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Electron-transfer reactivity of metalloproteins in folded, partially unfolded, and completely unfolded forms

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Electron-transfer reactivity of metalloproteins in folded, partially unfolded, and completely unfolded forms

by

Scott Michael Tremain

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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2002
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For the Major Program
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CHAPTER 1. GENERAL INTRODUCTION

General Overview

With the sequencing of the human genome complete, attention has shifted to studying the structure and stability of proteins, protein-protein and protein-ligand interactions, structure-function relationships, and how these relate to disease and drug discovery. The biological function and reactivity of a protein depends critically on its ability to adopt a specific and unique three-dimensional structure. When a protein is synthesized in the cell, its conformation approximates a random-coil, lacking most secondary and tertiary structure. Utilizing only its sequence of amino acids, proteins spontaneously fold into their native conformations under physiological conditions. A protein can efficiently fold from many unfolded states to one native state on the time scale of milliseconds to seconds. Understanding how this process occurs is one of the great challenges in science.

Metal ions and organic moieties are essential components of proteins involved in biological processes, such as respiration, metabolism, and photosynthesis. Nearly one-third of all proteins require cofactors to bind and activate substrates, transfer atoms or groups, transfer electrons, and provide structure. A class of proteins utilizing a cofactor to perform its biological function is cytochrome c. C-type cytochromes are defined as electron transfer proteins which have an iron porphyrin (heme) covalently attached to the protein backbone through cysteine residues. Because of their biological roles and favorable chemical and spectroscopic properties, these proteins were used in many important biochemical and biophysical studies. Especially relevant are recent studies on the folding of horse heart
cytochrome c.\textsuperscript{9-11}

One strategy that can provide insight into the mechanism of protein folding is to detect and characterize the intermediates in the folding pathway. One such intermediate is the molten globule, a compact denatured form with a significant amount of nativelike secondary structure, but largely flexible and disordered tertiary structure. In comparison with the native folded form, the internal hydrophobic core of the molten globule is more exposed to solvent and the side chains are more mobile.\textsuperscript{12} Characterization of these intermediates by traditional crystallographic or NMR methods is difficult because of the dynamic, heterogeneous, and often transient nature of these nonnative states.

Our approach involves measuring the intrinsic chemical reactivity of proteins in various conformational forms. Cytochrome c is our protein of choice because its folding mechanism has been thoroughly investigated and the covalently-bound heme prosthetic group provides an excellent optical marker. Various conformational forms can be captured in solution by simply changing the conditions (pH, presence of anions, presence of denaturants). At pH 2 in the presence of stabilizing anions, the molten-globule form of cytochrome c can be obtained.

The replacement of redox-active heme iron by redox-inactive zinc(II) in cytochrome c, allows study of reactions between heme proteins, which otherwise have overlapping absorption spectra. The long-lived triplet state of the zinc porphyrin, designated $^{3}$\textsubscript{Zncyt}, is easily produced by laser flash and is a strong reductant. Moreover, we can study photoinduced electron transfer from $^{3}$\textsubscript{Zncyt} to an electron acceptor without the complications of external reductants.
As a prerequisite to studying the reactivity of proteins in partially and completely unfolded forms, we investigate photoinduced electron-transfer reactions of folded $^{3}\text{Zncyt}$ with various cytochrome c derivatives. There are three possible mechanisms for quenching of $^{3}\text{Zncyt}$: enhancement of radiationless decay, Förster (dipole-dipole) energy transfer, and electron transfer. Our work will provide insight into the mechanism for quenching of $^{3}\text{Zncyt}$ by the iron(III), iron(II), iron-free, and heme-free forms of cytochrome c under conditions in which these proteins are folded.

To study the effect of protein conformation on reactivity, we compare the electron-transfer properties of $^{3}\text{Zncyt}$ in the folded forms at low ($F_{\text{low}}$) and high ($F_{\text{high}}$) ionic strength, molten-globule (MG), and forms unfolded by acid ($U_{\text{acid}}$) and by urea ($U_{\text{urea}}$) toward the following four oxidative quenchers: $\text{Fe(CN)}_6^{3-}$, $\text{Co(acac)}_3$, $\text{Co(phen)}_3^{3+}$, and iron(III) cytochrome c. The observed bimolecular rate constants will provide insights into the effect of the protein matrix in modulating electron-transfer reactivity.

**Dissertation Organization**

Chapter 2 explores the fate of the excited triplet state of zinc cytochrome c in the presence of iron(III), iron(II), iron-free, and heme-free forms of cytochrome c. The mechanism for quenching of the excited triplet state of zinc cytochrome c in the completely folded form is determined. Chapter 3 is a comprehensive study of the effect of protein conformation on electron-transfer reactivity. An electroneutral transition metal complex assesses the consequences of porphyrin exposure on electron-transfer reactivity. Chapters 2 and 3 represent papers that have been published in peer-reviewed journals. The dissertation
ends with Chapter 4, which gives the overall conclusions. The Appendix provides details on
the expression and isolation of the soluble form of turnip cytochrome $f$ for use in future
studies.

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CHAPTER 2. FATE OF THE EXCITED TRIPLLET STATE OF ZINC CYTOCHROME C IN THE PRESENCE OF IRON(III), IRON(II), IRON-FREE, AND HEME-FREE FORMS OF CYTOCHROME C

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Scott M. Tremain and Nenad M. Kostić

Abstract

We study, by laser flash photolysis, the mechanism of quenching the triplet state of zinc cytochrome c, $^3\text{Zncyt}$, by the iron(III), iron(II), iron-free, and heme-free forms of cytochrome c. The method of inserting zinc(II) ion into the heme group in a protein overcomes the difficulties in studying redox reactions between two heme proteins having similar absorption spectra. Quenching of positively charged $^3\text{Zncyt}$ by these positively charged reactants is promoted at high ionic strength, $\mu = 1500$ mM, which shields electrostatic charge and weakens protein-protein repulsion. Upon addition of potential quenchers, the rate constant for $^3\text{Zncyt}$ decay becomes higher in the following order: ferrocytochrome c < iron-free cytochrome c << ferricytochrome c. However, upon addition of as much as 350 $\mu$M heme-free cytochrome c, the rate of $^3\text{Zncyt}$ decay remains unchanged. The bimolecular rate constants for the reactions of $^3\text{Zncyt}$ are as follows: $(1.5 \pm 0.2) \times 10^4$
$M^{-1} s^{-1}$ with ferrocytochrome $c$, $(3.3 \pm 0.2) \times 10^4 M^{-1} s^{-1}$ with iron-free cytochrome $c$, and $(5.1 \pm 0.3) \times 10^6 M^{-1} s^{-1}$ with ferricytochrome $c$. Oxidative quenching of $^3Zncyt$ by ferricytochrome $c$ is proven by the observed formation of zinc cytochrome $c$ cation radical.

Energy transfer is responsible for the weak quenching of $^3Zncyt$ by iron-free cytochrome $c$ and ferrocytochrome $c$, as evident in the lack of any observable transient radical-ions $Zncyt^+$ and $Zncyt^-$ and the good overlap of the emission band of $^3Zncyt$ with the absorption bands of ferrocytochrome $c$ and iron-free cytochrome $c$.

**Introduction**

Because metalloproteins act as electron carriers and redox enzymes in many biological processes, chemical mechanisms of their electron-transfer reactions are being studied vigorously [1-5]. Various cytochromes $c$ are ubiquitous in biological systems as prototypical electron carriers. Because of their biological roles and favorable chemical and spectroscopic properties, mammalian cytochromes $c$ were used in numerous biochemical and biophysical studies of general importance [6-8]. Their oxidoreduction reactions with various chemical and biological agents have been investigated. Three-dimensional structures of iron(III) and iron(II) forms of cytochromes $c$ in both crystal and solution are known in detail [7,9-11]. The highly positively charged protein reacts with external redox partners via an exposed heme edge, which is surrounded by positively charged lysine residues [7].

Motivation for this work comes from the problem of studying the thermal redox reaction between two heme proteins. Because their absorption spectra overlap, changes in their oxidation states cannot be easily monitored spectrophotometrically [12,13]. A solution
to this problem is to replace redox-active iron in the heme by redox-inactive zinc(II), so that
the redox step becomes photoinduced. Replacement of iron(II) by zinc(II) does not
significantly perturb the structure of cytochrome c [14-16] and its interactions with other
proteins [17-19]. Derivatives of cytochrome c containing various metals in the place of iron
have been prepared and characterized [20]. This method has proven useful in the study of
some other physiological partners, notably cytochrome c and its peroxidase [2]. Cytochrome
c noninvasively reconstituted with zinc(II), called simply zinc cytochrome c and designated
Zncyt [14-17,21], has been used in kinetic studies of electron-transfer reactions in our and
other laboratories [16,22-40]. It has absorption maxima at 423 (Soret), 549, and 585 nm; a
fluorescent (singlet) excited state with the lifetime of 3.2 ns; and fluorescence maxima at 590
and 640 nm. Especially useful for kinetic studies is the lowest-lying triplet excited state,
designated $^3Zncyt$. Its lifetime is between 7 and 15 ms, depending on the protein purity.
Because this lifetime is very long, electron-transfer reactions of the triplet state can be studied
relatively easily. The $^3Zncyt$ state is a much stronger reductant than the native protein.
Because this state can be created simply by a laser pulse, no external reducing agents are
needed, and kinetic treatments can be accurate.

In this work we explore the effects of different possible quenchers on the lifetime of
$^3Zncyt$. Photoinduced reactions of $^3Zncyt$ with iron(III) cytochrome c (Fe$^{III}$cyt), iron(II)
cytochrome c (Fe$^{II}$cyt), iron-free cytochrome c (H$_2$cyt), and heme-free cytochrome c (apocyct),
at increasing ionic strength provide insight into the mechanism of possible quenching for
each of these reactants. Although these are not biological reactions in the narrow sense, this
work will be relevant to the future study of reactions between heme proteins.
Experimental

Chemicals. Distilled water was demineralized to a resistivity greater than 17 M\(\Omega\)·cm by the Barnsted Nanopure II apparatus. Chromatography resins and gels were purchased from Sigma Chemical Co., Pharmacia, and Bio-Rad. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fisher Chemical Co. and used as received.

Buffers. All the buffers were prepared fresh from the solid salts Na\(\text{H}_2\text{PO}_4\)·H\(_2\)O and Na\(_2\)HPO\(_4\)·7H\(_2\)O and had pH values of 7.00±0.05. The ionic strengths (\(\mu\)) higher than 10 mM were achieved by addition of solid NaCl. All buffers are characterized with ionic strength, not concentration. Buffers were degassed thoroughly prior to use by bubbling with wet ultrapure argon.

Proteins. Horse-heart cytochrome \(c\) was obtained from Sigma Chemical Co. Iron(III) and iron(II) forms were prepared simultaneously by treatment with enough ascorbic acid to reduce approximately one half of the protein in the sample. Separation was carried out with a CM-52 ion-exchange column; upon increasing the ionic strength, iron(II) form eluted first. The iron(III) and iron(II) forms were also prepared separately, by treatment with an excess of K\(_3\)[Fe(CN)\(_6\)] or ascorbic acid, and desalted with a Bio-Rad Econo-Pac 10 DG column. The rates of quenching \(^3\text{Zncyt}\) by Fe\(^{III}\)cyt and Fe\(^{II}\)cyt were independent of the method of preparation. The concentration of purified Fe\(^{II}\)cyt or Fe\(^{III}\)cyt was determined spectrophotometrically; the molar absorptivity difference between reduced and oxidized cytochrome \(c\) at 550 nm is 18.5 mM\(^{-1}\) cm\(^{-1}\).

Zinc cytochrome \(c\) was prepared and purified by a modification [16] of the original
procedure [17,40]. A saturated solution of Na$_2$HPO$_4$ was used instead of solid Na$_2$HPO$_4$ to adjust pH to 6.0 after reconstitution with zinc(II) ions. This method improves the yield of Zncyt by minimizing the loss of protein embedded within the precipitate. All procedures were done in the dark and as quickly as possible. Two of the criteria for purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. Another criterion for purity was the natural decay of $^3$Zncyt, $k_d < 110 \text{ s}^{-1}$. Iron-free (so called free-base) [14] and heme-free [41,42] forms of cytochrome c were prepared and purified by standard procedures. Concentration of apocyt was determined spectrophotometrically at 595 nm, with a Bio-Rad protein assay.

Kinetics. Laser-flash photolysis on the $\mu$s time scale was performed with a standard apparatus [27]. A Phase-R (now Luminex) DL 1100 laser contained a 50 $\mu$M solution of the dye rhodamine 590 in methanol and delivered 0.4-$\mu$s pulses of excitation light. The sample solution in a 10-mm cuvette was thoroughly deaerated by gentle flushing with wet ultrapure argon through a needle held 5 mm above the solution for at least 15 minutes after each addition of quencher or until the rate constant for $^3$Zncyt decay ceased changing. This procedure prevents denaturation of protein that might occur upon bubbling gas through the solution and ensures complete deoxygenation. Owing to some evaporation of the solvent in the argon stream, the sample was weighed before and after degassing to obtain accurate concentrations of Zncyt and quencher.

Decay of $^3$Zncyt was monitored at 460 nm, where its transient absorbance reaches a maximum. Eight to ten pulses were recorded for each addition of quencher. Appearance and disappearance of the zinc cytochrome c cation radical, Zncyt$^+$, was monitored at 675 nm,
where the difference in absorbance between Zncyt\(^+\) and \(^3\)Zncyt is greatest. Temperature was held by a circulating bath at 25.0 ± 0.1°C. The change of absorbance with time was analyzed with SigmaPlot v. 4.0. The results from separate fittings of the traces obtained by successive flashes were averaged by the least-squares method.

The concentration of Zncyt was always 10 \(\mu\)M. The concentration of \(^3\)Zncyt depended on the excitation power and was ca. 1.0 \(\mu\)M. The mole ratio of quencher to \(^3\)Zncyt was greater than 10:1, so that the conditions for pseudo-first-order reaction always prevailed. Second-order rate constants were obtained from the corresponding pseudo-first-order rate constants by least-squares fittings.

**Calculations.** The rate constant \(k_{en}\) for energy transfer by the Förster (dipole-dipole) mechanism was calculated \([43]\) according to Eq. (1), where \(\kappa\) is dipole-dipole orientation factor, \(n\) is refractive index of the medium, \(\tau\) is natural lifetime of the donor, and \(R\) is center-to-center distance between donor and acceptor (in cm). The overlap integral (in cm\(^6\) mol\(^{-1}\))

\[
k_{en} = \frac{(8.8 \times 10^{-38})\kappa^2}{n^2\tau R^6} \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda
\]  

(1)

describes the interaction between donor emission and acceptor absorption bands; \(F_D(\lambda)\) is the normalized emission probability of the donor; and \(\varepsilon_A(\lambda)\) is the molar decadic extinction spectrum of the acceptor (cm\(^2\) mol\(^{-1}\)). Numerical data for the donor (\(^3\)Zncyt) emission were obtained by scanning the published spectrum \([17]\) into a computer, manually obtaining the xy coordinates for 90 points (ca. one point per nm) with Adobe PhotoShop 4.0, and converting those xy coordinates into wavelengths and emission intensity. See Fig. S1 in the supplementary material. Numerical data for the acceptor (Fe\(^{III}\)cyt, Fe\(^{II}\)cyt, and H\(_2\)cyt)
absorption were obtained by converting their measured spectra into ASCII files and importing the data into SigmaPlot v. 4.0. The numerical value of the overlap integral, shown in Fig. S2 in the supplementary material, is calculated for H$_2$cyt, Fe$^{II}$cyt, and Fe$^{III}$cyt [44].

The effect of ionic strength is analyzed by Brønsted-Debye-Hückel theory, often used to estimate charges of small, globular proteins, as in Eq. (2), where symbols $k$ and $k_0$ are

$$\ln k = \ln k_0 - \frac{Z_1^2 \alpha \mu^{1/2}}{1 + \kappa R_1} - \frac{Z_2^2 \alpha \mu^{1/2}}{1 + \kappa R_2} + \frac{(Z_1 + Z_2)^2 \alpha \mu^{1/2}}{1 + \kappa R_i}$$

bimolecular rate constants at ionic strengths $\mu$ and zero; $Z_1$ and $Z_2$ are net charges of the reactants, and $R_1$ and $R_2$ are their radii; $R_i$ is the radius of the transition state for the bimolecular reaction; $\alpha = 1.17$ in water at 25°C; and $\kappa = 0.329$ $\mu$ $^{-1}$. Under assumption $R_1 = R_2 = R_v$, Eq. (2) reduces to the widely used Eq. (3), which is used also in this study. The radius of cytochrome c is 18.5 Å [45], so $R_v = 18.5$ Å.

Results and Discussion

Natural Decay of $^3$Zncyt. The excitation by laser of the porphyrin π-system can be considered a promotion of an electron with inversion of its spin. Because the excited (triplet) and ground (singlet) states differ in spin multiplicity, the lifetime of $^3$Zncyt is long. The rate constant $k_d$ for this simple decay in Eq. (4) was obtained from fittings of the traces to the

$$^3\text{Zncyt} \rightarrow k_d \rightarrow \text{Zncyt}$$

monoexponential function in Eq. (5). In the absence of added quencher, $k_d = 80 \pm 5$ s$^{-1}$.
\[ \Delta A = \alpha e^{-kt} \]  

(5)

independent of Zncyt concentration, ionic strength in the interval \(2.5 \text{ mM} \leq \mu \leq 1500 \text{ mM}\), and wavelength. This rate constant remained unchanged upon separate additions of 1.0 mM ascorbic acid and 1.2 mM sodium dithionite, as reductants.

**Effects of Possible Quenchers on Decay of \( ^3 \text{Zncyt}\).** To ensure complete removal of \([\text{Fe(CN)}_6]^{3-}\) ions, the unwanted quencher of \(^3\text{Zncyt}\), a control experiment was carried out with horse-heart cytochrome c containing 90\% Fe\(^{III}\)cyt and 10\% Fe\(^{II}\)cyt, as determined spectrophotometrically. Identical bimolecular rate constants for quenching of \(^3\text{Zncyt}\) were obtained in experiments with the commercial and desalted preparations of Fe\(^{III}\)cyt. Although the \([\text{Fe(CN)}_6]^{3-}\) ion is used as an oxidant for cytochrome c, it is completely removed by purification or desalting and will not interfere with the quenching of \(^3\text{Zncyt}\) in subsequent experiments.

At low ionic strength, \(\mu = 10 \text{ mM}\), in the presence of concentrations as high as 140 \(\mu\text{M Fe}^{III}\)cyt, 120 \(\mu\text{M Fe}^{II}\)cyt, 200 \(\mu\text{M H}_2\)cyt, and 200 \(\mu\text{M apocyt}\), the rate of \(^3\text{Zncyt}\) decay remained unchanged. At high ionic strength, \(\mu = 1500 \text{ mM}\), the \(^3\text{Zncyt}\) decay became faster and remained exponential upon incremental addition of Fe\(^{III}\)cyt up to 140 \(\mu\text{M}\), Fe\(^{II}\)cyt up to 150 \(\mu\text{M}\), and H\(_2\)cyt up to 240 \(\mu\text{M}\). However, upon addition of apocyt even up to 350 \(\mu\text{M}\), the rate of \(^3\text{Zncyt}\) decay remained unchanged. Typical traces are shown in Figs. S3 and S4 in the supplementary material. The pseudo-first-order rate constant was directly proportional to the quencher concentration and depended on ionic strength. In no case did we detect saturation behavior (leveling off), even at the highest concentrations of quencher mentioned above. The bimolecular rate constants \(k_{\text{bim}}\), given in Table 1, are obtained from the slopes of the linear
plots in Fig. 1.

**Possible Mechanisms of Quenching.** There are three possible mechanisms for quenching of $^3$Zncyt: enhancement of radiationless decay, Förster (dipole-dipole) energy transfer, and electron transfer. Enhanced radiationless decay is the slight increase in rate of $^3$Zncyt decay attributed, without evidence, to a slight conformational change in Zncyt upon association with another protein [30,46]. Energy transfer can occur if the emission bands of $^3$Zncyt (donor) at 590, 640, and 736 nm overlap with the absorption bands of the potential acceptor. Electron transfer can result in oxidative (Eq. (6)) or reductive (Eq. (7)) quenching

$$^3\text{Zncyt} + Q \rightarrow ^3\text{Zncyt}^* + Q^- \quad (6)$$

$$^3\text{Zncyt} + Q \rightarrow ^3\text{Zncyt}^- + Q^+ \quad (7)$$

by various quenchers, designated Q. The products of these reactions are the ion radicals $\text{Zncyt}^*$ and $\text{Zncyt}^-$. To our knowledge, the reaction in Eq. (7) has been studied only in this laboratory [20].

**Absence of Enhanced Radiationless Decay.** In order to determine if enhanced radiationless decay plays a role in the quenching of $^3$Zncyt, apocyt was used as a possible quencher. Lacking heme, this protein does not absorb wavelengths greater than 280 nm and is incapable of electron transfer and energy transfer. At low ionic strength, $\mu = 10$ mM, the rate of $^3$Zncyt decay does not increase as the concentration of apocyt is raised to 200 $\mu$M. The bimolecular rate constant remains $\leq 1 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, below the threshold of our kinetic method. At high ionic strength, $\mu = 1500$ mM, the rate of $^3$Zncyt decay still does not increase even upon addition of 350 $\mu$M apocyt (see Fig. 1), and the bimolecular rate constant remains
$1 \times 10^4 \text{M}^{-1} \text{s}^{-1}$. These control experiments with apocyt show that enhanced radiationless decay is not an efficient mechanism of quenching $^{3}\text{Zncyt}$. Even at high ionic strength, when electrostatic repulsions between positively charged Zncyt and apocyt are minimized, these proteins do not associate to an observable extent. Enhanced radiationless decay will not be an efficient mechanism of quenching in the cases of $\text{H}_2\text{cyt}$, $\text{Fe}^{II}\text{cyt}$, and $\text{Fe}^{III}\text{cyt}$, either. In the cases where enhanced radiationless decay of $^{3}\text{Zncyt}$ was observed, in our and other laboratories, this mechanism was proven by the same criteria applied in this study [26,29,46].

**Presence of Energy Transfer.** For energy transfer to be an efficient mechanism of quenching $^{3}\text{Zncyt}$, the emission spectrum of the $^{3}\text{Zncyt}$ (donor) and the absorbance spectrum of the acceptor must overlap. Both $\text{Fe}^{II}\text{cyt}$ and $\text{H}_2\text{cyt}$ have absorption bands that may overlap the emission bands of $^{3}\text{Zncyt}$ at 590, 640, and 736 nm. The sharp absorption band of $\text{Fe}^{II}\text{cyt}$ at 550 nm overlaps slightly with the emission band of $^{3}\text{Zncyt}$ at 590 nm. Broad absorption bands of $\text{H}_2\text{cyt}$ at 568 and 620 nm overlap well with the emission bands of $^{3}\text{Zncyt}$ at 590 and 640 nm. Therefore the rate of energy transfer is expected to be larger for $\text{H}_2\text{cyt}$ than for $\text{Fe}^{II}\text{cyt}$. At low ionic strength, $\mu = 20 \text{mM}$ for $\text{Fe}^{II}\text{cyt}$ and $\mu = 10 \text{mM}$ for $\text{H}_2\text{cyt}$, the rate of $^{3}\text{Zncyt}$ decay is not affected by the increasing concentration of $\text{Fe}^{II}\text{cyt}$ up to 120 $\mu\text{M}$ and of $\text{H}_2\text{cyt}$ up to 200 $\mu\text{M}$. However, at high ionic strength, $\mu = 1500 \text{mM}$, the rate of $^{3}\text{Zncyt}$ decay increases as the concentration of $\text{Fe}^{II}\text{cyt}$ or $\text{H}_2\text{cyt}$ is raised to 150 $\mu\text{M}$ and 240 $\mu\text{M}$, respectively; see Fig. 1. The bimolecular rate constant for $\text{H}_2\text{cyt}$ is twice as large as that for $\text{Fe}^{II}\text{cyt}$; although small, this difference is fully reproducible. At high ionic strength, electrostatic repulsions between these proteins are minimized, allowing the observed quenching of $^{3}\text{Zncyt}$ to occur. On the basis of these results and the control experiments with
apocyt, we conclude that energy transfer, not enhanced radiationless decay or electron transfer, is the most efficient mechanism of quenching $^{3}\text{Zncyt}$ by Fe$^{III}_\text{cyt}$ and H$_2\text{cyt}$.

To further verify energy transfer as the mechanism of quenching $^{3}\text{Zncyt}$, we calculated, using Eq. (1), the rate constant $k_\text{en}$ for Fe$^{III}_\text{cyt}$, Fe$^{II}_\text{cyt}$, and H$_2\text{cyt}$. We set $R = 25$ Å, $n = 1.4$, $\tau = 75$ ms [47], and $\kappa = 1$, and assumed 100% overlap between donor emission, which occurs from 570 to 670 nm [17] and acceptor absorption at room temperature (r.t.). The results ($k_\text{en}$ values) were 205, 38, and 385 s$^{-1}$ for Fe$^{III}_\text{cyt}$, Fe$^{II}_\text{cyt}$, and H$_2\text{cyt}$, respectively. The ability of Fe$^{II}_\text{cyt}$ and H$_2\text{cyt}$ to quench $^{3}\text{Zncyt}$ by energy transfer is confirmed. Moreover, energy transfer also contributes slightly to the quenching of $^{3}\text{Zncyt}$ by Fe$^{III}_\text{cyt}$.

A general mechanism for the reaction between electron donor D and the electron acceptor A is shown in Eq. (8). The quotient $k_\text{on}/k_\text{off}$ is the association constant $K_A$. Under

$$D + A \overset{k_\text{on}}{\underset{k_\text{off}}{\longleftrightarrow}} DA \overset{k_f}{\rightarrow} D^+A^- \quad (8)$$

the so-called improved steady-state approximation and the condition $[A] >> [D]$, Eq. (9) is obtained [48]. In the limiting case when $k_\text{off} > k_\text{on}[A] + k_f$, Eq. (9) yields Eq. (10). In this case, common in studies of protein reactions, the observed rate constant linearly depends on the concentration of the reactant present in excess. The symbol $k_\text{bim}$ represents the bimolecular rate constant for the reactions of $^{3}\text{Zncyt}$ with various quenchers.
Calculated values for $k_{on}$ in the reactions of $^{3}\text{Zncyt}$ with $\text{H}_2\text{cyt}$ and with Fe$^{II}$cyt are $5.1 \times 10^7$ s$^{-1}$ at $\mu = 2.5$ mM and $8.2 \times 10^9$ s$^{-1}$ at $\mu = 1500$ mM. We fitted to Eq. (9) experimental values of $k_{obs}$ for quenching of $^{3}\text{Zncyt}$ by $\text{H}_2\text{cyt}$ and Fe$^{II}$cyt. Justifiably, $k_{on}$ and $k_{off}$ were fixed. For these reactants, which bear large positive charge, the parameters $k_{off} > 2 \times 10^6$ s$^{-1}$ and $K_A < 4 \times 10^3$ M$^{-1}$ are quite reasonable. The $k_{on}$ values obtained by two methods, embodied in Eqs. (1) and (9), agreed well.

**The Question of Reductive Quenching of $^{3}\text{Zncyt}$.** The bimolecular rate constant for the reductive quenching reaction in Eq. (11) at pH 6.5 and ionic strength of 5.0 mM is

$$^{3}\text{Zncyt} + \text{EDTA} \rightarrow \text{Zncyt}^+ + \text{EDTA}^-$$

(11)

$1 \times 10^4$ M$^{-1}$ s$^{-1}$. Rapid decomposition of the cation radical EDTA$^+$ suppresses the reaction in Eq. (12), and the transient anion radical Zncyt$^-$ was detected at 690 nm [20]. It was previously shown [20] that Fe$^{II}$cyt does not quench $^{3}\text{Zncyt}$ at pH 7.0 and ionic strength of 40 mM, probably because both reactants bear the net charge of +6 under these conditions.

Furthermore, the electron self-exchange reaction of the native (iron) protein is relatively slow [7]. These studies were the background to this one.

We re-examined the reaction in Eq. (11) and found that the transient Zncyt$^-$ is detectable between 650 and 700 nm and best evident at 680 nm. Knowing this, we checked if $^{3}\text{Zncyt}$ is affected by the possible reductive quencher Fe$^{II}$cyt present in large excess. At low ionic strength, $\mu = 20$ mM, the rate of $^{3}\text{Zncyt}$ decay remains unchanged, and the transient Zncyt$^-$ is not observed. At high ionic strength, $\mu = 1500$ mM, the rate of $^{3}\text{Zncyt}$ decay
increases upon raising the concentration of Fe\textsuperscript{II} cyt to 150 μM. There is, however, no
evidence of the transient Zn cyt\textsuperscript− in the range 650–710 nm. These results argue against, but
do not conclusively refute, reductive quenching of \(3^2\)Zn cyt by Fe\textsuperscript{II} cyt. The more likely
mechanism of quenching \(3^2\)Zn cyt remains energy transfer.

**Oxidative Quenching of \(3^2\)Zn cyt.** We studied the reaction in Eq. (13) at pH 7.0 and

\[
3^2\text{Zn cyt} + \text{Fe}^{III} cyt \rightarrow \text{Zn cyt}^+ + \text{Fe}^{II} cyt
\]

at increasing ionic strengths. In the presence of Fe\textsuperscript{III} cyt the decay of \(3^2\)Zn cyt became faster as
ionic strength was raised from 2.5 to 1500 mM but remained monoexponential. The rate
constants for disappearance of \(3^2\)Zn cyt and for appearance of Zn cyt\textsuperscript+ were equal, within the
error margins. The formation of Zn cyt\textsuperscript+ is direct evidence for oxidative quenching, as in Eq.
(13). The transient Zn cyt\textsuperscript+ signal was evident between 660 and 690 nm, and 670 nm was the
best wavelength to record it. The pseudo-first-order rate constant for the reaction in Eq. (13)
is directly proportional to Fe\textsuperscript{III} cyt concentration and dependent on ionic strength, as shown in
Fig. S5 in the supplementary material. No saturation is observed at high Fe\textsuperscript{III} cyt
concentration, and the plots go through the origin. At low ionic strength, \(\mu = 2.5\) mM, there
is no increase in the rate of \(3^2\)Zn cyt decay as the concentration of Fe\textsuperscript{III} cyt is raised to 150 μM.
Evidently, the bimolecular rate constant is less than \(1 \times 10^4\) M\textsuperscript{−1} s\textsuperscript{−1}. There is no evidence for
the formation of the transient Zn cyt\textsuperscript+ at 670 nm, as shown in Fig. 2(a). These results show
that at low ionic strength, protein-protein repulsion dominates and electron transfer does not
occur. At high ionic strength, \(\mu = 1500\) mM, a large increase is observed in the rate of \(3^2\)Zn cyt
decay as the concentration of Fe\textsuperscript{III} cyt is raised to 140 μM. The bimolecular rate constant is
5.1 x 10^6 M^{-1} s^{-1}. Fig. 2(b) shows formation of the transient Zncyt^+, clear evidence for oxidative quenching. Although energy transfer is a viable mechanism of quenching, calculations show that it contributes only ca. 5% to the quenching of 3Zncyt by Fe^{III}cyt.

**Bimolecular Rate Constants.** Table 1 shows that as ionic strength increases, so does the rate of 3Zncyt decay. This trend is most evident in the reaction with Fe^{III}cyt. Shielding of the charged groups by counter ions allows for protein collision. The mechanism of quenching is purely diffusional, with no preformed protein-protein complexes present even at high concentration of the quencher. The near absence of quenching of 3Zncyt by Fe^{II}cyt, H_2cyt, and apocyt shows that oxidative quenching is more efficient than energy transfer, enhanced radiationless decay, and reductive quenching under these conditions.

We confirm a previous report that the reaction of 3Zncyt with Fe^{II}cyt in the presence of 10 mM phosphate buffer at pH 7, has an apparent bimolecular rate constant of 1 x 10^4 M^{-1} s^{-1} [49]. This previous study also found that the reaction of 3Zncyt with Fe^{III}cyt in the presence of 10 mM phosphate buffer at pH 7, had an apparent bimolecular rate constant of 2.4 x 10^6 M^{-1} s^{-1}. Our study, however, found no evidence of quenching at this low ionic strength, μ = 20 mM; our results indicate k_{bim} < 1 x 10^4 M^{-1} s^{-1}. The ionic strength must be close to 500 mM for the charges to be sufficiently shielded and the bimolecular rate constant to reach 2.0 x 10^6 M^{-1} s^{-1}. One possible explanation of the discrepancy is that [Fe(CN)_6]^{3-} ion, used as an oxidant in preparing Fe^{III}cyt, was incompletely removed before the kinetic experiments in the previous study. In our control experiments, [Fe(CN)_6]^{3-} concentration as low as 5 x 10^{-8} M could account for the large apparent bimolecular rate constant reported
before. This concentration is approximately 1% of that used for oxidizing cytochrome c prior to the kinetic experiments. In future studies of the interaction of $^3\text{Zncyt}$ with various electron acceptors, one should be aware of contaminants such as Fe$^{ll}\text{cyt}$ and $\text{H}_2\text{cyt}$, two redox-inactive proteins that could be present in preparations of Zncyt, since these proteins quench $^3\text{Zncyt}$ by energy transfer.

**Kinetic Effects of Ionic Strength.** The effect of ionic strength on the reaction of $^3\text{Zncyt}$ and Fe$^{lll}\text{cyt}$ (Table 1 and Fig. S5) is analyzed by Brønsted-Debye-Hückel theory, as in Eqs. (2) and (3). The plot in Fig. 3 has a slope of $60 \pm 2$. The fitted overall charges, $Z_1 = 5.6 \pm 0.2$ for Fe$^{lll}\text{cyt}$ and $Z_2 = 4.6 \pm 0.2$ for Zncyt, deviate from the known charges of +7 and +6, respectively. These results are typical for reaction of two positively charged molecules in which monopole-monopole interactions dominate.

**Conclusions**

Substitution of iron ion by the redox-inactive zinc(II) ion allows study of reactions between heme proteins, which have overlapping absorption spectra. Experiments with Fe$^{lll}\text{cyt}$, Fe$^{ll}\text{cyt}$, $\text{H}_2\text{cyt}$, and apocyt revealed the mechanism of quenching $^3\text{Zncyt}$. High ionic strength is necessary for avoidance of protein-protein repulsion. Electron transfer from $^3\text{Zncyt}$ to Fe$^{lll}\text{cyt}$ is a more efficient quenching mechanism than energy transfer and enhanced radiationless decay at high ionic strength. Iron-free $\text{H}_2\text{cyt}$ and also Fe$^{ll}\text{cyt}$ quenches $^3\text{Zncyt}$ by energy transfer; $\text{H}_2\text{cyt}$ is more efficient owing to a better overlap of its absorption bands with the emission bands of $^3\text{Zncyt}$. This work will be relevant to the future study of reactions between heme proteins.
Supplementary Material. Five Figures, showing kinetic traces of $^3$Zncyt in the absence and presence of Fe$^{III}cyt$, Fe$^{II}cyt$, H$_2$cyt, and apocyt; emission spectrum of $^3$Zncyt at r.t.; plots illustrating the determination of the overlap integral necessary for calculation of energy-transfer rate constant $k_{en}$ by Eq. (1); and the dependence of $k_{obs}$ on ionic strength.

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**Tables and Figures**

**Table 1.** Bimolecular Rate Constants \( (k_{\text{bim}} \times 10^{-4}, \text{M}^{-1} \text{ s}^{-1} ) \) for the Reaction \(^3\text{Zncyt}\) with Possible Quenchers at pH 7.0 and 25°C\(^a\)

<table>
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<tr>
<th>Quencher</th>
<th>2.5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>475</th>
<th>500</th>
<th>1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Fe}^{III}\text{cyt c} )</td>
<td>0.7±0.8</td>
<td>1±3</td>
<td>1±1</td>
<td>23±6</td>
<td>205±9</td>
<td>286±14</td>
<td>513±30</td>
</tr>
<tr>
<td>( \text{Fe}^{II}\text{cyt c} )</td>
<td></td>
<td>0.7±0.7</td>
<td></td>
<td></td>
<td>19±3</td>
<td></td>
<td>15±2</td>
</tr>
<tr>
<td>( \text{H}_{2}\text{cytc} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33±3</td>
</tr>
<tr>
<td>Apocyt c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.15±0.08</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[-1.0±0.1]</td>
</tr>
<tr>
<td>Sodium dithionite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[-0.2±0.7]</td>
</tr>
</tbody>
</table>

\(^a\) Small negative values arise when slopes of pseudo-first-order plots deviate slightly from the horizontal
Fig. 1. Dependence on protein concentration of the rate constant $k_{obs}$ for the reaction of $^3$Zncyt with Fe$^{III}$cyt, H$_2$cyt, Fe$^{II}$cyt, and apocyt at an ionic strength of 1.500 M, pH 7.0, and 25°C. Rate of natural decay of $^3$Zncyt $k_d$ is subtracted from $k_{obs}$. The solid lines are fittings to Eq. (10); their slopes yield $k_{bim}$. 
Fig. 2. Decay of $^3\text{Zncyt}$ in the presence of 80 μM Fe$^{III}$cyt at ionic strengths of (a) 2.5 mM and (b) 1.500 M. The solid lines are fittings to (a) monoexponential and (b) biexponential equations. In (a), $k_1 = 80$ s$^{-1}$, showing that $^3\text{Zncyt}$ is not quenched by Fe$^{III}$cyt at low ionic strength. In (b), $k_1 = 380$ s$^{-1}$ and $k_2 = 50$ s$^{-1}$, corresponding to fast growth and slow decay of transient Zncyt$^+$ formed upon reaction of $^3\text{Zncyt}$ with Fe$^{III}$cyt at high ionic strength.
Fig. 3. Dependence on ionic strength of the bimolecular rate constants $k_{\text{bim}}$ for the reaction of $^{3}\text{Zncyt}$ with $\text{Fe}^{\text{III}}\text{cyt}$. The solid line is the fitting to Eq. (3).
Fig. S1. Emission spectrum of $^3$Zncyt obtained manually by plotting light intensity versus wavelength (ca. 1 point per nm) using scanned picture of a published room temperature emission spectrum [17].
Figure S2. Plots of $F_D$ versus wavelength for determination of overlap integral, which is necessary for calculation, using Eq. (1), of long-range energy-transfer rate constant $k_{en}$. Plots show overlap of $^3\text{Zn} \text{cyt}$ emission spectrum with (a) $\text{H}_2\text{cyt}$, (b) $\text{Fe}^{II}\text{cy}$, and (c) $\text{Fe}^{III}\text{cyt}$ absorption spectra. Area under the curves, determined by integration, provides the overlap integral.
Fig. S3. Kinetic traces showing decay of $^{3}$Zncyt monitored at 460 nm in the (a) absence and (b) presence of 80 μM Fe$^{III}$ cyt. Experimental conditions are $\mu = 1500$ mM, pH 7, and 25°C.
Fig. S4. Kinetic traces showing decay of $^3$Zncyt monitored at 460 nm in the absence and presence of various potential quenchers: (a) apocyt, (b) absence of quencher, (c) Fe$^{II}$cyt, and (d) H$_2$cyt. Experimental conditions are protein concentration = 150 μM, μ = 1500 mM, pH 7.0, and 25°C. Smooth, solid line is monoexponential fitting of the decay of $^3$Zncyt in presence of 150 μM Fe$^{III}$cyt to illustrate the most efficient mechanism of quenching, oxidative electron transfer.
**Fig. S5.** Dependence on Fe$^{III}$ cyt concentration of $k_{obs}$ for the reaction of $^3$Zncyt with Fe$^{III}$ cyt at increasing ionic strength. Rate of natural decay of $^3$Zncyt $k_d$ is subtracted from $k_{obs}$. The solid lines are fittings to Eq. (10), whose slopes yield $k_{bim}$. 
CHAPTER 3. MOLTEN-GLOBULE AND OTHER CONFORMATIONAL FORMS OF ZINC CYTOCHROME C. EFFECT OF PARTIAL AND COMPLETE UNFOLDING OF THE PROTEIN ON ITS ELECTRON-TRANSFER REACTIVITY

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Abstract

To test the effect of protein conformation on reactivity, we use laser flash photolysis to compare the electron-transfer properties of the triplet state of zinc-substituted cytochrome c, designated $^3$Zncyt, in the folded forms at low ($F_{low}$) and high ($F_{high}$) ionic strength, molten-globule (MG) form, and the forms unfolded by acid ($U_{acid}$) and urea ($U_{urea}$) toward the following four oxidative quenchers: Fe(CN)$_6^{3-}$, Co(acac)$_3$, Co(phen)$_3^{3+}$, and iron(III) cytochrome c. We characterize the conformational forms of Zncyt on the basis of the far-UV circular dichroism, Soret absorption, and rate constant for natural decay of the triplet state. This rate constant in the absence of quencher increases in the order $F_{high} < F_{low} < MG < U_{acid} < U_{urea}$ because the exposure of porphyrin to solvent increases as Zncyt unfolds. Bimolecular
rate constants for the reaction of $^3$Zncyt with the four quenchers show significant effects on reactivity of electrostatic interactions and porphyrin exposure to solvent. This rate constant at the ionic strength of 20 mM increases upon unfolding by urea and acid, respectively, as follows: 1340-fold and 466-fold when the quencher is Co(phen)$_3^{3+}$ and 168-fold and 36-fold when the quencher is cyt(III). To compare reactivity of $^3$Zncyt in the $F_{\text{low}}, F_{\text{high}}, MG, U_{\text{acid}}$, and $U_{\text{urea}}$ forms without complicating effects of electrostatic interactions, we used the electroneutral quencher Co(acac)$_3$. Indeed, reactivity of folded $^3$Zncyt with Co(acac)$_3$ was independent of ionic strength. Reactivity of $^3$Zncyt with Co(acac)$_3$ upon partial and complete unfolding increases 10-fold, 54-fold, and 64-fold in the molten-globule, urea-unfolded, and acid-unfolded forms.

**Introduction**

Because metalloproteins act as electron carriers and redox enzymes in many biological processes, chemical mechanisms of their electron-transfer reactions are being studied vigorously.$^{1-6}$ Various cytochromes $c$ are ubiquitous in biological systems. Because of their biological roles and favorable chemical and spectroscopic properties, these proteins were used in many important biochemical and biophysical studies.$^{7-9}$

Conformational flexibility of proteins is essential for their function.$^{10,11}$ Despite much current research, however, little is known about the thermodynamics and kinetics of protein denaturation and unfolding.$^{12-14}$ Various experimental studies gave evidence for the conformational fluctuations and heterogeneity of proteins.$^{15-20}$ Partially unfolded forms are presumed intermediates in the folding of denatured proteins and seem to be implicated in
diseases caused by protein misfolding.\textsuperscript{21-24} We are interested in the effects of these conformational changes on the chemical reactivity of proteins.

Horse heart cytochrome $c$, whose oxidation states are designated cyt(II) and cyt(III), is often used because of its well-defined structure\textsuperscript{8,25-28} and suitable spectroscopic properties. The heme group, covalently attached to the backbone through cysteine residues 14 and 17, interacts with the surrounding protein. The six-coordinate low-spin iron binds His\textsubscript{18} and Met\textsubscript{80} as axial ligands. Polar interactions between the propionyl substituents in the heme and nearby polar side chains and hydrophobic interactions between the porphyrin ring and buried nonpolar side chains abound in the native structures. Electron transfer with external redox partners occurs via an edge of the heme that is partially exposed at the protein surface and surrounded by positively charged lysine residues.\textsuperscript{8}

At least five distinct forms of iron cyochrome $c$, dependent on pH, have been observed spectroscopically.\textsuperscript{29-31} At neutral pH, the folded low-spin form with Met\textsubscript{80} and His\textsubscript{18} as ligands dominates. At pH above 8–10, the dominant species is the low-spin so-called alkaline conformation, in which Met\textsubscript{80} is displaced from iron by a lysine residue.\textsuperscript{32,33} In the acid-unfolded form at pH 2, a high-spin electron configuration indicates that neither Met\textsubscript{80} nor His\textsubscript{18} remains coordinated to iron.\textsuperscript{34,35} At pH 2 in the presence of stabilizing anions, a partially folded form can be obtained.\textsuperscript{36} In the presence of high concentrations of denaturant (guanidine hydrochloride or urea), the protein conformation approximates a random-coil polypeptide devoid of a circular dichroism (CD) band at 222 nm. A recent NMR spectroscopic study of iron cytochrome $c$ in neutral solution detected His and Lys upon partial and His and His axial ligands upon complete denaturation with urea.\textsuperscript{37}
The molten-globule form, designated MG, is a thermodynamically distinct, compact denatured form with a significant amount of ordered secondary structure, but largely flexible, disordered, and fluctuating tertiary structure. In comparison with the native form, the internal hydrophobic core of the protein is more exposed to solvent, and side chains are more mobile.\(^{38,39}\) The MG form preserves many native internal hydration sites and is hydrated on the surface like the native form.\(^{40}\) Several structural studies found a major intermediate in protein folding\(^{41-43}\) that is similar in nature to the MG form.\(^{44-48}\) The MG form has been implicated in various biological processes,\(^ {49}\) such as interaction of nascent proteins with molecular chaperones\(^ {50,51}\) and interaction of proteins with membranes.\(^ {52}\)

Cytochrome c undergoes a transition from the acid-unfolded (\(U_{\text{acid}}\)) to the molten-globule (MG) form upon addition of anions (as salts).\(^ {33,38,53-55}\) At pH 2.0 in the absence of salt, cyt(III) is maximally unfolded because of electrostatic repulsions within the protein. Upon addition of salt, that is, at higher ionic strength, the added anions screen the cationic groups, and the protein cooperatively folds into a compact structure. This, the MG form of cyt(III), has been extensively characterized.\(^ {56-58}\) Its \(\alpha\)-helicity is comparable to that of the native form, but it has a fluctuating tertiary structure. The hydrophobic core, containing the N-terminal and C-terminal helicities and the heme group, is preserved in the MG form and still stabilized by nonbonding interactions, while the loop regions are fluctuating and partially disordered.\(^ {59,60}\) Of the two axial ligands, only His18 seems to remain attached to iron in the MG form, while Met80 is detached.\(^ {61}\) The radius of gyration of the native, MG, \(U_{\text{acid}}\), and \(U_{\text{urea}}\) forms of iron cytochrome c increases in the order 14.6 < 17.4 < 30.1 < 32.1 Å, respectively.\(^ {62}\)
The replacement of heme iron by zinc(II) in myoglobin, hemoglobin, cytochrome c peroxidase, and cytochrome c has successfully been used to probe the structure and reactivity of these proteins in the folded state. Spectroscopic studies of folded zinc-substituted cytochrome c, designated Zncyt, showed that metal substitution does not significantly alter the protein structure near the heme pocket. Ours is the first study of unfolded forms of this protein. We characterize them on the basis of far-UV circular dichroism spectra, Soret absorption spectra, their behavior upon unfolding by acid and by urea, and rate constant for natural decay of the triplet state.

Zinc cytochrome c offers many advantages over the native species. The long-lived triplet state of the zinc porphyrin, designated $^3Zncyt$, is easily produced by laser flash and is a strong reductant, suitable for exact kinetic studies. The triplet state is oxidatively quenched by electron acceptors Q according to eq 1; four such reactions are the subject of this study.

$$^3Zncyt + Q \rightarrow Zncyt^+ + Q^-$$ (1)

The resulting cation radical, designated Zncyt+, returns to the ground state, Zncyt, in the thermal (so-called back) electron-transfer reaction shown in eq 2. This reaction was the subject of a recent study in our laboratory.

$$Zncyt^+ + Q^- \rightarrow Zncyt + Q$$ (2)

Although overall structure and stability of cytochrome c in unfolded forms has recently been studied, reactivity of these forms remains almost unknown. We explore the effects of conformational change, by comparing electron-transfer kinetics of $^3Zncyt$ in folded and unfolded forms, in neutral and acidic solutions, and in the presence of urea, a denaturant. Because Zncyt and most of the quenchers are charged, we also control the electrostatic
interactions between them by using low and high ionic strength.

**Experimental Section**

**Chemicals.** Distilled water was demineralized to a resistivity greater than 17 MΩ·cm. Chromatography resins and gels were obtained from Sigma, Pharmacia, and Bio-Rad. Hydrogen fluoride, nitrogen, and ultrapure argon were obtained from Air Products. Tris(1,10-phenanthroline)cobalt(III) perchlorate dihydrate, [Co(phen)₃](ClO₄)₂·2H₂O, and the corresponding chloride salt, [Co(phen)₃]Cl₃·7H₂O, were prepared by published procedures.⁷⁵ (Caution, perchlorate salts of metal complexes containing organic ligands are potentially explosive! Only small amounts of them should be prepared and must be handled with great caution.) The Co(phen)₃³⁺ concentration was determined on the basis of the molar absorptivity: \( \varepsilon = 3.60 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) at 350 nm.⁷⁶ Tris(acetylacetonato)cobalt(III), Co(acac)₃, from Aldrich was 99.99% pure. Its concentration was determined on the basis of the molar absorptivity: \( \varepsilon = 133 \text{ M}^{-1} \text{ cm}^{-1} \) at 595 nm.⁷⁷ All other chemicals were of reagent grade, and were used as received from Fisher Scientific.

**Proteins.** Horse heart cytochrome c was obtained from Sigma. The iron(III) form was prepared by incubation with an excess of \( \text{K}_3[\text{Fe(CN)}_6] \), which was then removed with a Bio-Rad Econo-Pac 10 DG desalting column. The protein concentration was determined spectrophotometrically; the difference in molar absorptivity between reduced and oxidized forms at 550 nm is 18.5 mM⁻¹ cm⁻¹.

Zinc cytochrome c was prepared and purified by modification⁷⁰,⁷⁴ of the original procedure.⁷⁸,⁷⁹ All experiments were done in the dark, to prevent photodegradation of Zncyt
in the presence of dioxygen. Two of the criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. Another criterion of purity was that the rate constant $k_d$ for the natural decay of $^3$Zncyt be less than 110 s$^{-1}$. The concentration of Zncyt was determined on the basis of its molar absorptivity: $\varepsilon = 243 \text{ mM}^{-1} \text{ cm}^{-1}$ at 423 nm.\textsuperscript{80} The metal-free form of cytochrome $c$ is porphyrin cytochrome $c$, designated H$_2$cyt. It was prepared by a standard procedure.\textsuperscript{78}

**Preparation of Buffer and Denaturant Solutions.** The phosphate buffer had a concentration of 10 mM and pH value of 7.00 ± 0.05, determined by an Accumet 925 pH meter obtained from Fisher Scientific. Aqueous solutions of HCl were adjusted to pH 2.0. The ionic strength, $\mu$, of the buffer at pH 7.00 or of the HCl solution at pH 2.0 was adjusted to 20 mM and 1.500 M by addition of solid NaCl. Solutions of 8.0 M urea in the 10 mM phosphate buffer at pH 7.00 and $\mu = 20$ mM were prepared fresh by a published procedure\textsuperscript{81} and used within 8 h. Buffers and denaturant solutions were thoroughly degassed before use by bubbling with wet argon.

**Preparation of (Un)folded Forms of Zncyt.** The folded forms at low and high ionic strength are designated $F_{\text{low}}$ and $F_{\text{high}}$. They were obtained by dissolving Zncyt in 10 mM phosphate buffer at pH 7.00 and ionic strength of 20 mM and 1.500 M. The acid-unfolded form, designated $U_{\text{acid}}$, was obtained by dissolving Zncyt in the HCl solution at pH 2.0 and $\mu = 20$ mM. To obtain the molten-globule form, designated MG, the aforementioned solution was adjusted to 1.500 M by addition of solid NaCl, and pH was readjusted to 2.0 by HCl. Precautions were taken to avoid pH dropping below 2.0 lest zinc(II) ions irreversibly dissociate from porphyrin. This undesirable process can be detected, and thus avoided,
because the absorption spectrum is characteristic of H$_2$cyt. The urea-unfolded form, designated U$_{\text{urea}}$, was obtained by dissolving Zncyt in a solution of 8.0 M urea in 10 mM phosphate buffer at pH 7.00 and $\mu = 20$ mM. Transitions between the conformational forms were induced by acid-base titration, NaCl addition, or solvent exchange with Centricon-3 ultrafiltration units, obtained from Millipore.

**Circular Dichroism and Absorption Spectrophotometry.** Far-UV circular dichroism spectra of 10.0 $\mu$M Zncyt were obtained with a Jasco instrument, Model J-710. A 1-mm quartz cell was used for the $F_{\text{low}}$, $F_{\text{high}}$, MG, and $U_{\text{acid}}$ forms, but owing to the strong absorbance of urea, a 0.1-mm quartz cell was necessary for the $U_{\text{urea}}$ form. Each spectrum was an average of four measurements and was slightly corrected for a baseline contribution. The cell compartment was maintained at 20.0 $\pm$ 0.1 °C using a peltier thermoelectric cell PTC-348 W, equipped with an external sensor. The mean residue ellipticity, in deg cm$^2$/dmol$^{-1}$, is defined as $[\theta] = 100\theta_{\text{obs}}(c/l)^{-1}$, where $\theta_{\text{obs}}$ is observed intensity, $c$ is concentration in residue moles per liter, and $l$ is path length in centimeters. Helical content was calculated by an established method.$^{82}$

Absorption spectra of Zncyt in the $F_{\text{low}}$, $F_{\text{high}}$, MG, $U_{\text{acid}}$, and $U_{\text{urea}}$ forms were recorded with a Perkin-Elmer Lambda 18 spectrophotometer. To determine the stability of protein toward demetalation, 5.0 $\mu$M solutions of Zncyt or cyt(III) in solvents maintaining all five folded and unfolded forms were monitored over time.

**Equilibrium Unfolding of Zncyt.** Controlled denaturation of Zncyt was monitored by Soret absorption at 410 and 423 nm, at various concentrations of HCl (pH 1.1–7.0) and of urea (0.0–12.0 M). The unfolding transition at 20 °C was analyzed in terms of a two-state
model, and the optical data were converted into the free energy of unfolding ($\Delta G_U$) using eq 3, where $y$, $y_F$, and $y_U$ are the observed spectroscopic signal, folded-protein baseline, and unfolded-protein baseline, respectively. The midpoint of the unfolding transition ($C_m$) is obtained by fitting a four-parameter sigmoidal equation (SigmaPlot version 5.0, from Jandel Scientific) to the fraction of unfolded protein ($F_U$) and denaturant concentration. Linear least-squares regression was used to fit the data to eq 4, where $\Delta G_U(H_2O)$ is an estimate of the free energy of unfolding in aqueous solution, and $m$ is a measure of the dependence of $\Delta G_U(H_2O)$ on denaturant.  

$$\Delta G_U = \Delta G_U(H_2O) - m[\text{denaturant}]$$

**Laser Flash Photolysis.** Transient absorption of the triplet state, $^3\text{Zncyt}$, as a function of time was measured with a standard apparatus. The excitation source was a Continuum Minilite II Q-switched frequency-doubled Nd:YAG laser, which delivered 5-ns pulses at 532 nm. Incident energy was calibrated with a power meter (Scientech, model H410D) and kept at 1.0 mJ/pulse, to avoid Zncyt degradation. The probe source, perpendicular to the excitation beam, was a continuous 250 W QTH lamp with Aspherab condenser (Oriel Instruments, model 66198). A secondary lens focused the collimated light on a 1-mm aperture. Interference filters (Optometrics USA, half-bandwidth of ± 5 nm) placed before the sample holder and also before the detector minimized the entry of scattered light into the detector. Decay of the triplet state, $^3\text{Zncyt}$, was monitored at 458 nm, where its (transient) absorbance reaches a maximum. The appearance and disappearance of the
porphyrin cation radical, Zncyt⁺, was monitored at 676 nm, where the difference in absorbance between Zncyt⁺ and 3Zncyt is the greatest.80,84,85 Transient signals shorter than 1 μs were measured with a Hamamatsu R2949 photomultiplier tube equipped with a housing and voltage divider (Oriel Instruments, model 70680). The current was amplified (Oriel Instruments, model 70223) and digitized using a Lecroy Waverunner LT322 oscilloscope interfaced to a computer. Between 50 and 300 laser shots were accumulated, to enhance the signal. Transient signals longer than 1 μs were measured with a custom-built photodiode detector containing a Hamamatsu silicon PIN photodiode model S3071. The photodiode detector provided greater sensitivity and better signal-to-noise ratio than the photomultiplier tube did, for observing the small changes in absorbance of the transient species 3Zncyt and Zncyt⁺.

A 2.000-mL sample solution in a 10-mm cuvette was thoroughly deaerated by gentle flushing with wet argon for at least 15 minutes after each addition of quencher or until the rate constant for natural decay of 3Zncyt became constant. For experiments at pH 7.00, a deoxygenating solution containing glucose, glucose oxidase, and catalase was used, to ensure complete removal of dioxygen.80 Temperature was held at 25.0 ± 0.1 °C by a thermostated bath CH/P 2067, obtained from Forma Scientific.

The concentration of Zncyt was 3.0 μM in experiments done at pH 7.00 and 5.0 μM in those done at pH 2.0 or in the presence of urea. The concentration of the triplet state, 3Zncyt, depended on the excitation power and was ca. 0.3 to 0.5 μM. The mole ratio of quencher to 3Zncyt was greater than 10:1, so that the conditions for pseudo-first-order reaction always prevailed.
**Reaction Mechanism.** A general mechanism for the reaction between electron donor D and the electron acceptor A (the same as Q in eq 1) is shown in eq 5. The quotient \( \frac{k_{on}}{k_{off}} \) is the association constant \( K_A \) and \( k_f \) is the rate constant (for the reaction in the "forward" direction). Under the so-called improved steady-state approximation and the condition \([A] \gg [D]\), eq 6 is obtained. In the limiting case when \( k_{off} > k_{on}[A] + k_f \), eq 6 yields eq 7. In this case, common in studies of protein reactions, the observed rate constant \( k_{obs} \) linearly depends on the concentration of the reactant present in excess. The bimolecular rate constant \( k_{bim} \) corresponds to the reactions of \( ^3\text{Zncyt} \) with various quenchers A.

**Fittings of Kinetic Data.** The rate constants were obtained from changes in the absorbance at 458 and 676 nm with time. Exponential decay of \( ^3\text{Zncyt} \) in the absence and presence of quencher was fitted using nonlinear least-squares regression (SigmaPlot, version 5.0, from Jandel Scientific). In the absence of quencher, the rate constant for natural decay is designated \( k_d \). In the presence of quencher, the quenching rate constant \( k_q \) is obtained using eq 8. Plots of \( k_q \) against quencher concentration are linear and go through the origin. The bimolecular rate constant \( k_{bim} \) is determined from the slope by linear least-squares regression.
Results

Characterization of (Un)folded Forms of Zncyt by Absorption Spectroscopy.

Figure 1 and Table 1 show the absorption spectra of Zncyt in the F_low, F_high, MG, U_acid, and U_urea forms. Variation in the absorption is diagnostic of changes in conformation. Upon conversion of Zncyt from the F_low and F_high forms to the MG form, the Soret and Q bands move by only 1–3 nm. Upon unfolding of Zncyt to the U_acid form, the Soret band moves greatly, by 14 nm. The blue shift of the Q band is consistent with a change in zinc(II) ligation, an increase in the volume of the hydrophobic core, and an increase in porphyrin exposure to solvent. Upon unfolding of Zncyt to the U_urea form, the blue shift of the Soret maximum is less than that in the U_acid form. Soret absorption is the only optical property which can clearly distinguish the folded and unfolded forms of Zncyt.

Equilibrium Unfolding of Zncyt. Figure 2 and Table 2 describe unfolding of Zncyt. The protein is rather stable, with the midpoint of the unfolding transition (C_m) at pH 2.9 ± 0.1 with acid and at 7.1 ± 0.1 M with urea as a denaturant. As shown in Figure 2a, unfolded Zncyt refolds upon lowering pH below 2.0 with HCl. This refolding, having C_m at pH 1.5 ± 0.2, arises from the increase in the concentration of Cl^- ions, which induce conversion of Zncyt from the U_acid form to the MG form. The unfolding curves in this study of Zncyt, and those in previous studies of iron cytochrome c,38.54.57.83 confirm that the U_acid and U_urea forms of both proteins are completely unfolded at the conditions used in our kinetic studies, namely pH 2.0 and 8.0 M urea.

Characterization of (Un)folded Forms of Zncyt by Circular Dichroism Spectroscopy. Circular dichroism in the far-UV range is a property of the polypeptide
backbone and is a sensitive indicator of protein secondary structure. Circular dichroism spectra (Figure 3 and Table 1) of Zncyt in the F_{low}, F_{high}, and MG forms show minima at 222 and 210 nm and have similar \( \alpha \)-helical content, indicating similar backbone conformations. These are the same as those recently reported\(^7\)\(^4\),\(^8\)\(^6\) and also very similar to those reported for native (iron-containing) cyt(III).\(^5\)\(^4\) By contrast, the spectra of Zncyt in the U_{acid} and U_{urea} forms show minima below 210 nm and a great decrease in ellipticity at 222 nm. In lacking secondary structure, these forms resemble the corresponding unfolded forms of cyt(III)\(^3\)\(^8\)\(^5\)\(^4\) and Zncyt\(^8\)\(^6\) previously reported.

**Characterization of (Un)folded Forms of Zncyt by Natural Decay of the Triplet State.** Natural decay in the absence of quenchers, shown in Figure 4, usually is the simple process shown in eq 9 that obeys the monoexponential eq 10. When the MG and U_{acid} forms

\[ ^3 \text{Zncyt} \rightarrow \text{Zncyt} \]  
\[ \Delta A_{458\text{nm}} = a_1 \exp(-k_1t) + b \]

of Zncyt are left to age for several hours, a second phase in the natural decay arises gradually, so that the biexponential eq 11 is needed for the fitting. The new phase is faster but always

\[ \Delta A_{458\text{nm}} = a_1 \exp(-k_1t) + a_2 \exp(-k_2t) + b \]

remains a minor one.

Its rate constants \( (k_2) \) and amplitudes \( (a_2) \) are 710 ± 90 s\(^{-1}\) and 10 to 20\% for the MG form and 1230 ± 250 s\(^{-1}\) and 20 to 30\% for the U_{acid} form. An extreme example of this biphasic natural decay is shown in Figure 5. Natural decay of the U_{urea} form of \(^3\)Zncyt is always biexponential. For the major phase, \( k_1 = 1200 ± 220 \text{ s}^{-1} \) and \( a_1 = 82 ± 6\% \); for the minor phase, \( k_2 = 280 ± 80 \text{ s}^{-1} \) and \( a_2 = 18 ± 6\% \).
The $k_d$ values in Table 1 are those for the major phase. Our kinetic studies of
electron-transfer reactions were done with fresh samples, for which (except for the $U_{\text{urea}}$
form) the second phase is a very minor one. Moreover, the quenching reactions are much
faster than the natural decay. For these two reasons, biexponentiality of the natural decay is a
negligible factor in the kinetic experiments.

**Conformational Transitions Between (Un)folded Forms of Zncyt.** Various forms
of Zncyt were converted one into another as the solvents (specified previously for each
conformational form) were exchanged by ultrafiltration. The interconversions were followed
on the basis of the absorption and CD spectra and the natural decay of the triplet state.

Gradual conversion of the $F_{\text{high}}$ form into the MG form and the $F_{\text{low}}$ form into the $U_{\text{acid}}$
form was followed over 36 h. Attempts to reverse these processes and gradually, again over
36 h, reform the $F_{\text{high}}$ form from the MG form and the $F_{\text{low}}$ form from the $U_{\text{acid}}$ form failed.
The Soret bands of these folded forms and emission of the triplet state did not reappear.
Instead, absorption spectra with bands at 404, 506, 540, 568, and 620 nm were obtained (see
Figure S1 in the Supporting Information). These bands are characteristic of the metal-free
porphyrin cytochrome c, designated $H_{\text{2cyt}}$. Circular dichroism spectra shown in Figure S2
in the Supporting Information showed recovery of nativelike secondary structure in the $F_{\text{high}}$
and $F_{\text{low}}$ forms of $H_{\text{2cyt}}$. When, however, ultrafiltration was done rapidly, the MG form did
return to the $F_{\text{high}}$ form and the $U_{\text{acid}}$ form to the $F_{\text{low}}$ form, as the position of the Soret bands
(at 423 nm) and the rate constants $k_d$ for natural decay (ca. 75 s$^{-1}$) showed.

Conversion of the $U_{\text{acid}}$ form into the MG form was effected by adding NaCl to the
solution and monitored by measuring ellipticity $[\theta]$ at 222 nm and absorbance changes in the
Soret region. As the ionic strength is raised from 20 to 1000 mM, Zncyt recovers nativelike secondary structure (data not shown). As Figure S3 in the Supporting Information shows, isosbestic points at 398 and 417 nm persist as the ionic strength is raised from 20 to 110 mM, evidence for a single process. These isosbestic points become somewhat “smeared” upon going to 1000 mM, presumably because of processes occurring simultaneously.

Demetalation of Zncyt and Remetalation of H₂c cyt by Zinc(II) Ions. When the MG and Uₐcid forms of Zncyt are left to age for several hours, a second, faster phase in the natural decay arises gradually. To attribute this faster phase, the natural decay of the triplet state of the metal-free porphyrin cytochrome c, designated ³H₂c cyt, in the absence of quenchers was measured (see Table 1). The value of k₃ (1350 ± 70 s⁻¹) determined for this H₂c cyt sample is identical to the value of k₂ (1353 ± 30 s⁻¹) determined for the aged Zncyt sample in Figure 5.

To check whether the observed biphasic kinetics at pH 2.0 is indeed due to an increase in H₂c cyt concentration as the zinc(II) porphyrin is demetalated and to ensure that the appearance of H₂c cyt will have no effect on the rate constant for natural decay of ³Zncyt, increasing concentrations of H₂c cyt were added to a 10.0 µM solution of Zncyt for determination of k₄. Fitting of the kinetic traces to eq 11 yielded the rate constants k₁ and k₂ and the amplitudes a₁ and a₂ for the two phases. A plot of k₉ versus increasing concentration of H₂c cyt (data not shown) shows a very slight dependence of k₁ (slope = 0.23, 79 s⁻¹ ≤ k₁ ≤ 105 s⁻¹) and no dependence of k₂ (slope = 0.002, k₂ = 1440 ± 50 s⁻¹) on H₂c cyt concentration. Figure 6 shows that the faster phase, which arises over several hours, is indeed due to formation of H₂c cyt (see Scheme 1). The molecules Zncyt and H₂c cyt quench each other’s
triplet states slightly, if at all. Indeed, lack of a transient signal at 676 nm rules out the presence of Zncyt+, the intermediate that would have arisen in redox quenching of 3Zncyt by H2cyt. The slight quenching observed can be attributed to slight energy transfer.70

Our attempts to suppress loss of zinc(II) ions from Zncyt, that is, to remetalate H2cyt in situ by adding 1.0 mM zinc(II) acetate to the reaction mixture at pH 2.0 failed. See, however, Figure S4 in the Supporting Information for successful remetalation of H2cyt using standard procedures.74,78 Restoration of Zncyt was confirmed by the position of the Soret band (at 423 nm) and the rate constant k_d for natural decay (72 s⁻¹).

Effect of Zncyt Conformation on the Porphyrin Affinity for Zinc(II) Ions. To understand the stability toward demetalation of Zncyt and cyt(III) in the F_high, F_low, MG, U_acid, and U_urea forms, changes in their absorption spectra over time were monitored. As Figure 7 shows, the rate of demetalation at pH 2.0 is greatest when Zncyt adopts the U_acid form. Lack of change in the absorbance for the U_urea form (see Figure 7d) suggests that demetalation of Zncyt requires acidic conditions, presumably because hydrogen ions assist displacement of zinc(II) ions at pH 2.0 (see Scheme 1). No change in absorbance was observed for Zncyt in the F_high form after 7 days at pH 7.00 and 4 °C (data not shown) and for cyt(III) in the U_acid and MG forms after 2 and 5 days, respectively (see Figure S5 in the Supporting Information). Most importantly, these results show that flash photolysis experiments can be performed before loss of zinc(II) ions progresses enough to complicate analysis of the kinetics. By doing the experiments with fresh samples, we avoided these complications.

Oxidative Quenching of 3Zncyt by Inorganic Complexes and Cyt(III). To test the effect of the protein conformation on reactivity, we compared the electron-transfer properties
of Zncyt in the F\textsubscript{low}, F\textsubscript{high}, MG, U\textsubscript{acid}, and U\textsubscript{urea} forms. The probes of \(^{3}\)Zncyt reactivity were the following three inorganic and one protein electron acceptors: anionic \(\text{Fe(CN)}_6^{3-}\), cationic \(\text{Co(phen)}_3^{3+}\), electroneutral \(\text{Co(acac)}_3\), and cationic cyt(III). This native protein was chosen as a probe since both it and its zinc derivative will be subject to similar unfolding. Ionic strength and pH for obtaining the five folded and unfolded forms of Zncyt are chosen in some cases to bring out, but in most cases to minimize, effects of electrostatic interactions between this protein and the oxidizing agents, so that effects of conformation on reactivity become discernible. The results are shown in Figure 8.

In 18 out of 20 cases, presence of quenchers significantly increases the decay of the triplet state \(^{3}\)Zncyt. Only slight, but reproducible, increases occur upon addition of \(\text{Co(phen)}_3^{3+}\) and cyt(III) at low ionic strength. Evidently, electrostatic repulsions limit collisional quenching between two cationic species: the F\textsubscript{low} form of Zncyt and the quencher. When ionic strength is raised to 1.500 M, electrostatic interactions are suppressed, and diffusional quenching occurs. In the F\textsubscript{low} and F\textsubscript{high} forms, decay of the triplet state, \(^{3}\)Zncyt, remained monoexponential upon addition of all four quenchers. In the MG, U\textsubscript{acid}, and U\textsubscript{urea} forms, however, the decay was biexponential. Typically, the faster phase accounted for only ca. 15\% – 20\% of the total amplitude. In the U\textsubscript{urea} form, a slower phase accounted for only 10\% – 15\% of the total amplitude. Because these minor phases parallel the major ones in reactivity (upon addition of quenchers to different conformational forms of \(^{3}\)Zncyt) we will focus on the major phases in each reaction. The psuedo-first-order rate constants are directly proportional to quencher concentration and depend on ionic strength and conformation of Zncyt, as Figure 8 shows. The second-order rate constants, obtained from the slopes of the
linear plots, are listed in Table 3.

**Discussion**

**Structure and Reactivity of (Un)folded Forms of Cyt(III) and Zncyt.** The conformation of iron cytochrome c as a function of temperature, pH, denaturant, and salt concentration has been well studied. Recent investigations suggested that folded metal-substituted cytochrome c and iron cytochrome c have similar tertiary structures,\textsuperscript{72,74,83} but partially and completely unfolded forms of Zncyt have not yet been fully characterized.\textsuperscript{87} We characterize them in this study. Our goal is not to compare zinc and iron forms of cytochrome c, but to study electron-transfer reactivity of Zncyt in various conformational forms. Ours is the first study of its kind.

The reduction potential of heme proteins is influenced by identity, alignment, and basicity of the axial ligands; distortions of the heme; and local charges. Heme exposure to solvent is particularly important; decrease in this exposure correlates with an increase in reduction potential.\textsuperscript{88} Reduction potentials of the F\textsubscript{high} (+255 mV) and MG (+233 mV) forms of cyt(III) are similar,\textsuperscript{89} but that of the forms unfolded by guanidine hydrochloride\textsuperscript{90} or urea\textsuperscript{91} (ca. –150 mV) is much lower and close to the potential (ca. –200 mV) of bis(imidazole) iron(III) porphyrin in aqueous solution.\textsuperscript{92–94} (All the potentials are given versus NHE.)

**Absorption and Circular Dichroism Spectra of (Un)folded Forms of Zncyt.** The axial coordination to the zinc(II) ion in Zncyt and to the iron ion in unfolded forms of iron cytochrome c is still uncertain. A recent study suggested that zinc(II) is six-coordinate (with His18 and Met80 ligation) in the F\textsubscript{low} (pH 5, low salt concentration) form and five-coordinate
(with Met80 detached) in the MG (pH 2.0, 1.00 M NaClO₄) form.⁸⁶ Reconsideration⁷⁴ of NMR spectra⁷³ suggested that even the F₉low form of Zncyt is five-coordinate, that is, that Met80 is not coordinated. Similarity of the absorption spectra for the F₉low and MG forms of Zncyt (see Table 1 and Figure 1) suggests that these forms have similar ligation. Complete unfolding of Zncyt by acid or urea induces large changes in absorbance, presumably owing to changes in zinc(II) ligation and increases in porphyrin exposure to solvent. Interestingly, absorption spectra of our Zncyt in the Uacid form and of the Zncyt in the MG form induced by ClO₄⁻ are similar.⁸⁶ In cyt(III), small anions (such as Cl⁻, Br⁻, and NO₃⁻) induce formation of a compact, highly structured MG form, restoring the native Fe(III)–Met80 bond and nativelike redox properties. Large anions (such as I⁻, ClO₄⁻, and CCl₃COO⁻) induce formation of a compact MG form that has bis-His coordination to heme iron and a fluctuating tertiary structure and lacks nativelike redox properties. Chloride and ClO₄⁻ anions stabilize MG forms of iron cytochrome c having nativelike α-helical structures, but having distinct tertiary conformations in which highly flexible loop regions are responsible for different spectroscopic and redox properties.⁸⁹ Our results conclusively show that the MG form of Zncyt can be achieved, although it is unclear whether the axial ligands in it are the same as those in the MG form of iron cytochrome c.

Our experiments with acid and urea consistently show that identity of the metal ion in cytochrome c does not significantly affect the protein behavior in the completely unfolded (Uacid and Uurea) forms. The metal ion, its oxidation state, and its axial ligand(s) in cytochrome c affect the free energy of unfolding in aqueous solution, ΔGₒ(H₂O). Consequently, equilibrium unfolding of iron cytochrome c induced by guanidine
hydrochloride shows that cyt(II) is 42 kJ mol\(^{-1}\) more stable than cyt(III) towards unfolding.\(^9\) Values of \(\Delta G_U(H_2O)\) estimated by urea-induced unfolding follow the trend cyt(II) > Zncyt ≈ cyt(III) >> H\(_2\)cyt.\(^9\) We chose the conditions for the U\(_{\text{acid}}\) and U\(_{\text{urea}}\) forms, to ensure that Zncyt and iron cytochrome c behave the same under extreme conditions (pH 2.0 or 8.0 M urea). Most important, the biphasic kinetics observed in aged Zncyt samples cannot be attributed to heterogeneity due to incomplete unfolding of Zncyt.

Far-UV circular dichroism (see Figure 3) supports the conclusion that Zncyt and iron cytochrome c in the F\(_{\text{low}}\), F\(_{\text{high}}\), and MG forms have almost the same conformation.\(^5\)\(^4\)\(^,\)\(^7\)\(^2\)\(^,\)\(^7\)\(^4\)\(^,\)\(^8\)\(^6\) Marked loss of ellipticity at 222 nm for Zncyt in the U\(_{\text{acid}}\) and U\(_{\text{urea}}\) forms indicates lack of secondary structure, as with iron cytochrome c in U\(_{\text{acid}}\) and U\(_{\text{urea}}\) forms.\(^3\)\(^8\)\(^,\)\(^5\)\(^4\)

**Natural Decay of the Triplet State of Zncyt in Different (Un)folded Forms.** Table 1 shows that the rate constant \(k_d\) for natural decay of \(^3\)Zncyt increases in the order F\(_{\text{high}}\) < F\(_{\text{low}}\) < MG < U\(_{\text{acid}}\) < U\(_{\text{urea}}\). Independence of this rate constant on protein concentration confirms that unfolding is an intramolecular process. Excited-state energies of metalloporphyrins are strongly affected by axial ligation and solvent.\(^9\)\(^7\) The internal hydrophobic core in the MG form expands somewhat and becomes more exposed to solvent, hence, a slight increase in the rate constant for natural decay. Upon complete unfolding of Zncyt by acid or urea, and probable displacement of His18 and Met80 by solvent,\(^3\)\(^4\)\(^,\)\(^3\)\(^5\) this rate constant increases greatly. Because the rate constants for natural decay of \(^3\)Zncyt in the U\(_{\text{acid}}\) and U\(_{\text{urea}}\) forms are different, our use of two denaturing agents is justified. These results support the conclusion that in Zncyt the change in the rate constant for natural decay between the F\(_{\text{low}}\), F\(_{\text{high}}\), MG, U\(_{\text{acid}}\), and U\(_{\text{urea}}\) forms is due to differences in zinc(II) ligation, porphyrin accessibility to
solvent, and energy of the triplet state.

**Reactivity of $^3$Zncyt in the Five (Un)foided Forms.** Metal complexes, with their well-defined structural and chemical properties, are well suited as probes of the degree of Zncyt unfolding. Because cytochrome c uses essentially the same surface patches for interactions with “small” reactants and with proteins, the former have often been used as substitutes for the latter in informative studies. The reaction in eq 1 is bimolecular with all quenchers and under all conditions studied. The linear plots in Figure 8 indicate a simple collisional mechanism of quenching, which does not require the involvement of a persistent protein-quencher complex.

A) **Role of Electrostatic Interactions.** Electrostatic interactions have a dominant effect on $^3$Zncyt reactivity. Assuming normal pK$_a$ values, Zncyt and cyt(III) at pH 7.0 have overall charges of +6 and +7, respectively. At low ionic strength (20 mM), electrostatic repulsion between Zncyt on one side and Co(phen)$_3$$^{3+}$ or cyt(III) on the other hinders their association. The very slight dependence of $k_q$ on concentration of Co(phen)$_3$$^{3+}$ and cyt(III) is evident in the very small values of $k_{bin}$ in Table 3. At high ionic strength (1.500 M) the two cationic species overcome the repulsions and interact; the reactivity of $^3$Zncyt with Co(phen)$_3$$^{3+}$ and with cyt(III) increases 245-fold and 7.0-fold, respectively. Reaction between oppositely charged partners $^3$Zncyt and Fe(CN)$_6$$^{3-}$ is virtually diffusion-controlled at the low ionic strength, and is slowed down 20-fold at the high ionic strength.

Comparison of $^3$Zncyt reactivity in the MG ($\mu = 1500$ mM) and $U_{acid}$ ($\mu = 20$ mM) forms is only tentative because the effects of electrostatic interactions and porphyrin exposure may not be separable. In the case of Co(phen)$_3$$^{3+}$, the rate constant $k_{bin}$ decreases 2.0-fold as
Zncyt changes from the MG form to the U_{acid} form, presumably because the highly protonated latter form has a greater positive charge than the former form. In the case of cyt(III), however, \( k_{\text{bim}} \) increases 3.6-fold from the MG form to the U_{acid} form despite the enhanced electrostatic repulsion. The increased reactivity presumably is caused by a greater exposure of porphyrin in both Zncyt and cyt(III) upon unfolding.

To remove the ambiguities caused by electrostatic interactions and bring out the effects of (un)folding on reactivity, we chose the electroneutral oxidant Co(acac)_3. It shows the same rate constant for quenching the F_{low} and F_{high} forms of \(^3\text{Zncyt}\); indeed, this reaction is independent of ionic strength. Reactivity of \(^3\text{Zncyt}\) in the F_{high} form (under conditions that suppress electrostatic interactions) with Fe(CN)_6^{3-}, Co(phen)_3^{3+}, and Co(acac)_3 follows the decrease in the reduction potential of the inorganic quencher, respectively, in the order +410, +370, and −340 mV.

**B) Partial Unfolding of Zncyt: The Molten-Globule Form.** Although the MG form of Zncyt is extensively protonated, at high ionic strength the intramolecular electrostatic repulsions are suppressed, allowing the protein to maintain a compact, nativelike conformation. Direct comparisons between the F_{high} and MG forms of Zncyt, without complications from electrostatic interactions, are possible. In going from the F_{high} form to the MG form, the rate constant \( k_{\text{bim}} \) increases 1.4-fold, 1.6-fold, 4.0-fold, and 10-fold in the order cyt(III), Fe(CN)_6^{3-}, Co(phen)_3^{3+}, and Co(acac)_3. The increase is slight for the first two quenchers but larger for the last two, presumably because of their different surface characteristics. The basic recognition patch of cytochrome c and the cyano complexes are hydrophilic, whereas the phen and acac complexes are hydrophobic. The last two quenchers
can better take advantage of the enhanced hydrophobicity caused by the partial exposure of porphyrin in the MG form.

C) Complete Unfolding of Zncyt: The U_{acid} and U_{urea} Forms. Because both of these unfolded forms of Zncyt exist at low ionic strength (20 mM), they can be properly compared with the folded F_{low} form. Reactivity toward the anionic quencher Fe(CN)_6^{3-} is slightly lowered upon unfolding of the F_{low} form. The \( k_{bim} \) values for the U_{acid} and U_{urea} forms are virtually the same. Because these three forms of \(^3\)Zncyt react with Fe(CN)_6^{3-} at essentially diffusion-controlled rates, these reactions are least informative about the effects of unfolding.

Reactivity toward the cationic quenchers cyt(III) and Co(phen)_3^{3+} is enhanced more when the unfolding agent is urea (168-fold and 1340-fold, respectively) than when this agent is acid (36-fold and 466-fold, respectively) because Zncyt has a lower positive charge in the U_{urea} form than in the U_{acid} form. With either method of unfolding, the hydrophobic Co(phen)_3^{3+} benefits more than the hydrophilic cyt(III) from the greater accessibility of the hydrophobic porphyrin cofactor in Zncyt. The metal complex, being relatively small, also benefits more than cyt(III) does from the greater exposure of porphyrin in Zncyt.

The electroneutral complex Co(acac)_3 reacts at the same rate with the U_{acid} and U_{urea} forms of \(^3\)Zncyt. In other words, unfolding enhances the quenching reaction to virtually the same extent (62-fold and 54-fold, respectively) regardless of the agent used for the unfolding. When electrostatic interactions are eliminated, the effects on the reactivity of increased exposure of the porphyrin cofactor and of the closer approach by the quencher can be assessed.
Conclusions

Electron-transfer reactivity of the triplet state of zinc-substituted cytochrome c in the folded (at low and high ionic strength), molten-globule, and unfolded (by acid and urea) forms is probed with four different quenchers. This reactivity depends mostly on electrostatic interactions and the degree of porphyrin exposure. Using the electroneutral complex Co(acac)$_3$ as a quencher, we eliminated the electrostatic effects and assessed the consequences of porphyrin exposure for the electron-transfer reactivity. The rate constant for the photoinduced reaction in eq 1 increases 10-fold upon partial unfolding into the molten-globule form and again approximately 5-fold upon further, complete unfolding of zinc cytochrome c.

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Supporting Information Available: Five figures showing the absorption and far-UV circular dichroism spectra of Zncyt and H$_2$cyt illustrating Zncyt demetalation; change in absorption spectrum of Zncyt in the U$_{acid}$ form upon addition of NaCl stabilizing the MG form; change in absorption spectrum upon remetalation of H$_2$cyt forming Zncyt; and stability of cyt(III) in the MG and U$_{acid}$ form (5 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Table 1. Characterization of the Folded Forms (at Low and High Ionic Strength), Molten-Globule Form, and Forms Unfolded by Acid and by Urea of Zinc Cytochrome c and Metal-Free Porphyrin Cytochrome c at 25 °C and Different Values of pH and Ionic Strength (μ)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Form</th>
<th>pH</th>
<th>μ (mM)</th>
<th>Soret Band</th>
<th>Q Bands</th>
<th>( \lambda_{max} ) (nm)</th>
<th>Molar Ellipticity (-[\theta]_{222}) (deg cm² dmol⁻¹)</th>
<th>α-Helical Content (%)</th>
<th>Natural Decay ( k_d ) (s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Zncyt</td>
<td>Fₕₙₙ</td>
<td>7.00</td>
<td>20</td>
<td>423</td>
<td>549</td>
<td>585</td>
<td>10300</td>
<td>28.0</td>
<td>70 ± 4</td>
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</tr>
<tr>
<td>Fₙₕₕ</td>
<td>7.00</td>
<td>1500</td>
<td>423</td>
<td>549</td>
<td>585</td>
<td></td>
<td>11150</td>
<td>31.6</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>MG</td>
<td>2.0</td>
<td>1500</td>
<td>421</td>
<td>548</td>
<td>582</td>
<td></td>
<td>10530</td>
<td>28.1</td>
<td>190 ± 20</td>
</tr>
<tr>
<td>Uₐₛ₅</td>
<td>2.0</td>
<td>20</td>
<td>407</td>
<td>547</td>
<td>575</td>
<td></td>
<td>4120</td>
<td>16.4</td>
<td>440 ± 50</td>
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<tr>
<td>Uₜₛₖ</td>
<td>7.00</td>
<td>20</td>
<td>418</td>
<td>546</td>
<td>581</td>
<td></td>
<td>1880</td>
<td>4.4</td>
<td>1200 ± 220</td>
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<tr>
<td>H₂cₙₜ</td>
<td>Fₙₕₕ</td>
<td>7.00</td>
<td>1500</td>
<td>404</td>
<td>506</td>
<td>540 568</td>
<td>620</td>
<td>11070</td>
<td>32.6 1350 ± 70</td>
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</tr>
<tr>
<td>MG</td>
<td>2.0</td>
<td>1500</td>
<td>405</td>
<td>506</td>
<td>538</td>
<td>559</td>
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<tr>
<td>Uₐₛ₅</td>
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<td>20</td>
<td>406</td>
<td>552</td>
<td>594</td>
<td></td>
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<tr>
<td>Uₜₛₖ</td>
<td>7.00</td>
<td>20</td>
<td>400</td>
<td>504</td>
<td>540</td>
<td>565</td>
<td>616</td>
<td></td>
<td></td>
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</tbody>
</table>

*a\ Fₕₙₙ and Fₙₕₕ, folded forms at low and high ionic strength; MG, molten-globule form; and Uₐₛ₅ and Uₜ₄ₖ, forms unfolded by acid and by urea.*
Table 2. Thermodynamic Parameters for Urea-Induced Unfolding of Zinc Cytochrome c, Iron(III) Cytochrome c, and Metal-Free Porphyrin Cytochrome c at 20 °C

<table>
<thead>
<tr>
<th>protein</th>
<th>$\Delta G_u(H_2O)$ (kJ mol$^{-1}$)</th>
<th>$m$ (kJ mol$^{-1}$ M$^{-1}$)</th>
<th>$C_m$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zncyt$^a$</td>
<td>47.2 ± 3.3</td>
<td>6.6 ± 0.5</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>cyt(III)$^b$</td>
<td>37.5</td>
<td>5.0</td>
<td>7.5</td>
</tr>
<tr>
<td>H$_2$cyt$^b$</td>
<td>6.1</td>
<td>3.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

$^a$ This work.

$^b$ From ref 83; estimated errors are, at most, ±10%.

Table 3. Bimolecular Rate Constants for Reactions of the Triplet State of Zinc Cytochrome c in Different Conformational Forms with the Four Oxidative Quenchers Shown, at Different Values of pH and Ionic Strength ($\mu$)

<table>
<thead>
<tr>
<th>form$^a$</th>
<th>conditions</th>
<th>$k_{bim} \times 10^{-6}$, M$^{-1}$ s$^{-1}$ at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>$\mu$ (mM)</td>
</tr>
<tr>
<td>$F_{low}$</td>
<td>7.00</td>
<td>20</td>
</tr>
<tr>
<td>$F_{high}$</td>
<td>7.00</td>
<td>1500</td>
</tr>
<tr>
<td>MG</td>
<td>2.0</td>
<td>1500</td>
</tr>
<tr>
<td>$U_{acid}$</td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>$U_{urea}$</td>
<td>7.00</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ $F_{low}$ and $F_{high}$, folded forms at low and high ionic strength; MG, molten-globule form; and $U_{acid}$ and $U_{urea}$, forms unfolded by acid and by urea.
2 H⁺ + Zn^2+ \xrightarrow{\text{slow}} H₂^2 + Zn^2+

Zn^2+ \xrightarrow{hν} 3Zn^2+ \xrightarrow{k_1(\text{slow})} Zn^2+

H₂^2 \xrightarrow{hν} 3H₂^2 \xrightarrow{k_2(\text{fast})} H₂^2

Scheme 1
Figure 1. Absorption spectra in the (a) Soret region and (b) Q-band region of the folded form at high ionic strength (F_{high}), molten-globule (MG) form, and the forms unfolded by acid (U_{acid}) and by urea (U_{urea}) of 3.0 μM zinc cytochrome c. The absorption spectrum of the folded form at low ionic strength (F_{low}) of zinc cytochrome c (not shown) is identical to that of the F_{high} form. Conditions: F_{low}, at pH 7.00 and μ = 20 mM; F_{high}, at pH 7.00 and μ = 1.500 M; MG, at pH 2.0 and μ = 1.500 M; U_{acid}, at pH 2.0 and μ = 20 mM; and U_{urea}, at pH 7.00, μ = 20 mM, and 8.0 M urea.
Figure 2. (a) Equilibrium unfolding of 5.0 μM zinc cytochrome c at 20 °C, induced by HCl and monitored by Soret absorption, at 410 nm. Unbuffered aqueous protein solutions with pH adjusted from 7.0 to 1.1 are prepared by addition of dilute HCl or NaOH. The line is a fitting to four-parameter sigmoidal equations with the midpoint of the unfolding transition ($C_m$) at pH 2.9 ± 0.1 and the midpoint of the refolding transition ($C_m$) at pH 1.5 ± 0.2. (b) Equilibrium unfolding of 1.0 μM zinc cytochrome c at 20 °C, induced by urea and monitored by Soret absorption, at 423 nm. Urea concentrations from 0.0 to 12.0 M are prepared by dilution of 12.0 M urea into 10 mM phosphate buffer with pH 7.00 and μ = 20 mM. The line is a fitting to a four-parameter sigmoidal equation with the midpoint of the unfolding transition ($C_m$) at 7.1 ± 0.1 M. The fraction of unfolded protein ($F_U$) and free energy for unfolding ($\Delta G_U$) are calculated using eq 3. See Table 2 for the resulting thermodynamic parameters.
Figure 3. Circular dichroism spectra in the far-UV region of the folded forms at low ($F_{low}$) and high ($F_{high}$) ionic strength, molten-globule (MG) form, and the forms unfolded by acid ($U_{acid}$) and urea ($U_{urea}$) of 10.0 μM zinc cytochrome $c$. See Figure 1 for experimental conditions.
Figure 4. Natural decay (in the absence of quencher) of the triplet states of zinc cytochrome c (designated $^3$Zncyt, five traces) and metal-free porphyrin cytochrome c (designated $^3$H$_2$cyt, one trace), in the folded forms at low (F$_{low}$) and high (F$_{high}$) ionic strength, molten-globule (MG) form, and the forms unfolded by acid (U$_{acid}$) and urea (U$_{urea}$) of 10.0 μM zinc cytochrome c or 40.0 μM metal-free porphyrin cytochrome c. The lines are fittings to eq 10. The upper traces, truncated to the timescale of the figure, continue to decline exponentially. See Figure 1 for experimental conditions.
Figure 5. Natural decay (in the absence of quencher) of the triplet state of zinc cytochrome $c$ that was left for several hours in the $U_{acid}$ form (at pH 2.0 and $\mu = 20$ mM) before being returned by ultrafiltration to pH 7.00 and $\mu = 20$ mM. In this extreme case of the aged protein sample, fittings to eq 11 gave $k_1 = 86 \pm 3$ s$^{-1}$, $a_1 = 48.5 \pm 1.1\%$, $k_2 = 1350 \pm 30$ s$^{-1}$, and $a_2 = 51.5 \pm 0.8\%$. Quality of the fitting is shown in the plot of the residuals below.
Figure 6. Ratio of amplitudes $a_2$ (for the fast phase) and $a_1$ (for the slow phase) depends on the ratio of molar concentrations of the two sensitizers. Changes of transient absorbance with time upon laser excitation of solutions containing 10.0 μM zinc cytochrome c (Zncyt) and increasing concentrations of metal-free porphyrin cytochrome c (H$_2$cyt) are fitted to eq 11. Invisible error bars are smaller than the symbol. The solvent was 10 mM sodium phosphate buffer at pH 7.00 and $\mu = 1.500$ M.
Figure 7. Absorption spectra in the Soret and Q-band regions of the molten-globule (MG) form and the forms unfolded by acid (U$_{acid}$) and urea (U$_{urea}$) of zinc cytochrome c monitored over time. Conditions: (a) 4.5 μM Zncyt (MG) at pH 2.0 and μ = 1.500 M over a period of 31 h; (b) 10.0 μM Zncyt (MG) at pH 2.0 and μ = 0.500 M over 12 h; (c) 4.5 μM Zncyt (U$_{acid}$) at pH 2.0 and μ = 20 mM over 18 h; and (d) 5.0 μM Zncyt (U$_{urea}$) at pH 7.00, μ = 20 mM, and 8.0 M urea over 9 h. Note the break in the wavelength axis and the different absorbance scales.
Figure 8. Dependence on quencher concentration of the quenching rate constant $k_q$ obtained using eq 8 for the reactions at 25 °C of the triplet state of zinc cytochrome $c$ in the folded forms at ionic strengths of 20 mM ($F_{\text{low}}$) and 1.500 M ($F_{\text{high}}$), molten-globule (MG) form, and forms unfolded by acid ($U_{\text{acid}}$) and by urea ($U_{\text{urea}}$) with the four quenchers shown. The solid lines are obtained from linear least-squares regression; their slopes yield the bimolecular rate constants $k_{\text{bim}}$ given in Table 3. Invisible error bars are smaller than the symbol. The legend symbols are arranged from top to bottom in the same order as the slopes of the lines. Some plots overlap. See Figure 1 for experimental conditions.
Supporting Information

Figure S1. Absorption spectra in the Soret and Q band regions of 5.0 μM zinc cytochrome c (Zncyt) in the MG (pH 2.0 and μ = 1.500 M) and U_{acid} (pH 2.0 and μ = 20 mM) forms and the same two protein samples after several slow solvent exchanges by ultrafiltration into the folded F_{low} (10 mM phosphate buffer at pH 7.00 and μ = 20 mM) and F_{high} (10 mM phosphate buffer at pH 7.00 and μ = 1.500 M) forms. Absorption spectra of these protein samples after ultrafiltration is characteristic of metal-free porphyrin cytochrome c (H_{2}cyt) confirming that demetalation of zinc cytochrome c occurs.
Figure S2. Far-UV circular dichroism spectra in the far-UV region of 5.0 μM zinc cytochrome c (Zncyt) in the MG (pH 2.0 and μ = 1.500 M) and U_{acid} (pH 2.0 and μ = 20 mM) forms and the same two protein samples after several slow solvent exchanges by ultrafiltration into the folded F_{low} (10 mM phosphate buffer at pH 7.00 and μ = 20 mM) and F_{high} (10 mM phosphate buffer at pH 7.00 and μ = 1.500 M) forms. Circular dichroism spectra of these protein samples after ultrafiltration containing metal-free porphyrin cytochrome c (H_{2}cyt) show recovery of native-like secondary structure upon conversion of forms U_{acid} into F_{low} and MG into F_{high}. 
Figure S3. Conversion of 5.0 μM zinc cytochrome c from the U\textsubscript{acid} (pH 2.0 and μ = 20 mM) to MG (pH 2.0 and μ = 1.500 M) form effected by adding NaCl to the solution and monitored by absorbance changes in the Soret region. Clear isosbestic points persist at 398 nm and 417 nm as the ionic strength is raised from 20 to 110 mM. These isosbestic points become somewhat “smeared” upon going to 1.000 M. Spectra are corrected for dilution.
Figure S4. Conversion of 4.0 μM metal-free porphyrin cytochrome c (H₂cyt) into zinc cytochrome c (Zncyt) effected by adding 100 μM zinc(II) acetate to the protein solution acidified by 10% acetic acid at 50°C and monitored by absorbance changes in the Soret region. Metalation of metal-free porphyrin cytochrome c forming zinc cytochrome c (Soret band at 423 nm) is nearly quantitative.
Figure S5. Absorption spectra in Soret and Q band regions of non-native $U_{\text{acid}}$ (pH 2.0 and $\mu = 20$ mM) and MG (pH 2.0 and $\mu = 1.500$ M) forms of 10.0 $\mu$M iron(III) cytochrome $c$ monitored over time. No change in absorbance is observed over a period of 2 and 5 days in the $U_{\text{acid}}$ and MG forms, respectively. The stability of iron(III) cytochrome $c$ towards demetalation at pH 2 is evident and will not complicate analysis of the kinetics for reactions in which this protein is used as a quencher.
CHAPTER 4. GENERAL CONCLUSIONS

Because metalloproteins act as electron carriers in essential biological processes, such as respiration, photosynthesis, and metabolism, mechanisms of their electron-transfer reactions are being studied vigorously. The goal for many years has been to understand how protein structure and the structure of the redox center itself influence the rate constants for electron transfer between proteins. Toward this goal, we strive to understand how protein conformation modulates electron-transfer reactivity in heme proteins. We investigated photoinduced electron-transfer reactions of folded, partially unfolded, and completely unfolded zinc cytochrome c with various inorganic and protein electron acceptors.

In our studies of Zncyt in the folded conformation, photoinduced reactions of the excited $^3$Zncyt with iron(III) cytochrome c, iron(II) cytochrome c, metal-free porphyrin cytochrome c, and heme-free apocytochrome c revealed the mechanism of quenching. The replacement of heme iron by zinc(II) overcomes the difficulties in studying redox reactions between two heme proteins having similar absorption spectra. Quenching of the positively charged $^3$Zncyt by the positively charged reactants is promoted at high ionic strength, $\mu = 1500$ mM, which shields electrostatic charge and weakens protein-protein repulsion.

Electron transfer from $^3$Zncyt to iron(III) cytochrome c is a more efficient quenching mechanism than energy transfer and enhanced radiationless decay at high ionic strength. Oxidative quenching of $^3$Zncyt by iron(III) cytochrome c is proven by the observed formation of the electron-transfer intermediate Zncyt$^+$. Iron-free porphyrin cytochrome c and also iron(II) cytochrome c quenches $^3$Zncyt by energy transfer, owing to the lack of any observable
transient radical-ions Znocyty and Znocyty and the good overlap of their absorption bands with the emission bands of Znocyty. Enhanced radiationless decay will not be an efficient mechanism of quenching Znocyty by any cytochrome c derivative used in this study.

To study the effect of protein conformation on reactivity, we compared the electron-transfer properties of Znocyty in the folded, molten-globule, and completely unfolded forms toward the following four oxidative quenchers: Fe(CN)₆³⁻, Co(acac)₃, Co(phen)₃³⁺, and iron(III) cytochrome c. The observed bimolecular rate constants show electron-transfer reactivity depends mostly on electrostatic interactions and the degree of porphyrin exposure as the protein unfolds. Ionic strength and pH are chosen in some cases to bring out, but in most cases to minimize, effects of electrostatic interactions between Znocyty and the oxidative quenchers, so that effects of conformation on reactivity become discernible. The large increase in the observed bimolecular rate constants for the reactions of folded Znocyty and cationic quenchers Co(phen)₃³⁺ and iron(III) cytochrome c upon increasing ionic strength from 20 to 1500 mM shows the dominant role electrostatic repulsions play in controlling these reactions. Moreover, the even larger increase in the observed bimolecular rate constants for the reactions of Znocyty and cationic quenchers Co(phen)₃³⁺ and iron(III) cytochrome c at low ionic strength upon increasing the concentration of urea from 0 to 8 M shows the dominant role protein conformation plays in controlling these reactions. Using the electroneutral complex Co(acac)₃ as a quencher, we eliminated the electrostatic effects and assessed only the consequences of porphyrin exposure upon partial and complete unfolding of the protein for the electron-transfer reactivity. The bimolecular rate constant for the reaction of Znocyty and Co(acac)₃ increases 10-fold upon partial unfolding into the molten-
globule form and approximately 50-fold upon complete unfolding of Zncy.t. Electroneutral inorganic complexes are sensitive probes of the amount of partial and complete unfolding in zinc-substituted heme proteins. By measuring the chemical reactivity of proteins in various conformational forms, we can detect and characterize intermediates along the pathway of protein folding. A future application of our approach could be the detection of transient kinetic intermediates on the time scale of microseconds to seconds that arise during protein folding.
APPENDIX. OPTIMIZATION OF A BACTERIAL
EXPRESSION SYSTEM FOR THE SOLUBLE FORM OF
TURNIP CYTOCHROME F AND ITS ISOLATION

Introduction

Cytochrome $f$, designated cyt $f$, is an intrinsic membrane component of the
cytochrome $b_{6}f$ complex that transfers electrons from the Rieske iron-sulfur protein to
plastocyanin and cytochrome $c_{6}$. This 31-kDa protein is synthesized with a cleavable leader
sequence and is anchored in the thylakoid membrane by a single C-terminal transmembrane
helix. Cyt $f$ contains the characteristic fingerprint sequence Cys-X-Y-Cys-His of $c$-type
cytochromes, which is responsible for the covalent attachment of the heme group to the
protein backbone through two thioether bonds. The crystal structure of the soluble form of
cyt $f$ from turnip chloroplasts has revealed three unprecedented features for $c$-type
cytochromes (Martinez et al., 1994; Martinez et al., 1996). The overall structure of cyt $f$ is
elongated containing predominantly $\beta$-sheet character. Cyt $f$ also contains two readily
discernable domains, with the heme group covalently bound within the large domain. The
third unprecedented aspect of cyt $f$ is the heme ligation by the $\alpha$-amino group of the amino-
terminal residue Tyr1. This unusual mode of coordination may ensure a proper delay in the
timing of protein refolding, preventing cyt $f$ from properly folding until the signal peptide has
been cut off by a peptidase located in the lumen. In addition, crystal structures of cyt $f$ from
green alga Chlamydomonas reinhardtii (Chi et al., 2000; Sainz et al., 2000) and
cyanobacterium *Phormidium laminosum* (Carrell et. al, 1999) have been reported. Solution structures of the complex between cyt $f$ and plastocyanin have also been determined using NMR and rigid body molecular dynamics simulations (Ubbink et al., 1998; Crowley et al., 2001).

Cyt $f$ has been isolated from a variety of plant, algal, and cyanobacterial sources. For most higher plants (parsley, spinach, and tobacco) purification of cyt $f$ results in the production of oligomeric or aggregated forms of the protein (Gray et al., 1994; Bendall, 1982). However, monomeric forms of cyt $f$, with no tendency to aggregate, have been isolated from the *Cruciferae* family, which includes mustard, radish, charlock, cabbage, and turnip. (Beoku-Betts & Sykes, 1985; Gray, 1978).

At the present time, commercially available cyt $f$ is very expensive and large quantities needed for further NMR and kinetic experiments are difficult to obtain. Isolation of cyt $f$ from higher plants is a tedious process with the risk of possible plant contaminants. Therefore it would be desirable to produce higher plant cyt $f$ in a bacterial expression system, capable of producing large quantities of protein in shorter time. Furthermore, production of cyt $f$ mutants would be possible, leading to studies investigating electrostatic and hydrophobic interactions in protein-protein complexes.

At the time of this research (9/99 – 11/99), there were no published reports of expression systems for higher plant cyt $f$, although an expression system had already been developed for the cyanobacterial form of cyt $f$ (Schlarb et al., 1999). Prior to my arrival at Leiden University in Leiden, The Netherlands, development of a bacterial expression system for higher plant cyt $f$ had just begun (Cornvik & Ubbink, 1999). The goal was to construct an
expression system for the soluble part of turnip (*Brassica rapa*) cyt*f*, residues 45-296, in *Escherichia coli*. Since cyt*f* is a c-type cytochrome, heme lyase is required for covalent attachment of heme to the protein backbone. In *E. coli*, heme lyase is located in the periplasmic space, so it is essential to export cyt*f* there as well. This was achieved by insertion of the nucleotide sequence of an efficient periplasmic signal peptide from azurin in front of the gene encoding cyt*f*. Moreover, isolation of cyt*f* is much easier after export into the periplasmic space (Ubbink & van Beeaman, 1992).

This report summarizes the optimization of soluble turnip cyt*f* expression in *E. coli* and its subsequent isolation and purification. Previous work in the laboratory at Leiden University, Leiden, The Netherlands (Cornvik, T. & Ubbink, M.) showed plasmid pTC1 expressed in *E. coli* strain JM109 under semi-anaerobic conditions at 30°C provided the highest expression levels of turnip cyt*f*. In continuation of this work, I investigated the effect of growth time, level of dissolved oxygen, and presence of pEC86 (a cassette of cytochrome c maturation genes of *E. coli*) on cyt*f* expression levels in a 30-L fermentor. Highest expression yields of cyt*f* are achieved after 60-72 hours under semi-anaerobic conditions. Expression yields before isolation from growing cells are 0.9-1.0 mg/L. In addition, I optimized the recovery of cyt*f* after extraction from the periplasmic space by osmotic shock. Cyt*f* yields after extraction by osmotic shock are typically 0.6-0.8 mg/L. Finally, I developed a procedure to isolate cyt*f* from periplasmic extracts using a combination of ion-exchange and gel filtration chromatography. The purification involves a DEAE Sepharose (batch mode), DEAE Sepharose (pH 6, cyt*f* reduced), CM Sephadex (pH 6, cyt*f* reduced), and Superdex G75 (pH 6, cyt*f* reduced). The final yield from 30 L of culture was 10-12 mg cyt*f*. 
with an absorbance ratio of $A_{554}/A_{277}$ of 0.95. During my stay in Leiden, nearly 50 mg of pure cyt $f$ was obtained. After this research was completed, an article describing an *E. coli* expression system for soluble turnip cyt $f$ and its isolation was published by another laboratory (Gong et al., 2000).

**Experimental**

**Expression.** Plasmid pTC1 (Figure 1), containing the gene encoding the soluble form of cyt $f$, was obtained from Dr. M. Ubbink (Comvik & Ubbink, 1999). Plasmid pEC86, a cassette of cytochrome $c$ maturation genes of *E. coli*, was a gift from Dr. L. Thoeny-Meyer, Microbiology Institute, ETH Zurich, Switzerland (Arslan et al, 1998). Competent cells of *E. coli* strain JM109 were transformed with plasmid pTC1 and incubated overnight at 37°C in 1.5% Agar/LB plates supplemented with 100 μg/mL ampicillin. LB medium contained 10% Difco tryptone peptone, 5% Difco yeast extract, and 10% sodium chloride. A single white colony was picked and diluted into 3 mL LB (in 10 mL sterile tube) supplemented with ampicillin and incubated 4-5 hours with shaking at 30°C. Fermentor medium (30 L) containing 300 g Difco tryptone peptone, 150 g Difco yeast extract, 240 g sodium chloride, and 3.0 g potassium nitrate was sterilized. The fermentor was inoculated with 250 mL 0.5 M fumarate (filter sterilized), 5.0 g ampicillin, and 3 mL JM109/pTC1 preculture (slightly turbid). If available, 150 mL of a stock 0.2 g/50 mL Haemin solution was added (McConville & Charles, 1979). Culture was grown at 30°C and pH 6.8 under semi-anaerobic conditions with dissolved $O_2$ concentration regulated at 1.5%. When agitation rate reached 150 rpm (approximately 10 hours after inoculation), culture was induced with 1.5 g IPTG.
Figure 2 shows the fermentation conditions throughout the growth of the culture.

Periplasmic expression of cyt f during fermentation was determined via protein isolation by osmotic shock of the cells. A 10-mL sample from the fermentor was obtained (in 10-mL sterile tube) and centrifuged at 3300 rpm for 15 min. The pellet was resuspended in 1 mL sucrose buffer (30 mM Tris-HCl, 5 mM EDTA, 20% sucrose, pH 8) and incubated 20 minutes at room temperature. The cells were centrifuged at 3300 rpm for 15 min and absence of cyt f in supernatant (sucrose fraction) confirmed by absorbance spectroscopy. The pellet was resuspended in 1 mL cold Milli-Q water and incubated 20 min at 4°C. The cells were centrifuged at 3300 rpm for 15 min and presence of cyt f in supernatant (MilliQ fraction) confirmed by absorbance spectroscopy. Concentration of cyt f (mg/L) was obtained by using eq 1 where \( \Delta A_{554 \text{ nm}} \) is the difference between the maximum \( A_{554 \text{ nm}} \) value and the value of \( A_{554 \text{ nm}} \) obtained by extrapolating the baseline.

\[
\frac{\Delta A_{554 \text{ nm}}}{31500} \times 2.8 \times 10^7 = [\text{cyt f}] \text{ (in mg/L)}
\]  

Cells were harvested from the fermentor and concentrated after approximately 60-72 hours of growth. It was determined that freezing the harvested cells and later thawing them significantly lowered the recovery of cyt f. Therefore, immediately after harvest, the cells were centrifuged at 6000 rpm in a JA-20 rotor for 25 min and the pellets resuspended in a total of 3 L sucrose buffer (a 5-L beaker was used). The cells were stirred at room temperature for 15 min after addition of 1 \( \mu \)L DNase I. The cells were centrifuged at 6000 rpm for 25 min. The absence of cyt f in the supernatant (sucrose fraction) was confirmed by absorption spectroscopy. The pellets were resuspended in a total of 6 L cold Milli-Q water (use two 5-L beakers) and stirred vigorously in a cold room at 4°C for 30 min. The cells
were centrifuged at 6000 rpm for 25 min and the supernatant (MilliQ fraction) was collected. Figure 3 shows the absorption spectrum of cyt f present in the MilliQ fraction. Harvesting the cells and osmotic shock required about 6 hours.

**Purification.** Initial extraction of cyt f after osmotic shock was accomplished by adding 250 mL packed volume of Pharmacia DEAE Sepharose Fast Flow chromatography resin (pre-equilibrated in Milli-Q water, pH 6.5) to 6 L of the MilliQ fraction and stirring a few hours in a cold room. Cyt f was bound by the chromatography resin. Excess solution was removed from the chromatography resin through a glass filter. Absence of cyt f in the eluent was confirmed by absorption spectroscopy. A 350-mL slurry of the chromatography resin was prepared and poured into large batch chromatography column from Pharmacia (2.6 cm internal diameter). Bound cyt f was eluted using a solution of 10 mM MES buffer and 100 mM NaCl at pH 6.5. The flow rate was 3 mL/min. The best fractions of cyt f (as determined by the purity ratio $A_{554}/A_{277} > 0.03$) were pooled and 1 mM ascorbate was added to ensure cyt f remained in the reduced iron(II) form. Cyt f in the oxidized form decomposes readily. The solution of cyt f was exchanged into 10 mM MES buffer at pH 6.5 using an Amicon cell. After concentration of the cyt f solution using an Amicon cell, 1 mM ascorbate was added. The cyt f solution was frozen at -80°C until needed. Figure 4 shows the absorption spectrum of pooled cyt f after this DEAE batch column.

A proper DEAE ion exchange column (internal diameter 1.6 cm), pre-equilibrated with 10 mM MES buffer pH 6.5, was prepared. The cyt f solution recovered from the DEAE batch column was diluted with 10 mM MES buffer at pH 6.5 and 1 μL DNase I was added. The cyt f solution was loaded onto the DEAE column at a flow rate of 3 mL/min. Cyt f was
eluted overnight with a linear salt gradient at a flow rate of 3 mL/min (Buffer A: 2.2 L solution of 10 mM MES buffer at pH 6.5; Buffer B: 2.2 L solution of 10 mM MES buffer and 60 mM NaCl at pH 6.5). The best fractions of cyt (as determined by the purity ratio $A_{554}/A_{277} > 0.2$) were pooled and 1 mM ascorbate was added. The solution of cyt was exchanged into 10 mM MES buffer at pH 6.0 using an Amicon cell.

A proper Pharmacia CM Sephadex ion exchange column (1.6 cm internal diameter), pre-equilibrated with 10 mM MES buffer at pH 6.0, was prepared. Cyt was eluted overnight with a linear salt gradient at a flow rate of 3 mL/min (Buffer A: a 700 mL solution of 10 mM MES buffer at pH 6.0; Buffer B: a 1 L solution of 10 mM MES buffer and 300 mM NaCl at pH 6.5). The best fractions of cyt (as determined by the purity ratio $A_{554}/A_{277} > 0.4$) were pooled and 1 mM ascorbate was added. The solution of cyt was exchanged into 10 mM MES buffer at pH 6.0 using an Amicon cell. The CM Sephadex chromatography resin binds cyt very strongly, so short columns and higher salt gradients are recommended.

A Pharmacia Superdex G75 commercial FPLC gel filtration column, pre-equilibrated with a solution of 10 mM MES buffer, 100 mM NaCl, and 1mM ascorbate at pH 6.0 was used. Samples (2-mL sample loop) of concentrated cyt were injected onto the column and eluted at a flow rate of 1 mL/min. The best fractions of cyt (as determined by the purity ratio $A_{554}/A_{277} ~ 0.9-1.0$) were pooled and 1 mM ascorbate was added. The cyt solution was concentrated in Amicon cell.

Additional purification attempts used Pharmacia SP Sepharose and HiTrap Q Sepharose commercial FPLC ion exchange columns. After equilibrating a SP Sepharose column with 20 mM MES buffer at pH 6.5, cyt was loaded. However, cyt bound poorly to
the chromatography resin and eluted soon after increasing the flow rate to 3 mL/min. After equilibrating a Q Sepharose column with 10 mM MES buffer at pH 6.5, cyt f was loaded. A linear gradient of 0 to 60 mM NaCl was used to elute cyt f. At a flow rate of 5 mL/min, the cyt f band smeared, so flow rate was adjusted to 2 mL/min. Again, cyt f bound poorly to the column and quickly eluted at 24% 60 mM NaCl.

Results and Discussion

Expression. The expression yields of turnip cyt f in E. coli as the culture is growing in the fermentor are shown in the Figure 5. Remarkably, maximum cyt f expression yields plateau at approximately 50 to 55 hours. This suggests that in this expression system, the holoenzyme is produced during the stationary phase.

Tables 1 and 2 summarize cyt f expression and osmotic shock conditions. Both tables illustrate the difficulties in maximizing expression yields and isolating cyt f from cells by osmotic shock. In Table 1, the correlation of longer growth time to higher expression yield of cyt f in the growing culture is clear. However, growth conditions for this bacterial expression system may still be improved. Surprisingly, semi-anaerobic and aerobic growth conditions both show similar yields (~15 mg cyt f) at shorter growth times (30 hours). However, it is not clear whether this will be true at longer growth times. Moreover, incorporation of plasmid pEC86 could not improve yields of cyt f in this bacterial expression system. Actually, pEC86 increased formation of an unknown contaminating heme protein with Soret absorbance maximum at 408 nm (Figure 6).

The problem of low percent recovery of cyt f in the MilliQ fraction after osmotic
shock was troubling. It was observed that when cells are frozen after harvest, lower yields of cyt/f are recovered in the MilliQ fraction (~30% recovery). Cells may be disrupted upon freezing and when osmotic shock is subsequently performed, cyt/f was observed in both the sucrose and MilliQ fractions. In cases where cells were not frozen after harvest, higher yields of cyt/f were recovered in the MilliQ fraction (80-100% recovery), and no cyt/f was observed in the sucrose fraction. Additionally, larger volumes of Milli-Q water (6 L per 30-L fermentor instead of 3 L) helped to improve cyt/f recovery (see Table 2).

**Purification.** Initial extraction of cyt/f from the MilliQ fraction utilized CM or DEAE batch ion exchange columns. DEAE performs better than CM in binding cyt/f in the MilliQ fraction and in reproducibility. Tables 3 and 4 summarize the percent recovery and purity ratio for each fermentation. See Figure 4 for an absorption spectrum of cyt/f after the DEAE batch column.

The next step in purification utilized DEAE ion exchange chromatography. The first six and last four fermentations were pooled and purified using two different DEAE columns. Table 5 summarizes the results.

Further purification of cyt/f was done in two ways: A) Cyt/f recovered off DEAE column #1 (28 mg) was purified first using CM ion exchange chromatography and then gel filtration chromatography on the FPLC. B) Cyt/f recovered off DEAE column #2 (65 mg) was purified first by gel filtration chromatography on the FPLC and then by CM ion exchange chromatography.

Tables 6 and 7 summarize pathway A. A major problem with CM column #1 was that cyt/f bound strongly to column and could not be easily eluted. Percent recovery was low
since cyt $f$ oxidized and denatured on the column. CM column #2 utilized a shorter column and higher salt gradient to overcome these difficulties (Table 6).

The final step in pathway A is gel filtration chromatography. The best fractions of cyt $f$ (as determined by the purity ratio) were pooled and concentrated, loaded onto the gel filtration column, and eluted. Percent recovery is low owing to significant loss of protein upon injection into the sample loop. However, the purity ratio is excellent for future NMR and kinetic measurements (Table 7).

Tables 8 and 9 summarize pathway B. Three samples of cyt $f$ with different purity ratios were obtained from DEAE column #2: "best band", "tails best band", and "poor band". These samples of cyt $f$ were purified separately by gel filtration chromatography. In chromatography runs 2 through 5, 1 mM ascorbate was added to the buffer. Owing to the strong absorbance of ascorbate at 280 nm, purity ratios could not be determined after purification by gel filtration. See Figure 7 for absorption spectrum of cyt $f$ after gel filtration chromatography.

The final step in purification of cyt $f$ following pathway B was CM ion exchange chromatography. Samples of cyt $f$ with the highest purity ratios were pooled and concentrated, loaded onto the CM ion exchange column, and eluted. We obtained 47 mg of cyt $f$ with an excellent purity ratio for future NMR and kinetic experiments. See the Figure 8 for an absorption spectrum of pure cyt $f$.

Throughout the entire purification procedure, an unknown contaminating heme protein with a Soret band at 408 nm was present. Cation (CM and SP) and anion exchange (DEAE and Q) chromatography could not sufficiently separate cyt $f$ from this unknown
protein. In addition, gel filtration could not sufficiently separate these two proteins. The contaminating heme protein eluted right after cyt f. Based on the above results, the molecular weight, charge, and binding properties of these two proteins are very similar. Unfortunately, loss of cyt f during purification in fractions containing the contaminating protein is inevitable. Further characterization of this unknown heme protein should provide clues for more efficient separation from cyt f.

**Conclusions**

A bacterial expression system for the soluble form of turnip cyt f has been achieved. Expression yields before isolation from growing cells are 0.9-1.0 mg/L. However, cyt f yields after extraction from the periplasmic space by osmotic shock are typically 0.6-0.8 mg/L. Cyt f is purified from periplasmic extracts by a combination of ion exchange and gel filtration chromatography. The purification sequence is DEAE Sepharose (batch mode), DEAE Sepharose (pH 6, cyt f reduced), CM Sephadex (pH 6, cyt f reduced), and Superdex G75 (pH 6, cyt f reduced). The final yield from a 30-L culture is 10-12 mg cyt f with an absorbance ratio A554/A277 of 0.95.

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## Tables and Figures

**Table 1.** Fermentor yields and percent recovery of cyt $f$ in MilliQ fractions

<table>
<thead>
<tr>
<th>Date Harvested</th>
<th>Plasmid &amp; Conditions</th>
<th>Growth Time (hours)</th>
<th>Yield Cyt $f$ in Fermentor (mg)</th>
<th>Yield Cyt $f$ in MilliQ Fraction (mg)</th>
<th>% Recovery of Cyt $f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/02/99</td>
<td>pTC1+pEC86 semi-anaerobic</td>
<td>34</td>
<td>15.5</td>
<td>4.7</td>
<td>30.4</td>
</tr>
<tr>
<td>10/06/99</td>
<td>pTC1+pEC86 semi-anaerobic</td>
<td>33</td>
<td>19.2</td>
<td>6.2</td>
<td>32.3</td>
</tr>
<tr>
<td>10/08/99</td>
<td>pTC1+pEC86 aerobic</td>
<td>31</td>
<td>15.3</td>
<td>3.2</td>
<td>20.9</td>
</tr>
<tr>
<td>10/12/99</td>
<td>pTC1+pEC86 aerobic</td>
<td>30</td>
<td>13.2</td>
<td>3.6</td>
<td>27.1</td>
</tr>
<tr>
<td>10/15/99</td>
<td>pTC1 semi-anaerobic</td>
<td>32</td>
<td>12.4</td>
<td>6.1</td>
<td>72.0</td>
</tr>
<tr>
<td>10/22/99</td>
<td>pTC1 semi-anaerobic</td>
<td>45</td>
<td>20.0</td>
<td>16.4</td>
<td>81.9</td>
</tr>
<tr>
<td>10/25/99</td>
<td>pTC1 semi-anaerobic</td>
<td>71</td>
<td>20.2</td>
<td>20.1</td>
<td>99.4</td>
</tr>
<tr>
<td>10/29/99</td>
<td>pTC1 semi-anaerobic</td>
<td>68</td>
<td>29.3</td>
<td>24.6</td>
<td>84.0</td>
</tr>
<tr>
<td>11/02/99</td>
<td>pTC1 semi-anaerobic</td>
<td>74</td>
<td>29.0</td>
<td>26.5</td>
<td>91.4</td>
</tr>
<tr>
<td>11/05/99</td>
<td>pTC1 semi-anaerobic</td>
<td>71</td>
<td>23.7</td>
<td>25.9</td>
<td>109.2</td>
</tr>
<tr>
<td>11/08/99</td>
<td>pTC1 semi-anaerobic</td>
<td>73</td>
<td>21.8</td>
<td>10.0</td>
<td>45.9</td>
</tr>
</tbody>
</table>
Table 2. Osmotic shock conditions and yields of cyt $f$ in MilliQ Fractions

<table>
<thead>
<tr>
<th>Date Harvested</th>
<th>Plasmid</th>
<th>MilliQ Used (L)</th>
<th>Yield Cyt $f$ (mg)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/02/99</td>
<td>pTCl+pEC86</td>
<td>1</td>
<td>1.3</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.3</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2.2</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td>10/06/99</td>
<td>pTCl+pEC86</td>
<td>1</td>
<td>2.4</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2.0</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.9</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td>10/08/99</td>
<td>pTCl+pEC86</td>
<td>1</td>
<td>1.2</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.0</td>
<td>2/3 pellet frozen –80°C</td>
</tr>
<tr>
<td>10/12/99</td>
<td>pTCl+pEC86</td>
<td>2</td>
<td>3.1</td>
<td>2/3 pellet not frozen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.5</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td>10/15/99</td>
<td>pTCl</td>
<td>2</td>
<td>2.4</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.9</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.8</td>
<td>1/3 pellet frozen –80°C</td>
</tr>
<tr>
<td>10/22/99</td>
<td>pTCl</td>
<td>4</td>
<td>11.1</td>
<td>2/3 pellet not frozen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5.3</td>
<td>1/3 pellet frozen –20°C</td>
</tr>
<tr>
<td>10/25/99</td>
<td>pTCl</td>
<td>6</td>
<td>20.1</td>
<td>3/3 pellet not frozen</td>
</tr>
<tr>
<td>10/29/99</td>
<td>pTCl</td>
<td>6</td>
<td>24.6</td>
<td>3/3 pellet not frozen</td>
</tr>
<tr>
<td>11/01/99</td>
<td>pTCl</td>
<td>6</td>
<td>26.5</td>
<td>3/3 pellet not frozen</td>
</tr>
<tr>
<td>11/05/99</td>
<td>pTCl</td>
<td>6</td>
<td>25.9</td>
<td>3/3 pellet not frozen</td>
</tr>
<tr>
<td>11/08/99</td>
<td>pTCl</td>
<td>6</td>
<td>10.0</td>
<td>3/3 pellet not frozen</td>
</tr>
</tbody>
</table>
Table 3. Percent recovery of cyt f from MilliQ fraction after an initial DEAE batch column
(first six fermentation results combined)

<table>
<thead>
<tr>
<th>Date Harvested</th>
<th>Plasmid/Conditions</th>
<th>Cyt f added to columns (mg)</th>
<th>Cyt f eluted from columns (mg)</th>
<th>Purity Ratio A₅₅₄/A₂₇₇</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six Earlier Fermentors</td>
<td>pTC1 + pEC86</td>
<td>40.2</td>
<td>31.5</td>
<td></td>
<td>78.4</td>
</tr>
</tbody>
</table>

Table 4. Percent recovery of cyt f from MilliQ fraction after an initial DEAE batch column
(last 4 fermentations separately)

<table>
<thead>
<tr>
<th>Date Harvested</th>
<th>Plasmid/Conditions</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio A₅₅₄/A₂₇₇</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/25</td>
<td>PTC1 semi-anaerobic</td>
<td>20.1</td>
<td>18.0</td>
<td></td>
<td>89.6</td>
</tr>
<tr>
<td>10/29</td>
<td>PTC1 semi-anaerobic</td>
<td>24.6</td>
<td>19.6</td>
<td></td>
<td>79.7</td>
</tr>
<tr>
<td>11/02</td>
<td>PTC1 semi-anaerobic</td>
<td>26.5</td>
<td>22.7</td>
<td>0.03</td>
<td>85.7</td>
</tr>
<tr>
<td>11/05</td>
<td>PTC1 semi-anaerobic</td>
<td>25.9</td>
<td>20.7</td>
<td>0.02</td>
<td>79.9</td>
</tr>
</tbody>
</table>
Table 5. Percent recovery of cyt f after a proper DEAE column. The first six and last four fermentations were pooled and loaded onto separate columns.

<table>
<thead>
<tr>
<th>DEAE column</th>
<th>Cyt f Fermentations</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio $A_{554}/A_{277}$</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>First 6</td>
<td>31.5</td>
<td>27.8</td>
<td>0.2</td>
<td>88.3</td>
</tr>
<tr>
<td>2</td>
<td>Last 4</td>
<td>81</td>
<td>64.8</td>
<td>0.1 - 0.3</td>
<td>80.0</td>
</tr>
</tbody>
</table>

4.6 tails best band

8.4 poor band

0.05 - 0.08

0.02 - 0.03

Table 6. Percent recovery of cyt f from pathway A after a CM column.

<table>
<thead>
<tr>
<th>CM column</th>
<th>Cyt f Fermentations</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio $A_{554}/A_{277}$</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>First 6</td>
<td>27.8</td>
<td>11.7</td>
<td>0.15</td>
<td>42.1</td>
</tr>
<tr>
<td>2</td>
<td>First 6</td>
<td>11.7</td>
<td>9.0</td>
<td>0.4</td>
<td>76.9</td>
</tr>
</tbody>
</table>

9.0 best band
Table 7. Percent recovery of cyt f from pathway A after a final gel filtration column.

<table>
<thead>
<tr>
<th>Gel Filtration</th>
<th>Cyt f</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio A_{554}/A_{277}</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 mL best band</td>
<td>9.0</td>
<td>1.8</td>
<td>0.9 - 1.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Table 8. Percent recovery of cyt f from pathway B after a gel filtration column.

<table>
<thead>
<tr>
<th>Gel Filtration</th>
<th>Cyt f</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio A_{554}/A_{277}</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 mL best band</td>
<td>19.9</td>
<td>15.3</td>
<td>0.3 - 0.4</td>
<td>76.9</td>
</tr>
<tr>
<td>2</td>
<td>2 mL best band</td>
<td>26.9</td>
<td>21.9</td>
<td>Unk</td>
<td>81.4</td>
</tr>
<tr>
<td>3</td>
<td>1 mL best band + 1 mL tails of best band</td>
<td>15.6</td>
<td>11.8</td>
<td>Unk</td>
<td>75.6</td>
</tr>
<tr>
<td>4</td>
<td>2 mL tails best band</td>
<td>11.8</td>
<td>8.6</td>
<td>Unk</td>
<td>72.9</td>
</tr>
<tr>
<td>5</td>
<td>2 mL poor band</td>
<td>8.4</td>
<td>0.0</td>
<td>Unk</td>
<td>0.0</td>
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</table>
Table 9. Percent recovery of cyt f from pathway B after a final CM column.

<table>
<thead>
<tr>
<th>CM column</th>
<th>Cyt f</th>
<th>Cyt f added to column (mg)</th>
<th>Cyt f eluted from column (mg)</th>
<th>Purity Ratio $A_{554}/A_{277}$</th>
<th>% Recovery of Cyt f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>From gel filtration</td>
<td>57.6</td>
<td>47.0</td>
<td>0.87 - 0.95</td>
<td>81.5</td>
</tr>
</tbody>
</table>

2.4 0.4
Figure 1. Plasmid pTC1 containing the gene encoding the truncated soluble form of cyt f.
Figure 2. Semi-anaerobic fermentation conditions (pH, temperature, agitation rate, and dissolved oxygen) versus growth time for expression of cyt f in E. coli.
Figure 3. Absorption spectrum of cyt $f$ in MilliQ fraction after osmotic shock.
Figure 4. Absorption spectrum of cyt f after DEAE batch column.
Figure 5. Expression yields of cyt f in culture as growth time increases.
Figure 6. Absorption spectrum of MilliQ fraction after osmotic shock. Aerobic expression of plasmids pTC1 and pEC86 showing increased presence of unknown contaminating heme protein with Soret band at 408 nm.
Figure 7. Absorption spectrum of cyt f after gel filtration and before final CM column.
Figure 8. Absorption spectrum of pure turnip cyt f.
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