>
<td>3.46 ± 0.04</td>
<td>1.96 ± 0.06</td>
</tr>
<tr>
<td>RuA$_6$(dimethyl-3,5-pyridine dicarboxylate)</td>
<td>392</td>
<td>1.71 ± 0.21</td>
<td>0.96 ± 0.08</td>
</tr>
</tbody>
</table>

The driving-force dependence of the semi-log plots of $k_{obs}$ for electron-transfer oxidation of Cu(I)-C16C19GGY with a series of Ru(III) complexes is shown in Figure I.18 and Figure II.19 for the two emissive lifetime components, which reveals a striking parabolic dependence in the Marcus inverted region. The log $k_{et}$ value first increases with increasing the driving force and then decreases with further increase in the driving force. The $k_{et}$ value for the highly exergonic electron transfer from Cu(I) to Ru(III)A$_5$(dimethyl 3,5-pyridine dicarboxylate) is less than one third of the value for a much less exergonic electron transfer from Cu(I) to Ru(III)A$_6$. Such a pronounced decrease towards the highly exothermic region represents the existence of the Marcus inverted region in a truly bimolecular electron transfer.
Figure I.18 Plot of the electron transfer rates of the long lifetime component in Cu(I) C16C19GGGY adduct with Ru(III) complexes. The solid line is drawn based on the Marcus theory of electron transfer.

Figure I.19 Plot of the electron transfer rates of the short lifetime component in Cu(I) C16C19GGGY adduct with Ru(III) complexes. The solid line is drawn based on the Marcus theory of electron transfer.
Plotting of the data to the modified Marcus equation (Eq I.1) yields values of $\lambda = 1.7 \pm 0.1$ eV, $H_{DA} = 4.8 \pm 0.4$ cm$^{-1}$ for the short component, and $\lambda = 1.7 \pm 0.1$ eV, $H_{DA} = 3.9 \pm 0.4$ cm$^{-1}$ for the long component. The large reorganization energies combined with the observation of non-reversible redox behavior suggests that the one-electron oxidation of the $\text{Cu}_4\text{S}_4(\text{N/O})_4$ cluster might undergo an extreme conformational change involving the transformation of a trigonal Cu(I) center into a four-coordinate Cu(II) site. Assuming $\lambda_{11} = 1.20$ eV for the ruthenium pentaammine acceptors, the Marcus cross relation yields $\lambda_{22} = 2.2$ eV for Cu(I)-C16C19GGY. It is noted that the reorganization energy values obtained for both the two components are similar to that of inorganic copper complexes [Cu(phen)$_2$]$^{2+/+}$ ($\lambda = 2.4$ eV). In addition, the large value of $\lambda$ and small value of $H_{DA}$ apparently lowers the values of $k_{ET}$ below the diffusion limit permitting the inverted Marcus behavior to be observed.

I.3.6 Kinetic analysis for *Cu(I)/C16C19GGY quenching by Ru(III)

A kinetic scheme for excited-state quenching by electron transfer is shown in Scheme I.1 using an exemplary reductive quenching of Cu(I)* by Ru(III).
Scheme I.1 Kinetic scheme for excited-state quenching by electron transfer

\[
\begin{align*}
\text{Cu(I)} & \xrightleftharpoons[k_d]{k_a} \text{Cu(I)*} \\
\text{Cu(I)*} + \text{Ru(III)} & \xrightarrow[k_{\text{ET}}]{k_a} \left\{ \text{Cu(I)*Ru(III)} \right\} \\
\left\{ \text{Cu(I)*Ru(III)} \right\} & \xrightleftharpoons[k_{\text{ET}}]{k_{\text{ET}}} \left\{ \text{Cu(II)Ru(II)} \right\} \\
\left\{ \text{Cu(II)Ru(II)} \right\} & \xrightarrow[k_{\text{bET}}]{k'} \left\{ \text{Cu(II)Ru(III)} \right\} \\
\left\{ \text{Cu(II)Ru(II)} \right\} & \xrightarrow[k_d]{k_d} \text{Cu(II) + Ru(II)}
\end{align*}
\]

The scheme is based on an earlier one given by Rehm and Weller\(^{48}\) for the quenching of a series of aromatic excited states. In the scheme, \(k_a\) and \(k_a\) represent forward and reverse rate constants for the formation of an association complex between the excited state and quencher prior to electron transfer. In other words, \(k_a\) is the diffusion-controlled rate constant. The equilibrium constant for formation of the complex is \(K_a = k_a/k_{\text{a}}\). \(k_{\text{ET}}\) is the first-order rate constant for electron-transfer quenching of the excited state within the association complex; and \(k_{\text{ET}}\) is the rate constant for the corresponding back-electron transfer reaction to return to the excited state. The electron transfer product undergoes processes which include back electron transfer to give ground-state \((k_{\text{bET}})\) rather than excited state and a competitive step where the redox product, \(\text{Cu(II)Ru(II)}\), separate in solution \((k_d)\). \(k'\) is the combined rate constant for processes following the quenching step, which lead to net quenching rather than to repopulation.
of the excited state. According to Scheme I.1, the expression for \( k_q \) is shown below where \( K_a = k_a/k_{-a} \) and \( K_{ET} = k_{ET}/k_{-ET} \). This expression can be simplified to Equation I.7 and I.8, depending on the relative magnitude of \( k_{ET} \) to \( k' \).

\[
k_q = \frac{K_a k_{ET} k'}{k_{-ET} + k'} \quad \text{(Eq. I.6)}
\]

**Case I** \[ k_{ET} \ll k' \], \[ k_q = K_a k_{ET} \] \quad \text{(Eq. I.7)}

**Case II** \[ k_{ET} \gg k' \], \[ k_q = K_a K_{ET} k' \] \quad \text{(Eq. I.8)}

In the case of Cu(I)/C16C19-GGY with Ru(III), the precursor complex formation is a rapidly established preequilibrium with the subsequent electron transfer within this complex being rate determining. Under this condition, the observed second-order rate constant is equal to \( K_a k_{ET} \). The observed free energy dependence of \( k_q(\text{short}) \) and \( k_q(\text{long}) \) can be interpreted in terms of the inverted Marcus behavior on the condition that the assumption that the values of \( K_a \) remain relatively constant throughout this entire series of reactions is reasonable. To support this assumption, it is noted that the amino acid sequence of Cu(I)/C16C19GGY distributes four negatively-charged glutamic acid residues along the solvent-exposed surface of each chain of the four-stranded coiled coil, and that all but one of the ruthenium quenchers shown in Table I.1 have an overall charge of 3+. The only exception to this is \([\text{Ru(NH}_3)_5\text{Cl}]^{2+}\) whose smaller charge suggests that its value of \( K_a \) will be slightly smaller than those of the other ruthenium quenchers. However, this will cause its values of \( k_q(\text{short}) \) and \( k_q(\text{long}) \) to represent lower limits of their true bimolecular electron transfer rates and will not affect the observation of the inverted Marcus behavior.

**1.3.7 Temperature dependence of the observed bimolecular electron transfer rates**
Typical temperature-dependence data for $k_q$ of *Cu(I) quenching by [Ru(NH$_3$)$_6$]$_3^+$ are listed in Table I.2 and shown in Figure I.20 and I.21. It is apparent that the quenching rate constants for both the long lifetime component and the short lifetime component show positive temperature dependence.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$k_S$ ($10^9$ M$^{-1}$s$^{-1}$)</th>
<th>$k_L$ ($10^9$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>278.15</td>
<td>3.18 ± 0.09</td>
<td>1.75 ± 0.03</td>
</tr>
<tr>
<td>298.15</td>
<td>5.86 ± 0.21</td>
<td>3.47 ± 0.05</td>
</tr>
<tr>
<td>323.15</td>
<td>9.37 ± 0.34</td>
<td>5.55 ± 0.10</td>
</tr>
</tbody>
</table>

Table I.2. Temperature-dependence for bimolecular quenching rate constants of *Cu(I) quenching by [Ru(NH$_3$)$_6$]$_3^+$.

Tazuke et al.$^{71}$ have demonstrated that the positive temperature dependence corresponds to case I; case II will exhibit negative temperature dependence. The activation enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) of quenching can be calculated by the Eyring equation. Since in case I, $k_q = K_a k_{ET}$ and according to Eyring transition state theory, $k_{ET} = \langle \kappa \rangle \frac{k_BT}{h} \exp(-\Delta G^\ddagger / RT)$ where $\langle \kappa \rangle$ is the electronic transmission coefficient, the probability of electron transfer within the activated complex, the bimolecular rate constant for electron transfer is given by

$$k_q = K_a \langle \kappa \rangle \frac{k_BT}{h} \exp(-\Delta G^\ddagger / RT) \quad \text{(Eq. I.9)}$$
Figure I.20 Eyring plot for the quenching of *Cu(I)C16C19-GGY short lifetime component by [Ru(NH₃)₆]³⁺.

Figure I.21 Eyring plot for the quenching of *Cu(I)C16C19-GGY long lifetime component by [Ru(NH₃)₆]³⁺.
In biological systems, redox cofactors are usually separated by large distances in the protein matrix. Under this circumstance, the donor and acceptor are very weakly coupled, and the electron transfer reaction is considered to occur in a nonadiabatic regime. In most cases, and for nearly all reactions in solution, one takes $\kappa = 1$. By fitting the observed bimolecular quenching rate constants vs. temperatures to Eyring equation (Eq I.9), the activation enthalpy of quenching can be calculated and yields $\Delta H^\ddagger = 3.96$ kcal/mol and 3.69 kcal/mol for the long and short lifetime component respectively. These values of activation enthalpy are comparable to the $\Delta H^\ddagger$ found for Ru(NH$_3$)$_4$(bpy)$^{3+/2+}$ exchange reaction, which is 4 kcal/mol. It is feasible to obtain both enthalpy and entropy from the Eyring equation for first-order electron transfer reactions; however, in bimolecular electron transfer reactions, since both entropy and $K_a$ affect the temperature dependence of quenching rate constants in the same direction, it is impossible to obtain both entropy and $K_a$ values together by fitting $\ln k_q$ vs. temperature.

**I.3.9 Summary**

In summary, the α–helical coiled-coil motif serves as a scaffold for constructing synthetic electron-transfer metalloprotein. The designed miniature Cu(I) metalloprotein Cu(I)/C16C19GGY undergoes collisional photoinduced electron-transfer to a variety of ruthenium(III) acceptors in solution. Inverted Marcus behavior is observed as the buried Cu$_4$S$_4$ (N/O) cofactor which has a very high reorganization energy and experiences a weak electronic coupling to the aqueous acceptors. This latter effect might be due to the positioning of the cofactor within the hydrophobic core of the protein which is well away from the bulk solvent.
1. Calhoun, J. R.; Nastri, F.; Maglio, O.; Pavone, V.; Lombardi, A.; DeGrado, W. F.,

2. Lombardi, A.; Summa, C. M.; Geremia, S.; Randaccio, L.; Pavone, V.; DeGrado, W. F.,


    **1997**, *119*, 6195-6196.


CHAPTER II
INTERACTIONS BETWEEN METALLOPEPTIDES AND CYTOCHROME C

II.1 Introduction

Non-covalent molecular interactions in biological systems are ubiquitous and finely tuned. While enzyme-substrate complexes\(^1\)-\(^4\) are relatively well-understood and considerable knowledge has been gained about the way proteins interact with DNA,\(^5\)-\(^7\) understanding of protein-protein interactions on its structural features and configurational dynamics is relatively poor.\(^8\) This is partially due to the fact that many complexes involving proteins are large and intractable for structural studies. Another reason is the dynamic nature of the non-covalent protein-protein complexes, which are formed by electrostatic, hydrophobic, \textit{van der Waals} interactions, or hydrogen bonding. The structural features and configurational dynamics of protein-protein complexes are key factors in regulating biological processes. These include the way proteins interact with one another, the stoichiometry of the complexes, the distances between reactants involved in the biological processes, and the configurational rearrangements of the complexes.\(^9\) In view of this complexity, simple model systems must be designed to further develop the knowledge of the structural and dynamic features of more complicated protein-protein interactions. Often, design is aimed at mimicking protein structural elements in a smaller form.

\textit{II.1.1 Long-range electron transfer in biological systems}

In many biological systems, long range electron-transfer pathways between redox groups are dominated by “through space” coupling, as the electron transfer event occurs across a non-
covalent protein-protein interface. Electron transfer reactions have been widely studied for over half a century and the semiclassical theory of Marcus has greatly advanced this field by defining many factors that control the electron transfer rates, such as the reorganization energy and electronic coupling. By determining the electron-transfer reaction rate under different conditions, much useful structural information about protein-protein interactions, such as the distance between redox centers, can be obtained. Thus, the study of biological electron transfer can be used to provide detailed information on the formation and dynamics of these non-covalent protein-protein complexes on a molecular level.

There are three approaches that have been applied to study the electron-transfer reactions in metalloproteins: (i) native and hybrid multisite protein; (ii) protein-protein and protein-small molecule ion pairs; and (iii) synthetic multisite proteins. The labeling of proteins with transition metal complexes to study long-range electron transfer interactions was first accomplished by Gray\textsuperscript{11-15} and Isied,\textsuperscript{16-18} and now is routinely utilized for electron transfer studies in many peptide and protein systems. In 1982, Gray and co-workers\textsuperscript{10} first reported the synthesis of the multisite protein ruthenium pentaammine histidine-33-ferricytochrome c (Ru-Fe-Cyt c) and the kinetics of the intraprotein electron transfer from Ru(II) to Fe(III).\textsuperscript{15} Compared to organic substrates, using metal complexes as redox cofactors enables the study of electron transfer processes occurring with high driving forces (up to 1.5 V), while organic molecules are typically limited to the lower driving force region.\textsuperscript{20} They labeled a number of heme-containing native proteins with ruthenium complexes in order to probe different regions of proteins for electron transfer tunneling. Subsequent work in Gray’s laboratory has focused on the elucidation of distant electronic couplings between redox sites in several Ru-proteins.\textsuperscript{14,21,22} In particular, work on Ru-azurin has provided a reference point for electron tunneling through folded polypeptide structures.\textsuperscript{23} Their
studies have shown that Cu(I) to Ru(III) electron transfer rates in labeled azurin crystals are nearly identical with solutions values for each donor-acceptor pair.

In early work by Isied and co-workers, they developed a system in which long-distance intramolecular electron transfer occurs in a series of metal-derivatized oligoproline peptides of the type M₁-(Pro)ₙ-M₂, where n = 0–9 and M₁ and M₂ are various complexes of ruthenium, osmium, or cobalt. These oligoproline peptides exist in the helical polyproline II structure. Intramolecular electron transfer studies demonstrated the distance dependence of the electron transfer rate versus the number of helical prolines bridging a donor and acceptor ruthenium site to a metal-to-metal distance ≈ 40 Å. The dependence of rate versus the number of prolines observed for n = 6, 7, and 9 is very similar to that observed earlier for [(bpy)₂Ru²⁺L-(Pro)ₙ-Co³⁺(NH₃)₅], n = 4–6. The rates observed at these long distances show that long-range electron transfer can be observed between an appropriate donor and acceptor directly connected to the proline bridge via peptide bonds at distances similar to the diameter of a small protein. Further, a series of ruthenium and osmium bipyridine derivatives with hydrogen bonding molecular recognition sites capable of forming noncovalent donor acceptor complexes were used to determine the dependence of the rate of intramolecular electron transfer on the reaction driving force across a hydrogen bonding interface. At similar distance, electron transfer rates across H-bonding interfaces are only modestly slower than that observed in the covalently bonded donor-acceptor complexes and can be as effective a bridge for electron transfer as covalently bound bridges. Subsequently, they observed the transition of electron transfer reaction from electron superexchange to hopping by examining the synthesized [(bpy)₂Ru²⁺L-Proₙ-apyRu³⁺(NH₃)₅]⁵⁺ where the number of proline residues (n) varies from 0 to 9. The distance
dependence of long-range electron transfer rate constants shows a clear transition from a super-exchange mechanism \((n=0-3)\) to a hopping mechanism \((n=5-9)\).

Besides the approaches used by Gray’s and Isied’s laboratories, another approach of studying native and hybrid multisite proteins can also be used to study fixed-site electron transfer reaction in metalloproteins. Biological electron transfer takes place among and within proteins over considerable distances between active sites containing transition metal ions or organic cofactors and is generally characterized by relatively weak electronic interactions among them.\(^{26}\)

At least three elementary steps are required to complete redox reaction between soluble proteins: (i) formation of an active donor-acceptor complex; (ii) electron tunneling within the donor-acceptor complex; (iii) dissociation of the oxidized and reduced products.\(^{23}\) Electron tunneling in proteins occurs in reactions where the electronic interaction between redox sites is relatively weak.

As mentioned above, an important property of protein-protein complexes is that they are not static but dynamic compared to the covalently bound donor-acceptor complexes. Large proteins have numerous possible conformations, and as a result, protein-protein complexes also have many possible configurations. However, the thermodynamically stable configuration might not be able to perform the electron transfer reaction, and then a configurational rearrangement will be required to achieve the more reactive configurations for electron transfer to occur. In this case, the configurational dynamics of protein-protein complexes will be an important factor in determining the rate constant of the total electron transfer process. As a result, there are multiple binding sites in many protein-protein complexes and it is not uncommon to observe that the electron transfer kinetics is gated by the dynamics of conformational changes within the complexes.\(^{27,28}\)
Since both the donor-acceptor docking geometries and the conformations of these protein-protein complexes are unknown, it has been difficult to measure rates of long-range electron transfer between redox sites in protein-protein complexes until rapid triggering methods were available. The laser flash photolysis is a useful method for the investigation of reactive intermediates generated by a laser pulse. As shown in Scheme II.1, the singlet state of the electron donor is obtained by flash excitation and transfer to the triplet state through intersystem-crossing (ISC). In the presence of an electron acceptor, the triplet emission is quenched by electron transfer from the triplet state of electron donor to acceptor. Thus, by measuring the emission lifetime decay, the electron transfer rate can be obtained in the dynamic protein-protein complexes.

Scheme II.1 Emission lifetime experiments to study electron transfer kinetics. (Adapted from reference 9)

The electron transfer reaction between cyt $c$ and cyt $b_5$ has been the subject of investigation for over forty years. The structural model proposed by Salemme$^{29}$ in 1976 showed the existence of four principal complementary charge interactions within cytochrome $c$ (cyt $c$)/cyt $b_5$ complex. In addition, this structural model proposed for the precursor complex of this
protein pair stimulated a great deal of work on cyt c related protein-protein complexes. Several heme proteins, such as cyt c, cytochrome c peroxidase (ccp), cyt b5, cyt f, myoglobin (Mb), et al. have been widely used for electron transfer and protein-protein interaction studies. Meyer et al.\textsuperscript{30} used Ru-modified cyt b5 to examine the kinetics of electron transfer in cyt c/cyt b5 complexes. Two concentration-independent electron transfer rates were observed, demonstrating that two cyt c/cyt b5 species are present in the solution and the slower Fe(III)-cyt c reduction phase is limited by conformational changes within one of the complexes.

Kinetics measurements on crystallographically characterized metal-substituted hemoglobin (Hb) hybrids by Hoffman and co-workers provided some of the earliest insights into interprotein electron transfer rates.\textsuperscript{31, 32} Since Hb is a very strongly bound complex of four polypeptide subunits, dynamic problems are not complicating factors for electron transfer measurements. Thus, the first example of long-range electron transfer between chromophores that are rigidly held at a fixed and crystallographically known distance and orientation were obtained by replacement of the native Fe center in the β–subunits of Hb with Zn or Mg to make the photoinduced electron transfer possible. Electron transfer from a triplet-excited Zn-porphyrin in the β–subunit to an Fe (III) center in the α–subunit is initiated by flash photoexcitation, which forms the long-lived (over 10 ms) ZnP triplet state. The time constant for the electron transfer is around 16 ms.\textsuperscript{32}

Subsequent work of Hoffman’s group\textsuperscript{33} focused on using metal-substituted ccp and cyt c to probe the electron transfer kinetics between these two proteins. The water soluble, heme protein cyt c is one of the widely studied electron-transfer proteins since its three dimensional structures of both the reduced and oxidized state\textsuperscript{34, 35} are known in detail. The electron transfer reactions involving these two physiological redox partners have been studied in great details. At
low ionic strength, acidic ccp and basic cyt c form a stable complex. A model of a 1:1 binding complex of ccp and cyt c was proposed and the crystal structure of the 1:1 complex of the two proteins was reported by Kraut et al.\textsuperscript{36} Hoffman and co-workers used quenching studies and species variations to provide evidence for two distinct cyt c binding sites on ccp: a tight-binding but less-reactive domain and a weak binding but more-reactive domain. More efficient electron transfer occurs through the weak-binding site, forming a 2:1 complex. But the tight-binding site, which is observed in X-ray structure, was assumed to be the electron transfer pathway by Kraut et al. because of its high association affinity. Further, through the study of electron transfer between Zn-Mb and cyt b\textsubscript{5},\textsuperscript{37} a new “dynamic docking” paradigm for protein-protein reactions was proposed. Numerous weakly bound conformations of the docked complex contribute to the binding of cyt b\textsubscript{5} to Mb and Hb, but only a very small subset of these are electron transfer active. This subset does not include the conformations that is most favorable for binding.\textsuperscript{37} The studies discussed above, together with some other results,\textsuperscript{38-41} have shed much light on the mechanisms of protein-protein electron transfer processes.\textsuperscript{23}

As evident from the above discussion on electron transfer reactions in biological systems, studies conducted with simple models can provide valuable guidelines for study of the electron transfer theory. At the same time, by studying electron transfer reactions between protein-protein complexes, much useful information on protein-protein interaction can be obtained.

\textit{II.1.2 Electron transfer to probe the configurational dynamics of protein--protein complexes}

Widespread studies on the interactions between zinc cyt c and plastocyanin have been carried out by Kostic’s group.\textsuperscript{42-45} It was found that at low ionic strength, at which the protein-protein complex persists, electron transfer reaction undergoes unimolecularly within a preformed
complex as well as via a bimolecular encounter complex. Since the electron transfer rate constant decreases with increasing solution viscosity, the electron transfer reaction within the preformed complex is gated by the configurational rearrangement. The rate limiting step, configurational rearrangement, seems to be configurational fluctuation, a process during which the donor and the acceptor remain docked in the same general orientation but wiggle with respect to each other.\textsuperscript{46} Based on these results, Kostic and co-workers drew the conclusion that both the preformed and encounter complexes have the same electron transfer distance and both have the configurational rearrangement as the rate limiting step.

In other work, a similar kinetic mechanism has also been observed in the interactions between a linear, negatively charged metallopeptide RuCE\textsubscript{5}G and cyt c. Lasey et al.\textsuperscript{47} designed a small negatively-charged ruthenium metallopeptide, RuCE\textsubscript{5}G, which can form stable electrostatic complexes with ferricytochrome c. Computer representation of cyt c and the RuCE\textsubscript{5}G metallopeptide are shown in Figure II.1.

\textbf{Figure II.1} Computer representation of cyt c (left) and the RuCE\textsubscript{5}G metallopeptide (right), (Adapted from reference 47).
The singlet state of Ru is obtained by exciting the metal-to-ligand charge-transfer (MLCT) band and transfers to the triplet state through intersystem conversion (ISC). Triplet state emission measurements showed that the triplet lifetime of RuCE$_5$G is shortened and decays via biexponential kinetics in the presence of cyt c. These results indicate the existence of two excited-state populations of ruthenium peptides, both of which undergo photoinduced electron-transfer to the iron heme. The faster decay component displays concentration-independent kinetics, which demonstrates the presence of a preformed peptide-protein complex. This preformed complex undergoes intramolecular electron-transfer with a rate constant of $k_{\text{ET}} = (2.7 \pm 0.4) \times 10^6$ s$^{-1}$. The slower decay component displays concentration-dependent kinetics which saturate at high concentrations of cyt c. Rapid equilibrium formation of an encounter complex which then undergoes unimolecular electron-transfer yields $k_{\text{ET}} = (7 \pm 3) \times 10^5$ s$^{-1}$. The smaller value of $k_{\text{ET}}$ suggests that a somewhat longer donor-acceptor distance exists in the encounter complex. The kinetic mechanism for the interactions between RuCE$_5$G and cyt c are shown in Scheme II.2.
Scheme II.2 Kinetic mechanism for the interactions between RuCE$_5$G and cyt c (Adapted from reference 47).

Significantly, the magnitude of $k^\text{obs}_{\text{ET}}$ decreases with increasing solvent viscosity and the behavior can be fit to the expression $k^\text{obs}_{\text{ET}} \propto \eta^{-\alpha}$ to give $\alpha = 0.59 \pm 0.05$. The electron-transfer process occurring in the preformed complex is therefore gated by a rate-limiting configurational change of the complex. Interestingly, the value of $k^\text{obs'}_{\text{ET}}$ is also viscosity dependent showing that this reaction is also gated. However, a value of $\alpha = 0.98 \pm 0.14$ was observed, which emphasized the very dynamic nature of the encounter complex.

Subsequent work of Liu et al.$^{48}$ showed how a small modification of the metal peptide could produce significant changes in the dynamics of its preformed complex. A new metalloprotein was prepared in which a ruthenium polypyridyl complex was coupled directly to the CE$_5$G peptide to yield RuCE$_5$G-short. This new metallopeptide differs from the original one only by the absence of the flexible acetyl linker joining the metal complex to the cysteine side-chain of the peptide. Photoinduced electron-transfer experiments demonstrated that RuCE$_5$G-short also forms an electrostatic complex with cyt c within which intra-complex electron-transfer can occur. In addition, viscosity studies showed that this process is also gated by rate-limiting configurational changes of the complex. However, it was seen that the preformed complex involving RuCE$_5$G-short was more mobile ($\alpha = 0.97 \pm 0.03$) than the one involving the longer peptide and had a higher binding constant. These observations were rationalized by molecular modeling studies which indicated that the two peptides likely adopt different conformations (Figure II.2).
Figure II.2. Energy-minimized structures of the metal-peptides: a) RuCE₅G; and b) RuCE₅G-short which differ by the method of attaching the ruthenium center to the cysteine side-chain. (Adapted from reference 48)

Whereas the short peptide has a roughly linear rod-like geometry, the flexible acetamido linker of RuCE₅G allows it to form a hairpin-like structure in which the bulky ruthenium polypyridyl cation is placed in closer proximity to the negatively-charged glutamate chain. It was speculated that the higher mobility of the RuCE₅G-short:cyt c complex may due to its rod-like conformation, and the lower binding constant of the RuCE₅G complex may arise from partial charge compensation occurring between the oppositely charged portions of the metal peptide as they are brought closer together in the hairpin structure. These results demonstrated how gated electron-transfer experiments can be used to directly probe the dynamics of peptide-protein complexes, and how apparently subtle changes made to the peptide sequence may produce large changes in the dynamics of the complexes that they form.

The first part of this chapter focused on using electron transfer reactions to study the interactions between a cyclic peptide, which is linked to a ruthenium complex, and cyt c.
Different behavior is expected since the binding interactions might be different by selective modification of the peptide sequence. With different peptide sequence, the total charge on the peptide and the charge distribution along the peptide chain will be altered, thus results in the different binding behavior between the ruthenium complexes linked peptide to cyt c. These different binding behaviors might include the different binding domain on the surface of cyt c, conformations for the electrostatic protein-protein complexes, and the configurational change for different conformations of protein-protein complexes before the electron transfer reaction can occur. In the second part, metallopeptides with different redox potentials but similar conformations were compared. The reorganization energy and donor-acceptor distance of the preformed complexes between metalloproteins and cyt c could be obtained by measuring the true electron transfer rates and fitting to the Marcus equation.

II.2 Experimental

II.2.1 Materials

N-methylpyrrolidone, 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), piperidine, anhydrous 1-hydroxybenzotriazole (HOBT) and the Fmoc-protected L- and D-amino acid derivatives except for Fmoc-Glu-OAll (α-allyl ester) were purchased from Peptides International, Inc. (Louisville, KY) and PE Biosystems (Foster City, CA). Fmoc-Glu-OAll (α-allyl ester) were prepared by methods described previously. Sodium dihydrogen phosphate and sodium monohydrogen phosphate were obtained from Fisher Scientific (Pittsburg, PA) and used to prepare buffers with pH = 7.0. Dichloromethane, β-mercaptoethanol, trifluoroacetic acid (HTFA), tetrakis(triphenylphosphine)palladium(0) and phenol were obtained from Aldrich Chemicals (Milwaukee, WI), and triisopropylsilane was from
Lancaster Chemicals (Windham, NH). Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$, where bpy = 2,2’-bipyridine and 5-chloro-phen-AcCl = 5-chloroacetamido-1,10-phenanthroline, was prepared as previously described.$^{49,50}$ Ferricytochrome c (cyt c) from horse heart was purchased from Sigma (St. Louis, MO) and used as received.

II.2.2 General Methods

Reverse-phase HPLC runs were performed on a preparative (21.2×250 mm) Zorbax 300SB-C18 column. A two-pump system (Waters Model 515) equipped with a Waters Model 996 diode array detector/spectrophotometer with a 1-cm path length cell was used. Linear gradients of acetonitrile and water, each containing 0.1% (v/v) trifluoroacetic acid, were used as the mobile phase. Absorption spectra were recorded on a Hewlett-Packard 8452A spectrophotometer. Static luminescence spectra were obtained with a single photon counting spectrofluorimeter from Edinburgh Analytical Instruments (FL/FS 900). MALDI mass spectrometry was performed on Bruker OmniFLEX MALDI-TOF mass spectrometer.

II.2.3 Synthesis of Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$

[Ru(bpy)$_2$(phen-NH)$_2$](PF$_6$)$_2$ was synthesized according to literature procedure.$^{51}$ Typically, a 10% molar excess of 5-amino-1,10-phenanthroline (0.21 g, 1.08 mmol) dissolved in 100 mL of hot ethanol was added to 0.5 g (0.96 mmol) of Ru(bpy)$_2$Cl$_2$·2H$_2$O dissolved in 50 mL of hot H$_2$O. The solution was deaerated with argon for 20 min and refluxed for 3 hours under argon. Most of the ethanol was removed by rotary evaporation, and a 5-fold molar excess of NH$_4$PF$_6$ (0.98 g, 4.8 mmol) dissolved in H$_2$O was added to produce a red-orange precipitate. The product was collected by suction filtration and was purified by elution chromatography on an
alumina column with a 2:1 toluene-acetonitrile solution. After elution and evaporation, the product was re-dissolved in a minimum amount of acetonitrile (<5 mL) and dropped into 350 mL of rapidly stirred diethyl ether. The red-orange powder was collected by suction filtration, washed with diethyl ether, and dried overnight under vacuum.

\[ \text{[Ru(bpy)\textsubscript{2}(phen-ACl)](PF\textsubscript{6})\textsubscript{2}} \text{ was prepared through literature methods.}^{49} \text{ Typically, } N,N'\text{-dicyclohexylcarbodiimide (DCC) (2.5 g, 1.2 mmol) was added to a solution of chloroacetic acid (2.55 g, 2.7 mmol) dissolved in anhydrous ethyl acetate (75 mL). A white precipitate formed immediately, and the mixture was stirred at ambient temperature for 2 h. The reaction mixture was filtered, and the filtrate was evaporated to yield chloroacetic anhydride as viscous yellow oil. The oil was dissolved in acetonitrile (30 mL) and added to a solution of [Ru(bpy)\textsubscript{2}(phen-NH\textsubscript{2})](PF\textsubscript{6})\textsubscript{2} (2 g, 2 mmol) in acetonitrile (100 mL). The mixture was stirred overnight, and the acetonitrile was then removed by rotary evaporation. The resulting solid was redissolved in a minimum amount of methanol and precipitated with the addition of saturated NH\textsubscript{4}PF\textsubscript{6} (aq). The [Ru(bpy)\textsubscript{2}-(phen-ClA)](PF\textsubscript{6})\textsubscript{2} was collected by vacuum filtration as an orange solid, washed with water followed by diethyl ether, and stored in the dark under vacuum.} \]

**II.2.4. Synthesis of Ru(5-chloro-phen)\textsubscript{2}(phen-ACl)(PF\textsubscript{6})\textsubscript{2}**

The precursor Ru(5-chloro-phen)\textsubscript{2}C\textsubscript{12} used to synthesize [Ru(5-chloro-phen)\textsubscript{2}(phen-NH\textsubscript{2})](PF\textsubscript{6})\textsubscript{2} were prepared according literature procedure.\textsuperscript{52} As an example, RuC\textsubscript{13}·3H\textsubscript{2}O (1 g, 4.82 mmol), 5-chloro-1,10-phenanthroline (2.07 g, 9.64 mmol), and LiCl (13 mg, 0.5 mmol) were heated at reflux in dimethylformamide (9.6 mL) for 8 h. The reaction was stirred magnetically throughout this period. After the reaction mixture was cooled to room temperature, 50 mL of reagent grade acetone was added and the resultant solution cooled at 0 °C overnight.
Filtering yielded a red-orange solution and a black micro-crystalline product. The solid was washed with water (3 × 8 mL) followed by diethyl ether (3 × 25 mL), and then it was dried by suction. Subsequent procedures were similar to that applied to make Ru(bpy)$_2$(phen-ACl)(PF$_6$)$_2$.

II.2.5 Synthesis of cyclic peptide cyclo[Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu]

The 7-residue linear peptide was synthesized on a PE Biosystems (Foster City, CA) model 433A peptide synthesizer using the Fmoc N-terminal protection strategy and the manufacturer’s HMP resin. Side-chain anchoring of Fmoc-Glu(OH)-OAll (α-allyl ester) to the HMP resin was followed by stepwise solid phase assembly of the linear sequence. Activation was achieved using HBTU/HOBT in DMF. After the synthesis was complete, the resin was dried under vacuum overnight and Allyl- and Fmoc- protecting group were removed using the methods described in literature.$^5$ Typically, dried peptide-resin (40mg, 0.27mmol/g) was washed with DMF (5×0.5min), suspended in 3mL DMSO-THF-0.5M aqueous HCl-morpholine (2:2:1:0.1). The C-terminal allyl ester was cleaved by treatment with 5 equivalent Pd(PPh$_3$)$_4$ (62 mg, yellow powder) under argon at room temperature for three hours. The peptide-resin was collected by filtration and washed with: THF (3×2min), DMF (3×2min), DCM (3×2min), DIEA-DCM (1:19, 3×2min), DCM (3×2min), 0.03M Sodium N.N-diethylthiocarbamate in DMF (3×15min) to ensure complete removal of Pd(PPh$_3$)$_4$, DMF (5×2min), DCM (3×2min), and DMF (3×1min). The Fmoc group was removed with piperidine-DMF (1:4, 3×1min+2×5min+2×1min),
followed by brief washing with 0.4% (v/v) concentrated HCl in DMF to ensure complete removal of piperidine, and final washing with DMF (5×0.5min) and DCM (5×0.5min).

On-resin cyclization was achieved through activation of the Cα-carboxyl group using BOP/HOBT and DIEA in DMF and its condensation with a free Nα-amino group to close the “head-to-tail” ring. Typically, 40 mg peptide-resin was suspended in 0.5mL DMF, following which 5 equivalent BOP (23.88 mg), 5 equivalent HOBt (7.29 mg), and 10 equiv DIEA (13.96 mg, 17.84 μL) in 0.5mL DMF were added and stirred at room temperature for 2~5 hours.

Final deprotection and cleavage of the free cyclic peptide from the resin was achieved using a cleavage cocktail comprised of trifluoroacetic acid (88% (v/v)), phenol (5% (v/v)), triisopropylsilane (2% (v/v)), and water (5% (v/v)). The solution containing the resin and crude peptide was then filtered through glass wool and the filtrate was added dropwise to cold diethyl ether set in a dry ice-acetone bath. The white precipitate was then collected by vacuum filtration, washed with cold diethyl ether. The cyclic peptide was used without further purification.

**II.2.6 Synthesis of Ru(bpy)$_2$(phen-am)-cyclo[Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu]**

The synthesis of the [Ru(bpy)$_2$(phen-am)-cyclo[Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu]]$^{3-}$ metallopeptide, called Rucyclic, was prepared by the following general procedure: 5 mg of cyclic apopeptide was dissolved in 250 μL 50 mM phosphate buffer (pH 7). To this was added 7 mg tris-(2-carboxyethyl)phosphine (TCEP), and the solution was stirred for 15 min. 5% NaOH solution was added dropwise to this solution until pH 9~10. 18 mg of Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$ was dissolved in 250 μL DMF and added to the reaction mixture. The resulting solution was heated for one hour at 65°C. The crude reaction mixture was applied directly onto
an ion-exchange column (Sephadex-SP C-25) that was previously equilibrated with deion water. The collected fraction was further purified by reverse-phase HPLC using the gradient starting at 10% acetonitrile and increasing to 30% acetonitrile in 40 minutes. The faction was collected, lyophilized, and analyzed by MALDI.

II.2.7 Emission Lifetime Measurements

Emission lifetimes were measured with a nitrogen-pumped broadband dye laser (2-3 nm fwhm). The excitation wavelength was set to 450 nm using Coumarin 460, and the ruthenium emission was monitored at 610 nm. Unless otherwise noted, measurements were performed on argon-saturated solutions of the metallopeptide and cyt c dissolved in a low ionic strength phosphate buffer solution (typically 0.5 mM). The concentrations of stock solutions of the metallopeptide (ca. 60 μM in water) and cyt c (ca. 1 mM protein dissolved in 5 mM phosphate buffer) were determined by UV-Vis spectroscopy using values of \( \varepsilon_{450} = 16,600 \text{ M}^{-1}\text{cm}^{-1} \) for the ruthenium polypyrindyl center of the metallopeptide and \( \varepsilon_{530} = 10,100 \text{ M}^{-1}\text{cm}^{-1} \) for cyt c.

II.2.8 NMR Measurements

Most NMR samples were prepared by dissolving the metallopeptide in 90% H\(_2\)O/10% D\(_2\)O at 5-15 mM concentrations with one exception that a COSY experiment was performed by using 90% D\(_2\)O/10% H\(_2\)O as the solvent in order to exclude the interference of HDO, which has a proton chemical shift of 4.75. TMS was added for a chemical shift reference. One- and two-dimensional \(^1\text{H} \) NMR spectra were recorded on a 400-MHz Varian Unity spectrometer. Solvent suppression was achieved in all cases with a presaturation pulse.
II.3 Results and Discussion

II.3.1 Synthesis and characterization

The head-to-tail cyclic peptide *cyclo*[Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu] was synthesized according to a three-dimensional orthogonal strategy which was described previously in literature.\(^54\) A glutamic acid with a C-terminal allyl ester was anchored to HMP resin through its side-chain carboxyl group. Stepwise solid-phase assembly of the linear peptide was performed on a peptide synthesizer using Fmoc chemistry and the resin was collected. To obtain the desired cyclic peptide, the following deprotections and cyclization were performed on resin until final cleavage. The allyl-protected C\(^\alpha\)-carboxyl group of the first loaded glutamic acid residue was selectively removed by treatment with palladium (0) under nearly neutral conditions. The Fmoc protected N-terminal amino group was liberated by using 20% piperidine in DMF. Head-to-tail cyclization was achieved intramolecularly in the resin-bound peptide. Compared to solution phase procedures, on-resin deprotections and cyclization have the advantages of clean ring closure and easy purification, which can be achieved by filtration.\(^55\) Release of the desired cyclic peptide was achieved by final deprotection and resin cleavage using TFA. The structure of the cyclic peptide and the synthetic strategy are shown in Scheme II.3. The collected peptide was analyzed by MALDI (Figure I.3, m/z: [M]\(^+\) calculated 845.85; found 846.48).
Scheme II.3 Structure of head-to-tail cyclic peptide CPE$_5$ and synthetic strategies.
The negatively-charged metallopeptide [Ru(bpy)$_2$(phen-am)-cyclo[Cys-Glu-D-Glu-Glu-Pro-Glu-D-Glu]$_3^{3-}$, called Rucyclic, whose structure is shown below, was designed to model a negatively-charged metalloprotein. An acetamido linker was used to attach the ruthenium polypyridine complex to the cysteine side chain of the head-to-tail cyclic peptide CPE$_5$. 

Figure II.4 Structure of the metallopeptide, Rucyclic.
The apopeptide was dissolved in 50 mM pH 7.0 phosphate buffer, and TCEP was added to inhibit oxidation of cysteine to form the disulfide bond. The pH of the solution was adjusted carefully to a value of 9~10 by adding 5% NaOH solution dropwise to facilitate the deprotonation of cystein side-chain, which has a pKa of 8.33. Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$ solution in DMF was added to the reaction mixture, and the resulting solution was heated for one hour at 65°C in the dark.

Scheme II.5 Synthesis of the mtallopeptide, Rucyclic.

The crude reaction mixture was applied directly onto an ion-exchange column (Sephadex-SP C-25) to remove unreacted Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$, which has two positive charges, thus it exchanged with the Sephadex-SP C-25 resin and remained in the column. However, the side chain carboxyl groups of the five glutamic acid residues are deprotonated at
neutral or basic condition since the pKa of the carboxyl group is 4.25. This results in five negative charges for the apopeptide and three negative charges for the metallopeptide in the basic reaction mixture. Thus, the negatively-charged product passed through the cation exchange column and was able to be crudely separated from the unreacted excess starting material.

Figure II.5 HPLC chromatogram of Rucyclic synthesis reaction mixture with absorbance monitored at 210 nm (red) and 450 nm (black).

By applying cation-exchange chromatography, further purification by reversed-phase HPLC was simplified, especially when Rucyclic and Ru(bpy)$_2$(phen-AcCl)(PF$_6$)$_2$ have close retention times (Figure II.6). The resulting metallopeptide, Rucyclic, was purified by preparative reverse-phase HPLC, using a linear gradient starting at 10% acetonitrile with 0.1% HTFA and
increasing to 30% in 40 minutes. The metallopeptide Rucyclic was identified by MALDI mass spectroscopy (m/z: [M]$^+$ calculated 1494.59; found 1494.28).

![Figure II.6 MALDI mass spectrum of metallopeptide Rucyclic.](image)

The UV-Vis absorption spectrum of Rucyclic displays an absorption band centered at 450 nm, the metal-to-ligand-charge-transfer (MLCT) of ruthenium (II) polypyridine complexes with an extinction coefficient of $\varepsilon_{450} = 16,600$ M$^{-1}$cm$^{-1}$. The emission of ruthenium polypyridine complex has a maximum at 610 nm and decays following single-exponential kinetics. The singlet MLCT state of Ru polypyridine complexes was excited at 450 nm, producing the excited singlet state, which converses to the triplet state through intersystem crossing (ISC) and returned to the ground state via both a radiative process (phosphorescence) and a radiationless deactivation (thermal). At 298 K, pH 7.0, the emission of Rucyclic at 610 nm decays with a
lifetime of $\tau_0 = 1.17\mu s$ and the first-order rate constant of $k_0 = (8.74 \pm 0.31) \times 10^5 \text{s}^{-1}$ in argon-saturated aqueous solution (Figure II.7).

![Graph showing triplet decay](image)

**Figure II.7** Triplet decay trace of Rucyclic in argon-saturated solution, [Rucyclic] = 7.0 μM in 0.5 mM pH 7.0 phosphate buffer at 298 K. The solid line represents fitting to a single exponential kinetics. The intensity is normalized at time zero.

The kinetic behavior and lifetime of the Rucyclic triplet decay are consistent with other Ru complexes reported previously. The emission lifetime of Ru(bpy)$_2$(phen-AcCl)(TFA)$_2$ was measured at the same condition with a lifetime of $\tau_0 = 1.01\mu s$.

### II.3.2. Effect of cyt c on the triplet decay kinetics

The emission lifetime of Rucyclic shortens with increasing cyt c concentration. The emission decay measured from a solution containing both Rucyclic and cyt c can be accurately fit to a double exponential decay kinetics (Eq.I.3),
\[ I(t) = A_S \exp(-k_S t) + A_L \exp(-k_L t) \]  \hspace{1cm} \text{(Eq.I.3)}

in which \( A_S, k_S \) and \( A_L, k_L \) are the amplitudes and rate constants of the shorter and longer lifetime components, respectively (Figure II.8). Turro et al.\textsuperscript{57} have demonstrated that cyt c quenched the luminescence of several ruthenium polypyridine complexes exclusively by photoinduced electron transfer. Lasey et al.\textsuperscript{47} also showed that the luminescence of a designed metallopeptide, RuCE\(_5\)G, was also quenched by cyt c via an electron transfer mechanism. The mechanism has been confirmed in both cases by the assignment of the 550 nm as the reduced form of cyt c in transient absorption spectroscopy.

**Figure II.8** Triplet decay traces of Rucyclic in (a) absence and (b) presence of cyt c in argon-saturated solution. [Rucyclic] = 7.0 µM, [cyt c] = 40 µM in 0.5 mM, pH 7.0 phosphate buffer at 298 K. The traces are fit to a single exponential and a biexponential kinetics, respectively. For comparison, the intensities are normalized at time zero.

As the concentration of cyt c in solution is increased from 7.5 µM to 80 µM, the magnitudes of \( k_S \) and \( k_L \) both increased, with \( k_S \) approaching saturation above 40 µM and \( k_L \)
above 80 µM (Figure II.9). These results indicate there are two populations of photoexcited [Rucyclic]^* exist when interact with cyt c.

**Figure II.9** Observed rate constants of the fast and slow component of the triplet decay of Rucyclic (7.0 µM) as a function of the concentration of cyt c in 0.5 mM pH 7.0 phosphate buffer. The solid line represents the fit of the data to Eq. II.1.

\[
k = k_0 + \frac{k_{ET}K_{b}[cytc]}{1 + K_{b}[cytc]}
\]  

(Eq.II.1)

In Eq. II.1, \(k_0\) is the decay rate constant measured in the absence of cyt c, \(k_{ET}\) is the observed rate constants for electron transfer occurring within the encounter complexes, and \(K_b\) is the binding constant for the formation of encounter complexes which was defined as \(K_b = k_{on} / k_{off}\). The kinetics of the bimolecular electron transfer reaction can be described by Eq. II.1, in which a prior equilibrium and steady-state approximation were assumed. For the scheme
in which the first step is bimolecular, if the first step is much faster than the second, then [I] can be regarded as being equilibrium. Consider the case where [B] >> [A], with rate constants such that the buildup of P follows first-order kinetics. The approximate solutions are

\[
\text{Case I} \quad k_{obs} = \frac{k_1 k_2 [B]}{k_{-1} + k_2}
\]

\[
\text{Case II} \quad k_{obs} = \frac{k_1 k_2 [B]}{k_1 [B] + k_{-1} + k_2}
\]

The rate constant will be either a linear (case I) or a plateauing (case II) function of [B], depending on the magnitude of \(k_1[B]\) compared to \(k_{-1} + k_2\). In case II, if the rate constant does level off at a high [B], it approaches \(k_2\), which is the maximum value. On the other hand, if case I applies, the rate never attains a maximum. In the system of \(*\text{Ru(II)Cyclic}\) and cyt c, \([*\text{Ru(II)Cyclic}]\) is much smaller compared to [cyt c], and the electron transfer reaction is the rate-limiting step compared to the formation of the encounter complexes, which involves a very rapid collisional mechanism. The observed rate constant in case II can be further simplified as

\[
k_{obs} = \frac{K_b k_2 [B]}{K_b [B] + 1}
\]

since \(k_2\) is much smaller than \(k_{-1}\), thus making \(k_2/k_{-1}\) negligible.

The observed quenching rate constants for both the two populations can be fit well into Eq.II.1 and yield \(k_0 = (8.43 \pm 0.20) \times 10^5 \text{ s}^{-1}\), which is comparable to \(k_0 = (8.74 \pm 0.31) \times 10^5 \text{ s}^{-1}\), which was measured directly from the lifetime of Rucyclic alone. Binding constants values obtained for the two populations by fitting to Eq. II.1 are \(9.4 \times 10^3 \text{ M}^{-1}\) for the longer lifetime.
component, and $2.7 \times 10^5 \text{M}^{-1}$ for the short lifetime component. The relative amplitudes of the two decay components, $A_S$ and $A_L$, can be used to determine the fractional population of the short lifetime ruthenium peptide encounter complex according to

$$f_S = \frac{A_S}{A_S + A_L}$$

![Graph showing the dependence of the fractional population of the short lifetime component with increasing cyt c concentration.](image)

**Figure II.10.** Fraction of the short lifetime component as a function of [cyt c] in solution, [Rucyclic] = 7 µM, 0.5 mM pH 7.0 phosphate buffer at 298 K.

Figure II.10 shows the dependence of the fractional population of the short lifetime component with increasing cyt c concentration. According to Figure II.10, the population of the short lifetime component increases with the increasing cyt c concentration. This indicates that the quenching mechanism of the excited Rucyclic by cyt c is more complicated than the simple model involving two conformations of Rucyclic quenched separately by cyt c. If Rucyclic has two conformations and these two conformations, which form two encounter complexes with cyt
c, are quenched separately by cyt c, the fractional population of the two encounter complexes should be constant, thus independent of cyt c concentration.

II.3.3. Viscosity dependence of the electron transfer kinetics

Upon adding sucrose as viscogen, the emission of Rucyclic decays via a biexponential kinetics, which is consistent with the behavior seen in the absence of sucrose. From the kinetics analysis above, it was shown that if the rate constant does level off at a high [cyt c], it approaches \( k_{ET} \), which is the maximum value. In this case, the true electron transfer rates for the short lifetime component were measured using [cyt c] = 60 \( \mu \)M, where the observed rate constants reach plateau. In these experiments, solutions were prepared by mixing different amounts of sucrose and phosphate buffer in such a way as to maintain a constant pH and ionic strength. As shown in Figure II.11, the electron transfer rate constants for the short lifetime component decrease smoothly with increasing solution viscosity (4, 14, 24, 32, 44, 48, and 56% sucrose by weight, for \( \eta = 1.1, 1.5, 2.3, 3.6, 8.5, 12.4, \) and 33.1 cP, respectively) and can be fit to an empirical exponential relationship (Eq. II.2) to yield values of \( A = (1.75 \pm 0.08) \times 10^6 \) s\(^{-1} \), \( C = (1.00 \pm 0.09) \times 10^6 \) s\(^{-1} \), and \( \alpha = 0.89 \pm 0.04 \).

\[
k_{ET}^{obs} = A \eta^{-\alpha} + C
\]

(Eq. II.2)

The value obtained for the exponent is lower in magnitude than the value of \( \alpha = 1 \) predicted by Kramers’ theory \(^{59}\) for reactions of small molecules controlled by the molecular collision in solutions. However, it is comparable to those observed for some related protein-protein studies for which values of \( \alpha \) have been reported to be in the range of 0.6 - 0.7. \(^{60, 61}\) This result indicates that some internal friction within the complex works together with the friction
between the complex and the solvent to retard the configurational reorientation process. The viscosity dependence data was further fit with the modified Kramers’ equation (Eq II.3),

\[ k_{ET}^{obs} = \frac{k_B T (1 + \sigma)}{h (\eta + \sigma)} \exp(-\Delta G^e / RT) + k_\infty \]  

(Eq. II.3)
in which \( \eta \) is the solution viscosity, \( \sigma \) is the internal protein friction, \( \Delta G^e \) is the activation free energy, and \( k_\infty \) is the rate constant at infinite viscosity where configurational motions are prohibited.\(^{62-64}\)

**Figure II.11** Viscosity dependence of \( k_{ET}^{obs} \), the rate constant for electron-transfer occurring within the encounter complex with the short lifetime component. Conditions: [Rucyclic] = 7 \( \mu \)M, [cyt c] = 60 \( \mu \)M in 0.5 mM pH 7.0 phosphate buffer at 298 K. The solid line represents a fit of the data to Eq II.2 as described in the text. The error bars reflect the standard deviation of results obtained from the average of three independent experiments performed for each point.
The fitting trace is very similar to Figure II.11, and gives $\sigma = 0.15$ cP, $\Delta G^\ddagger = 37.2$ kJ mol$^{-1}$, and $k_\infty = (1.30 \pm 0.02) \times 10^6$ s$^{-1}$. The value obtained for the activation free energy is comparable to that of RuCE$_5$G ($\Delta G^\ddagger = 37.0$ kJ mol$^{-1}$). The $\sigma$ value obtained here indicates that the effect of the internal friction within the complex to the configurational reorientation process is comparable to that of the environment viscosity of 0.15 cP. The $k_\infty$ obtained here can be treated as the real electron transfer rate of the complex with the initial thermodynamic stable but less electron transfer active conformation.

The viscosity experiments show that the observed values of electron transfer do not reflect the rate of a true electron transfer event; instead, the ET events are gated by a rate-limiting conformational change of the complexes: $k_{ET}^{obs} = k_{gate} < k_{ET}^{True}$.

**Figure II.12** Viscosity dependence of $k_{ET}^{obs}$, the rate constant for electron-transfer occurring within the encounter complex with the long lifetime component. Conditions: [Rucyclic] = 7 µM, [cyt $c$] = 60 µM in 0.5 mM pH 7.0 phosphate buffer at 298 K. The solid line represents a fit of the data to Eq II.2 as described in the text. The error bars reflect the standard deviation of results.
obtained from the average of three independent experiments performed for each point. Inset: linear fit of ln(kET-C) vs. ln(η) for both lifetime components.

When 44% sucrose was present, the magnitude of ks increased with the increasing cyt c concentration (Figure II.13), which is similar to what observed without sucrose. The observed quenching rate constants for the short lifetime component can be fit well into Eq. II.1 and yield $k_0 = (1.22 \pm 0.15) \times 10^6 \text{ s}^{-1}$ and a binding constant value of $2.2\times10^4 \text{ M}^{-1}$, which is ten fold smaller compared to the value without sucrose.

![Figure II.13](image)

**Figure II.13** Observed rate constants of the fast component of the triplet decay of Rucyclic (7.0 µM) as a function of the concentration of cyt c in 0.5 mM pH 7.0 phosphate buffer with 44% sucrose. The error bars reflect the standard deviation of results obtained from the average of three independent experiments.

**II.3.4 NMR assignment of the Rucyclic conformations**
In order to confirm the existence of two different conformations of Rucyclic and to identify them, NMR experiments were performed. The identification of the connectivities and the assignments of the proton resonances of Rucyclic were determined from the 2D correlated spectroscopy. The cis/trans interconversion of amide and peptide can be observed separately if a sufficient spectral resolution is available. When correct NMR parameters have been obtained they must be assigned to a molecular constitution before their interpretation with respect to molecular conformation can start. Most of the common amino acids exhibit typical patterns resulting from distinct chemical shift values. The amide NH signal region overlaps with the aromatic signals in the range of 7-9 ppm in 1-D $^1$H NMR. In COSY, by examining the amide resonances for each amino acid, information about how many amino acids are presented can be obtained. Figure II.14 shows the resonance region between amide protons and $\alpha$ protons.

Figure II.14 400-MHz $^1$H-$^1$H correlation of Rucyclic (amide and $\alpha$ protons regions).

Seven signals were observed unambiguously, yet there is still a possibility that some signals were unobserved because of the heavy overlap with water resonance suppression. In this
case, double-quantum filtered COSY was performed to further illustrate the number of amino acids presented. Figure II.15 shows one more signal with an amide proton chemical shift of 8.16 ppm and an α proton chemical shift of 4.62 ppm. In COSY, the α and β proton crosspeak corresponding to this signal is relatively weak with a β proton chemical shift of 3.13 ppm shown in Figure II.15. In this case, this signal was assigned as Cys with more information obtained from HMBC. In an HMBC experiment, a signal with a carbonyl carbon chemical shift of 174 ppm is shown to be coupled to the α proton chemical shift of 4.62 ppm that we are monitoring and a proton signal with a chemical shift of 3.13 ppm. Based on the information from both COSY and HMBC this amide and α proton crosspeak can be unambiguously assigned to a Cys residue.

**Figure II.15** 400-MHz $^1$H-$^1$H double-quantum filtered correlation of Rucyclic (amide and α proton region)

It is obvious that, if only one conformation exists, there should be only six crosspeaks found in this region with the absence of proline, which has no amide proton. The finding that
eight crosspeaks were found strongly suggests the existence of two or even three conformations. In order to confirm this, the identification of these eight signals needs to be determined. The identification of each amide proton signal was obtained by analyzing the other areas of COSY: the couplings between $\alpha$ and $\beta$, $\beta$ and $\gamma$, as well as $\gamma$ and $\delta$ protons, which are shown in Figures II.16 and II.17. To exclude the interference of the huge water peak, the COSY areas shown in Figures I.16 and I.17 were obtained by using 90% D$_2$O/10% H$_2$O with other experiment conditions unchanged.

**Figure 11.16** 400-MHz $^1$H-$^1$H double-quantum filtered correlation of Rucyclic (amide and aliphatic proton regions)
Each amino acid residue exhibits distinct chemical shift values, and the assignment of all eight amide and α proton resonances was accomplished. Table II.1 summarizes the assignment of each of the eight amide and α proton resonances and α, β, γ and δ protons of proline. It is found that, except for the signal obtained in double-quantum filtered COSY, all the other seven amide and α proton signals result from glutamic acids.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amide</th>
<th>α</th>
<th>β</th>
<th>γ</th>
<th>δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Glu</td>
<td>7.40</td>
<td>4.38</td>
<td>2.02</td>
<td>2.18</td>
<td></td>
</tr>
<tr>
<td>2 D-Glu (1)</td>
<td>8.82</td>
<td>4.38</td>
<td>1.92</td>
<td>2.42</td>
<td></td>
</tr>
<tr>
<td>2 D-Glu (2)</td>
<td>8.76</td>
<td>4.33</td>
<td>1.88</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td>3 Glu (1)</td>
<td>8.72</td>
<td>4.16</td>
<td>2.06</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>3 Glu (2)</td>
<td>8.70</td>
<td>4.14</td>
<td>2.00</td>
<td>2.40</td>
<td></td>
</tr>
<tr>
<td>4 Cys</td>
<td>8.16</td>
<td>4.64</td>
<td>3.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 D-Glu</td>
<td>8.42</td>
<td>4.48</td>
<td>1.87</td>
<td>2.22</td>
<td></td>
</tr>
<tr>
<td>6 Glu</td>
<td>7.43</td>
<td>4.40</td>
<td>2.02</td>
<td>2.30</td>
<td></td>
</tr>
<tr>
<td>7 Pro (1)</td>
<td>4.24</td>
<td>2.40</td>
<td>1.90</td>
<td>2.14,2.06</td>
<td></td>
</tr>
<tr>
<td>7 Pro (2)</td>
<td>4.20</td>
<td>2.12</td>
<td>2.00</td>
<td>1.98</td>
<td>3.62, 3.74</td>
</tr>
</tbody>
</table>

Table II.1 Chemical shifts of amide, α, β, γ, and δ protons in Rucyclic peptide

Further evidence is needed to assign the peptide sequence in order to better understand the structure of two conformations of the encounter complexes and their different binding behaviors with cyt c observed in the emission lifetime experiments. For sequence analysis, through-space (NOE) or through-bond (coupling) effects across the amide bonds must be used. The conventional way to get the connectivity between the amino acids is to measure the NOE effects. The most common effects are observed between NH protons of one amino acid and the α or β protons of the amino acid preceding the sequence. Overhauser enhancements between α protons of adjacent amino acids occur across cis-peptide bonds.
When interpreting the NOESY results, some of the signals were lost. It seems that the coupling to the \( \alpha \) proton within the same amino acid is always weaker than that to the \( \alpha \) proton of the residue following in the sequence. Starting from a crosspeak arising from the NH proton and the \( \alpha \) proton within the same amino acid, which has the NH proton chemical shift of 8.82 ppm and the \( \alpha \) proton chemical shift of 4.38 ppm, and which has been assigned as a Glu in COSY and numbered as a.a.2 (1) in table 1, an NOE signal corresponding to the NH proton chemical shift of 8.82 ppm and the \( \alpha \) proton chemical shift of 4.16 ppm was found. This NOE signal indicates the Glu with an NH chemical shift of 8.72 ppm and an \( \alpha \) proton chemical shift of 4.16 ppm, which was numbered as a.a 3(1), is coupled to the starting Glu a.a 2(1). The sequence therefore is Glu-Glu, which can be extended to the full sequence. Due to the absence of the amide proton in proline, the sequence terminates when it comes to proline. The sequence observed should be
Glu-D-Glu-Glu-Cys-D-Glu-Glu-Pro. Through the NOESY experiment, each amino acid residue, which has already been identified in COSY, can have its position in the cyclic peptide sequence determined. The sequence is summarized in Table II.1. The numbers before each amino acid indicate its position in the sequence, which should be observed in NOESY, with proline as the end. For six out of ten amino acid residues observed, a second number in brackets indicates their presence in different conformations. It is clearly shown that two conformations exist according to the peptide sequence assignment.

According to NMR analysis, the conclusion can be drawn that two conformations exist for Rucyclic metallopeptide and the existence of two conformations result in the two encounter complexes observed when Rucyclic interact with cyt c.

II.3.5 Computer modeling of Rucyclic

Molecular modeling of the Rucyclic peptide (Figure II.19) was built to better understand the different binding behavior of Rucyclic with cyt c. Compared to the extended linear conformation of RuCE$_5$G, Rucyclic has a more compact structure and the charge density on the negatively-charged peptide chain is smaller than that of RuCE$_5$G due to the addition of a neutral praline residue. It is speculated that the more compact structure of Rucyclic together with the smaller charge density might lead to the absence of preformed complex when interacts with cyt c.
In summary, Rucyclic forms two encounter complexes when interact with cyt c. Compared to the linear metallopeptide RuCE$_5$G, this different binding behavior may be due to the two different conformations of Rucyclic, which has been confirmed by NMR experiments. Molecular modeling of Rucyclic indicates that it may adopt a more compact structure; together with the smaller charge density along the peptide chain result in the absence of the electrostatic preformed complex with cyt c. Small metalloprotein models, such as RuCE$_5$G, RuCE$_5$G-short, and Rucyclic, might bind to different binding domains on the protein surface due to their different conformations. Thus, small metalloprotein models can serve as a tool to explore the more complicated natural proteins whose crystal structures and conformations are still unknown. In addition, the dynamic docking of protein-protein interactions may be illustrated by studying the gated electron transfer reactions between small metalloprotein models and the proteins examined.
II.3.6 5-Cl-PhenRuCE₅G: Investigation of the reorganization energy and donor-acceptor distance

Gated electron transfer processes occur within the preformed and encounter complexes of RuCE₅G/cyt c. In order to achieve the meaningful values of reorganization energy and donor-acceptor distance, studies on the true electron transfer rates are required. The fitting of $k_{ET}^{obs}$ to the modified Kramers’ equation (Eq.II.3) for the viscosity dependence of gated electron transfer rates can be used to determine the electron transfer rate at infinite viscosity, $k_{\infty}$. Since no configurational reorientation is allowed at infinite viscosity, the $k_{\infty}$ value can be treated as the true electron transfer rate within the peptide-protein complex at the initial thermodynamically stable configuration. Useful information on dynamic peptide-protein complexes, such as the reorganization energy and donor-acceptor distance in the preformed complex, could be obtained by investigating the driving force dependence of $k_{\infty}$, using different metallopeptides with different redox potentials.

Liu⁹ has synthesized RuCE₅G, bisPhenRuCE₅G, and DMPRuCE₅G, with redox potentials of 1.36 V, 1.29 V, and 1.20 V vs. NHE respectively. These metallopeptides were synthesized by coupling different ruthenium chromophores, [Ru(phen)₂(phen-am)]²⁺ and [Ru(dimethyl-phen)₂(phen-am)]²⁺ with the same apopeptide, CE₅G. The electron withdrawing or donating abilities of the substituents on the parent ligand, 1, 10-phenanthroline, provide a wide range of redox potentials for the ruthenium center, resulting in different driving forces for the electron transfer reaction. Redox potentials, observed electron transfer rates, $k_{\infty}$, and driving forces of preformed complexes of RuCE₅G, bisPhenRuCE₅G and DMPRuCE₅G are listed below in Table II.2.
Table II.2 Comparison of redox potentials, observed electron transfer rates, $k_\infty$, and driving forces of preformed complexes of RuCE$_5$G, bisPhenRuCE$_5$G and DMPRuCE$_5$G. (Adapted from reference 9)

<table>
<thead>
<tr>
<th></th>
<th>$E^0$ (eV)</th>
<th>$-\Delta G^0$ (eV)</th>
<th>$k_\infty$ ($10^6$ s$^{-1}$)</th>
<th>$k^{\text{obs}}_E$ ($10^6$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuCE$_5$G</td>
<td>1.36</td>
<td>1.07</td>
<td>0.85 ± 0.04</td>
<td>2.7</td>
</tr>
<tr>
<td>bisPhenRuCE$_5$G</td>
<td>1.29</td>
<td>1.18</td>
<td>1.05 ± 0.02</td>
<td>2.7</td>
</tr>
<tr>
<td>DMPRuCE$_5$G</td>
<td>1.20</td>
<td>1.23</td>
<td>1.08 ± 0.03</td>
<td>2.1</td>
</tr>
</tbody>
</table>

The ruthenium complex, [Ru(5-Cl-phen)$_2$(phen-ACl)](PF$_6$)$_2$ used to prepare the metallopeptide, 5-Cl-PhenRuCE$_5$G, was made by the same procedures described in the experimental section. However, the coupling with the apopeptide was much more difficult than the reaction of [Ru(bpy)$_2$(phen-ACl)](PF$_6$)$_2$ with CE$_5$G. A much harsher condition was required: pH 9-10, heat at 65 °C for 1 hour. The crude reaction mixture was purified by reverse-phase HPLC using a linear gradient starting at 10% acetonitrile with 0.1% HTFA and increasing to 30% in 40 minutes, shown in Figure II.20, with absorbance monitored at 450 nm. The purified 5-Cl-PhenRuCE$_5$G was analyzed by MALDI-TOF mass spectroscopy (Figure II.21, m/z: [M]$^+$ calculated 1589.50; found 1589.65).
Figure II.20 HPLC chromatogram of 5-Cl-PhenRuCE$_5$G synthesis reaction mixture with absorbance monitored at 450 nm.
The UV-Vis spectrum of 5-Cl-PhenRuCE$_5$G is very similar to that of its ruthenium precursor, which shows MLCT band at 450 nm. The extinction coefficient, $1.25 \times 10^4$ M$^{-1}$ cm$^{-1}$, was obtained from the UV-Vis absorption working plot. This value was used to determine the concentrations of the metallopeptide. The reduction potential of 5-Cl-PhenRuCE$_5$G was obtained by measuring the reduction potential of [Ru(5-Cl-phen)$_2$(phen-NHAc)]$^{2+}$, a model compound. Differential-pulse polarography (DPP) gave the $E^0$ (Ru$^{III}$/Ru$^{II}$) = 1.47 V vs. NHE. This number is comparable with values of various Ru complexes reported.$^{56}$ The larger conjugated system of the phenanthroline ligand stabilizes the higher oxidation state of the ruthenium center, which should result in a lower reduction potential of 5-Cl-PhenRuCE$_5$G compared to RuCE$_5$G. However, the electron withdrawing inductive effect of the chloro group increases its reduction potential and
results in a higher redox potential of 5-Cl-PhenRuCE5G as compared to RuCE5G, bisPhenRuCE5G, or DMPRuCE5G.

![Figure II.22](Image)

**Figure II.22** Differential-pulse polarography (DPP) of [Ru(5-Cl-phen)₂(phen-NHAc)]²⁺ measured at room temperature in water solution with 0.1 M pH 7.0 phosphate buffer as the support electrolyte.

The emission of 5-Cl-PhenRuCE5G follows a single exponential decay kinetics with a lifetime of $\tau_0 = 1.2 \mu$s, which is slightly longer than that of RuCE5G ($\tau_0 = 1.1 \mu$s). The result implies that the electronic structure of this new metallopeptide is very similar to that of RuCE5G. The triplet state emission decay of 5-Cl-PhenRuCE5G is very similar to those observed for RuCE5G, bisPhenRuCE5G and DMPRuCE5G as described in Scheme I.1. The observed electron transfer rates, $k_{ET}^{obs}$ of the preformed complexes of 5-Cl-PhenRuCE5G is $(3.1 \pm 0.2) \times 10^6 \text{ s}^{-1}$. Compared to the rate constant observed for RuCE5G, $k_{ET}^{obs} = (2.7 \pm 0.1) \times 10^6 \text{ s}^{-1}$, the preformed 5-Cl-PhenRuCE5G/Cyt c complex exhibits a rate constant that is about 15% larger than that of
RuCE₅G. Figure II.23 shows the concentration dependence of the observed rate constants for both the short lifetime component and the long lifetime component.

**Figure II.23** Observed rate constants of the fast and slow component of the triplet decay of 5-Cl-PhenRuCE₅G (7.0 µM) as a function of the concentration of cyt c in 0.5 mM pH 7.0 phosphate buffer.

Figure II.24 shows the cyt c concentration dependence of the preformed complex faction of 5-Cl-PhenRuCE₅G, which can be fit to the 1:1 binding isotherm very well.

\[
f_{\text{complexed}} = \frac{1/K_a + [Ru]_0 + [Cyt c] - \sqrt{(1/K_a + [Ru]_0 + [Cyt c])^2 - 4[Ru]_0[Cyt c]}}{2[Ru]_0} \quad (\text{Eq. II.4})
\]

In Eq II.4, \(K_a\) is the equilibrium binding constant and \([Ru]_0 = 7\)µM is the initial concentration of RuCE₅G. The solid line in Figure I.20 is a non-linear least squares fit of the data to Eq II.4, which gives a binding constant of \(K_a = (2.9 \pm 0.1) \times 10^4 \) M⁻¹ in 0.5 mM phosphate buffer, pH = 7.
at 298 K. Compared to the binding constants of the preformed RuCE₅G/cyt c complex ($K_a = 3.5 \times 10^4 \text{M}^{-1}$), bisPhenRuCE₅G/cyt c ($K_a = 2.9 \times 10^4 \text{M}^{-1}$), DMPRuCE₅G/cyt c ($K_a = 1.8 \times 10^4 \text{M}^{-1}$), the preformed 5-Cl-PhenRuCE₅G/cyt c complex has a slightly lower binding affinity than RuCE₅G, similar to that of the bisPhenRuCE₅G and about 60% larger than that of the DMPRuCE₅G/cyt c complex.

![Fractional population of bound 5-Cl-PhenRuCE₅G as a function of total cyt c concentration](image)

**Figure II.24** Fractional population of bound 5-Cl-PhenRuCE₅G as a function of total cyt c concentration, [Ru-peptide] = 7 µM, in 0.5 mM pH 7.0 phosphate buffer at room temperature.

As discussed in the introduction, the viscosity dependence of the $k^{\text{obs}}_{ET}$ of the preformed RuCE₅G/cyt c complex shows that the electron transfer process is gated by the configurational reorientation and that the observed rate constant is not the real electron transfer rate, but the reorientation rate. The viscosity dependence of the observed electron transfer rates of the preformed 5-Cl-PhenRuCE₅G/cyt c complex was examined using the same procedures used for the RuCE₅G/cyt c complex. Figure II.25 shows that the observed electron transfer rate constants
of the preformed 5-Cl-PhenRuCE$_5$G/cyt $c$ complex decrease as solvent viscosity increases. Electron transfer process in this complex is also gated by the reorientation.

**Figure II.25** Viscosity dependence of the observed intracomplex electron-transfer rate constant for 5-Cl-PhenRuCE$_5$G, [Ru-peptide] = 7 µM, [cyt $c$] = 20 µM, 0.5 mM pH 7.0 phosphate buffer at 298 K.

The solid line represents a fit of the data to Eq. II.3 as described in the text. The error bars reflect the standard deviation of results obtained from the average of three independent experiments performed for each point. The $k_\infty$ value obtained from the fitting according to the modified Kramers’ equations is $(6.13 \pm 0.83) \times 10^5$ s$^{-1}$ for 5-ClPhenRuCE$_5$G. Combined with the $k_\infty$ values obtained by Liu$^9$ for RuCE$_5$G, bisPhenRuCE$_5$G and DMPRuCE$_5$G, the driving force dependence of $k_\infty$ can be fit to the Marcus equation (Eqs. I.1 and I.2) to obtain the electronic coupling constant, the reorganization energy and donor-acceptor distance.

$$k_{ET} = \frac{2\pi^2}{h}\frac{H^2_{AB}}{\sqrt{\pi \lambda k_B T}} \exp\left[-\frac{(\Delta G^0 + \lambda)^2}{4\lambda k_B T}\right]$$  \hspace{1cm} (Eq. I.1)
\[ k_{ET} = k_0 \exp[-\beta(r - r_0)] \exp[-\frac{(\Delta G^0 + \lambda)^2}{4\lambda RT}] \]  

(Eq. I.2)

<table>
<thead>
<tr>
<th></th>
<th>(E^0) (eV)</th>
<th>(-\Delta G^0) (eV)</th>
<th>(k_\infty ) (10(^6) s(^{-1}))</th>
<th>(k_{ET}^{\text{obs}} ) (10(^6) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-ClPhenRuCE(_5)G</td>
<td>1.47</td>
<td>0.98</td>
<td>0.61 ± 0.08</td>
<td>3.1</td>
</tr>
<tr>
<td>RuCE(_5)G</td>
<td>1.36</td>
<td>1.07</td>
<td>0.85 ± 0.04</td>
<td>2.7</td>
</tr>
<tr>
<td>bisPhenRuCE(_5)G</td>
<td>1.29</td>
<td>1.18</td>
<td>1.05 ± 0.02</td>
<td>2.7</td>
</tr>
<tr>
<td>DMPRuCE(_5)G</td>
<td>1.20</td>
<td>1.23</td>
<td>1.08 ± 0.03</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table II.3** Comparison of redox potentials, observed electron transfer rates, \(k_\infty\), and driving forces of preformed complexes of 5-ClPhenRuCE\(_5\)G, RuCE\(_5\)G, bisPhenRuCE\(_5\)G and DMPRuCE\(_5\)G. (Partly adapted from reference 9)

**Figure II.26** Marcus curve fit for the dependence of \(\ln k_\infty\) on driving force of the four metallopeptides.
Figure II.26 shows the relationship between $k_\infty$ and the driving force of the excited-state electron transfer reaction. The $\ln k_\infty$ vs. $-\Delta G^0$ plot was perfectly fit to the modified Marcus equation (Eq. I.2), which yields a reorganization energy ($\lambda$) equal to 1.25 eV, the electronic coupling constant ($H_{AB}$) equal to 0.069 cm$^{-1}$, and a donor-acceptor distance ($r$) equal to 16.4 Å. This reorganization energy is consistent with the $\lambda$ value found for electron-transfer reactions within single metalloproteins labeled with ruthenium complexes, which have been found to be 1.2 and 1.3 eV, respectively, for cyt $c$ and Mb,$^{11}$ and for reactions involving pairs of metalloproteins, such as protein-protein complex for the hemoglobin and cyt $b_5$, which is 1.4 eV.$^{68-70}$ The $\lambda$ value obtained is higher than the driving forces of all the four electron transfer reactions. All the four reactions are thus, located in the normal region of the Marcus curve, where the electron transfer rate increases with the increasing driving force. In the fit of the four different metallopeptides, it is assumed that the differences in reorganization energy and electronic coupling constant are negligible. A similar assumption was made in the investigations of electron transfer in protein-protein complexes,$^{71-78}$ protein-uroporphyrin complexes,$^{79}$ and bimolecular electron transfer between cyt $c$ and several positively-charged ruthenium complexes.$^{57}$ These results show that the de novo designed metallopeptide can thus be useful probes of the dynamics of protein-protein complexes.

REFERENCES


