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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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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_{low}, F_{high}, MG, U_{acid}, and U_{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

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presumed intermediates in the folding of denatured proteins and seem to be implicated in diseases caused by protein misfolding.[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[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 His18 and Met80 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.[8]

At least five distinct forms of iron cyochrome *c*, dependent on pH, have been observed spectroscopically.[29−31] At neutral pH, the folded low-spin form with Met80 and His18 as ligands dominates. At pH above 8−10, the dominant species is the low-spin so-called alkaline conformation, in which Met80 is displaced from iron by a lysine residue.[32,33] In the acid-unfolded form at pH 2, a high-spin electron configuration indicates that neither Met80 nor His18 remains coordinated to iron.[34,35] At pH 2 in the presence of stabilizing anions, a partially folded form can be obtained.[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.[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_{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 α-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 helices 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_{acid}, and U_{urea} forms of iron

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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.[63−71] 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.[72−74] 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 the 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 ^{3}Zncyt, 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. 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.[69]

$$^3\text{Zncyt} + \text{Q} \rightarrow \text{Zncyt}^+ + \text{Q}^- \quad (1)$$

$$\text{Zncyt}^+ + \text{Q}^- \rightarrow \text{Zncyt} + \text{Q} \quad (2)$$

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 ^{3}Zncyt 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, $[Co(phen)_3](ClO_4)_3 \cdot 2H_2O$, and the corresponding chloride salt, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 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 $Co(phen)_3^{3+}$ concentration was determined on the basis of the molar absorptivity: $\epsilon = 3.60 \times 10^3$ M^{-1} 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: $\epsilon = 133$ M^{-1} 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 $K_3[Fe(CN)_6]$, 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 modification[70,74] 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: $\epsilon = 243$ mM^{-1} cm^{-1} at 423 nm.[80] The metal-free form of cytochrome *c* is porphyrin cytochrome *c*, designated H_2cyt. It was prepared by a standard procedure.[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, μ, 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[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_{low} and F_{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_{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 with 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

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avoided, because the absorption spectrum is characteristic of H_2-cyt. The urea-unfolded form, designated U_{urea}, was obtained by dissolving Zncyt in a solution of 8.0 M urea in 10 mM phosphate buffer at pH 7.00 and μ = 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 μM Zncyt were obtained with a Jasco instrument, model J-710. A 1-mm quartz cell was used for the F_{low}, F_{high}, MG, and U_{acid} forms, but owing to the strong absorbance of urea, a 0.1-mm quartz cell was necessary for the U_{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 ± 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 $[\theta] = 100\theta_{obs}(cl)^{-1}$, where θ_{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_{low}, F_{high}, MG, U_{acid}, and U_{urea} forms were recorded with a Perkin-Elmer Lambda 18 spectrophotometer. To determine the stability of protein toward demetalation, 5.0 μM 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 (ΔG_U) using eq 3:

$$\Delta G_U = -RT \ln\left(\frac{y - y_F}{y_U - y}\right) \quad (3)$$

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:

$$\Delta G_U = \Delta G_U(H_2O) - m[\text{denaturant}] \quad (4)$$

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 ΔG_U-(H_2O) on denaturant.[81,83]

Laser Flash Photolysis. Transient absorption of the triplet state, ^{3}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 aperature. 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}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 ^{3}Zncyt 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 ^{3}Zncyt 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 min after each addition of quencher or until the rate constant for natural decay of ^{3}Zncyt 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, ^{3}Zncyt, depended on the excitation power and was ca. 0.3−0.5 μM. The mole ratio of quencher to ^{3}Zncyt 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 k_{on}/k_{off} is the association constant K_A

$$D + A \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} DA \xrightarrow{k_f} D^+A^- \quad (5)$$

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] ≫ [D], eq 6 is obtained. In the limiting case when $k_{off} > k_{on}[A] + k_f$, eq 6 yields eq 7.

$$k_{obs} = \frac{k_f k_{on}[A]}{k_{on}[A] + k_{off} + k_f} \quad (6)$$

$$k_{obs} = K_A k_f [A] = k_{bim}[A] \quad (7)$$

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}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}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

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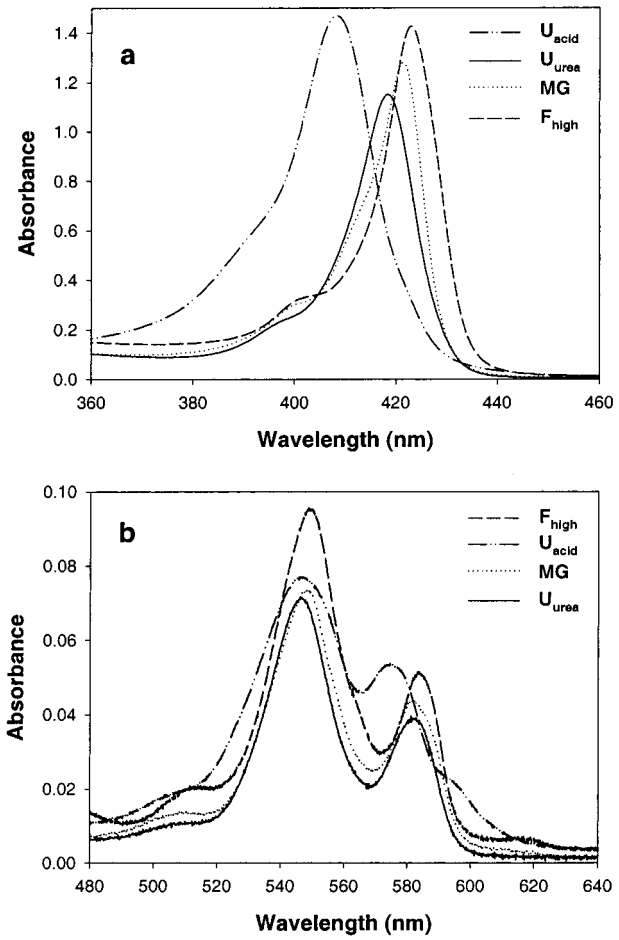

Figure 1. Absorption spectra in the (a) Soret region and (b) *Q*-band region of the folded form at high ionic strength (F_{high}), molten-globule (MG) form, and the forms unfolded by acid (U_{acid}) and by urea (U_{urea}) of 3.0 μM zinc cytochrome *c*. The absorption spectrum of the folded form at low 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 $\mu = 20$ mM; F_{high}, at pH 7.00 and $\mu = 1.500$ M; MG, at pH 2.0 and $\mu = 1.500$ M; U_{acid}, at pH 2.0 and $\mu = 20$ mM; and U_{urea}, at pH 7.00, $\mu = 20$ mM, and 8.0 M urea.

quenching rate constant k_q is obtained using eq 8.

$$k_q = k_{obs} - k_d \tag{8}$$

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 the 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 α-helical content, indicating similar backbone conformations. These are the same as those recently reported[74,86] and also very similar to those reported for native (iron-containing) cyt(III).[54] 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)[38,54] and Zncyt[86] 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 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 remains a minor one.

$$^3\text{Zncyt} \xrightarrow{k_d} \text{Zncyt} \tag{9}$$

$$\Delta A_{458nm} = a_1 \exp(-k_d t) + b \tag{10}$$

$$\Delta A_{458nm} = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t) + b \tag{11}$$

Its rate constants (k_2) and amplitudes (a_2) are 710 ± 90 s^{-1} and 10%−20% for the MG form and 1230 ± 250 s^{-1} and 20%−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, $k_1 = 1200 \pm 220$ s^{-1} and $a_1 = 82 \pm 6\%$; for the minor phase, $k_2 = 280 \pm 80$ s^{-1} and $a_2 = 18 \pm 6\%$.

The k_d values in Table 1 are those for the major phase. Our kinetic studies of electron-transfer reactions were done

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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)

				absorption maxima, λ_{max} (nm)					molar ellipticity $-[\theta]_{222}$	α-helical	natural decay
protein	form[a]	pH	μ (mM)	Soret band		*Q* bands			(deg cm^2 dmol^{-1})	content (%)	k_d (s^{-1})
Zncyt	F_{low}	7.00	20	423	549	585			10300	28.0	70 ± 4
	F_{high}	7.00	1500	423	549	585			11150	31.6	63 ± 3
	MG	2.0	1500	421	548	582			10530	28.1	190 ± 20
	U_{acid}	2.0	20	407	547	575			4120	16.4	440 ± 50
	U_{urea}	7.00	20	418	546	581			1880	4.4	1200 ± 220
H_2cyt	F_{high}	7.00	1500	404	506	540	568	620	11070	32.6	1350 ± 70
	MG	2.0	1500	405	506	538	559				
	U_{acid}	2.0	20	406	552	594					
	U_{urea}	7.00	20	400	504	540	565	616			

[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.

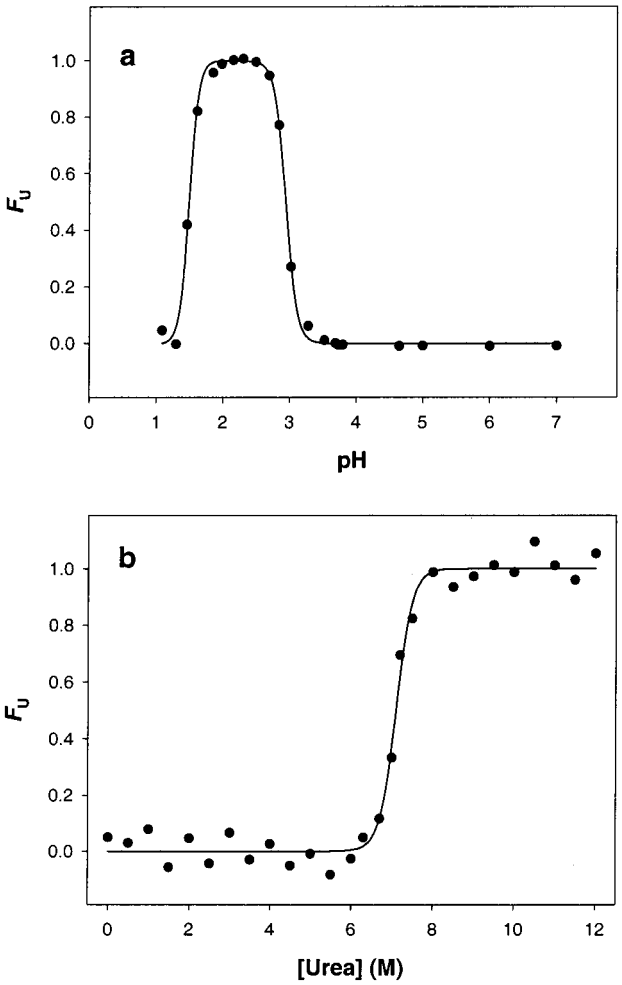

Figure 2. (a) Equilibrium unfolding of 5.0 μM zinc cytochrome *c* at 20 °C, induced by HCl and monitored by Soret absorption, at 410 nm. Unbuffered aqueous protein solutions with pH adjusted from 7.0 to 1.1 are prepared by addition of dilute HCl or NaOH. The line is a fitting to four-parameter sigmoidal 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 ± 0.02. (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 (ΔG_U) are calculated using eq 3. See Table 2 for the resulting thermodynamic parameters.

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

protein	$\Delta G_U(H_2O)$ (kJ mol^{-1})	*m* (kJ mol^{-1} M^{-1})	C_m (M)
Zncyt[a]	47.2 ± 3.3	6.6 ± 0.5	7.1 ± 0.1
cyt(III)[b]	37.5	5.0	7.5
H_2cyt[b]	6.1	3.6	1.7

[a] This work. [b] From ref 83; estimated errors are, at most, ±10%.

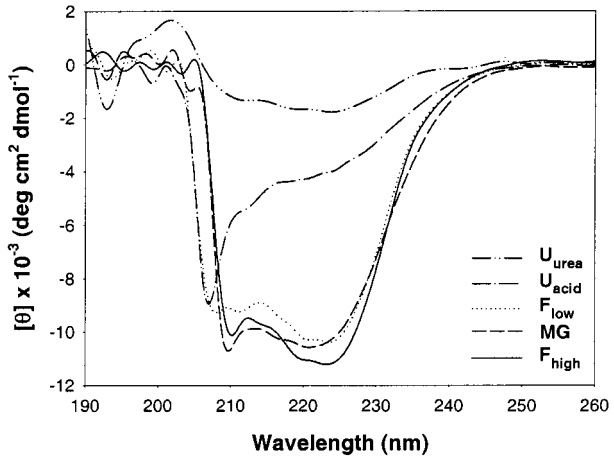

Figure 3. Circular dichroism spectra in the far-UV region of the folded forms at low (F_{low}) and high (F_{high}) ionic strength, molten-globule (MG) form, and the forms unfolded by acid (U_{acid}) and by urea (U_{urea}) of 10.0 μM zinc cytochrome *c*. See Figure 1 for experimental conditions.

with fresh samples, for which (except for the U_{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_{high} form into the MG form and of the F_{low} form into the U_{acid} form was followed over 36 h. Attempts to reverse these processes and gradually, again over 36 h, reform the F_{high} form from the MG form and the F_{low} form from the U_{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

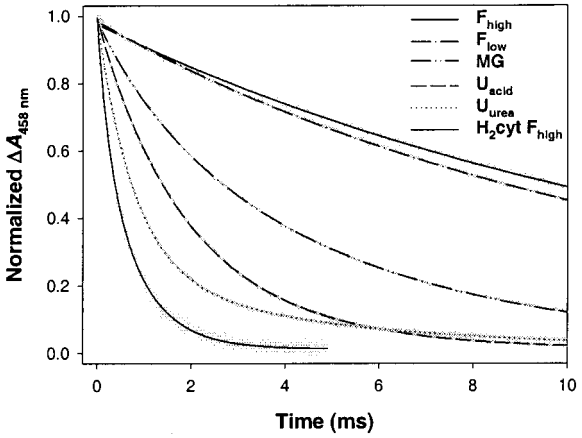

Figure 4. Natural decay (in the absence of quencher) of the triplet states of zinc cytochrome *c* (designated ^{3}Zncyt, five traces) and metal-free porphyrin cytochrome *c* (designated $^3H_2$cyt, one trace), in the folded forms at low (F_{low}) and high (F_{high}) ionic strength, molten-globule (MG) form, and the forms unfolded by acid (U_{acid}) and by urea (U_{urea}) of 10.0 μM zinc cytochrome *c* or 40.0 μM metal-free porphyrin cytochome *c*. The lines are fittings to eq 10. The upper traces, truncated to the time scale of the figure, continue to decline exponentially. See Figure 1 for experimental conditions.

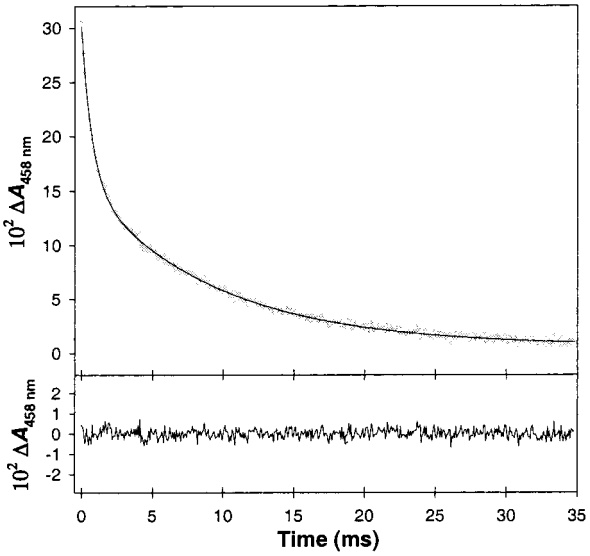

Figure 5. Natural decay (in the absence of quencher) of the triplet state of zinc cytochrome *c* that was left for several hours in the U_{acid} form (at pH 2.0 and $\mu = 20$ mM) before being returned by ultrafiltration to pH 7.00 and $\mu = 20$ mM. In this extreme case of the aged protein sample, fittings 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.

the metal-free porphyrin cytochrome *c*, designated H_2cyt.[78] Circular dichroism spectra shown in Figure S2 in the Supporting Information showed recovery of nativelike secondary structure in the F_{high} and F_{low} forms of H_2cyt. When, however, ultrafiltration was done rapidly, the MG form did return to the F_{high} form and the U_{acid} form to the F_{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_{acid} form into the MG form was effected by adding NaCl to the solution and monitored by measuring ellipticity [θ] 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

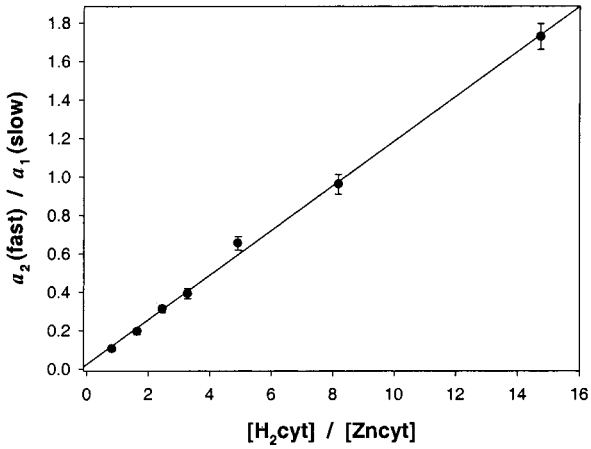

Figure 6. Ratio of amplitudes a_2 (for the fast phase) and a_1 (for the slow phase) depends on the ratio of molar concentrations of the two sensitizers. Changes of transient absorbance with time upon laser excitation of solutions containing 10.0 μM zinc cytochrome *c* (Zncyt) and increasing concentrations of metal-free porphyrin cytochrome *c* (H_2cyt) 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.

Scheme 1

$$2\,H^+ + \text{Zncyt} \xrightarrow{\text{slow}} H_2\text{cyt} + Zn^{2+}$$

$$\text{Zncyt} \xrightarrow{h\nu} {}^3\text{Zncyt} \xrightarrow{k_1\,(\text{slow})} \text{Zncyt}$$

$$H_2\text{cyt} \xrightarrow{h\nu} {}^3H_2\text{cyt} \xrightarrow{k_2\,(\text{fast})} H_2\text{cyt}$$

(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 H_2cyt by Zinc(II) Ions. When the MG and U_{acid} 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 $^3H_2$cyt, in the absence of quenchers was measured (see Table 1). The value of k_d ($1350 \pm 70\ s^{-1}$) determined for this H_2cyt sample is identical to the value of k_2 ($1353 \pm 30\ s^{-1}$) 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 H_2cyt concentration as the zinc(II) porphyrin is demetalated and to ensure that the appearance of H_2cyt will have no effect on the rate constant for natural decay of ^{3}Zncyt, increasing concentrations of H_2-cyt were added to a 10.0 μM solution of Zncyt for determination of k_d. Fitting of the kinetic traces to eq 11 yielded the rate constants k_1 and k_2 and the amplitudes a_1 and a_2 for the two phases. A plot of k_q versus increasing concentration of H_2cyt (data not shown) shows a very slight dependence of k_1 (slope = 0.23, $79\ s^{-1} \le k_1 \le 105\ s^{-1}$) and no dependence of k_2 (slope = 0.002, $k_2 = 1440 \pm 50\ s^{-1}$) on H_2cyt concentration. Figure 6 shows that the faster phase, which arises over several hours, is indeed due to formation of H_2cyt (see Scheme 1). The molecules Zncyt and H_2cyt quench each other's triplet states slightly, if at all. Indeed,

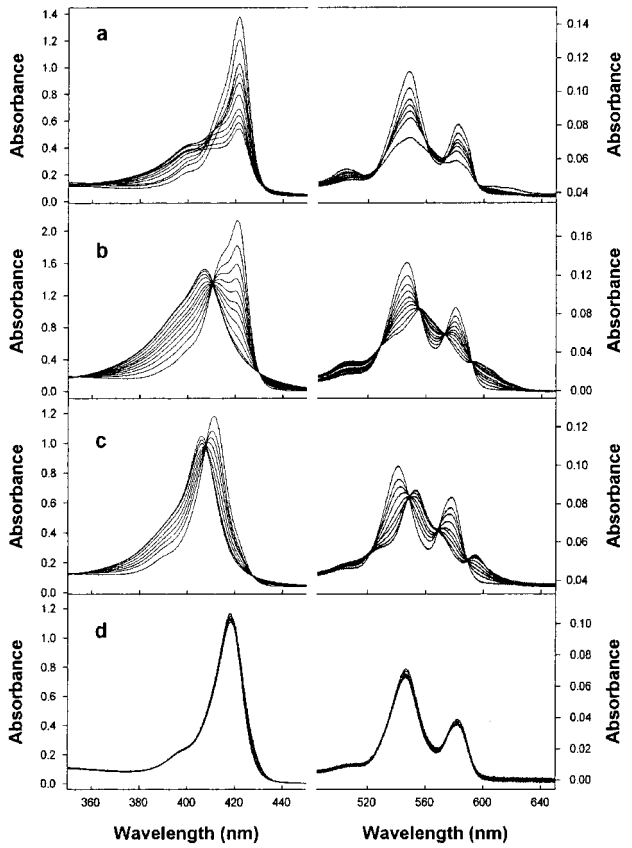

Figure 7. Absorption spectra in the Soret and *Q*-band regions of the molten-globule (MG) form and the forms unfolded by acid (U_{acid}) and by urea (U_{urea}) of zinc cytochrome *c* monitored over time. Conditions: (a) 4.5 μM Zncyt (MG) at pH 2.0 and $\mu = 1.500$ M over a period of 31 h; (b) 10.0 μM Zncyt (MG) at pH 2.0 and $\mu = 0.500$ M over 12 h; (c) 4.5 μM Zncyt (U_{acid}) at pH 2.0 and $\mu = 20$ mM over 18 h; and (d) 5.0 μM Zncyt (U_{urea}) at pH 7.00, $\mu = 20$ mM, and 8.0 M urea over 9 h. Note the break in the wavelength axis and the different absorbance scales.

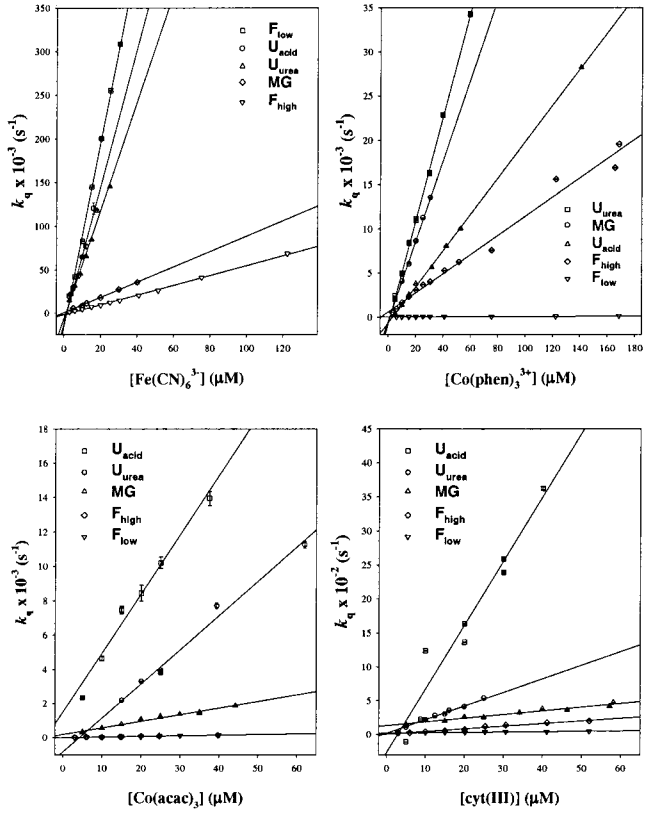

Figure 8. Dependence on quencher concentration of the quenching rate constant k_q obtained using eq 8 for the reactions at 25 °C of the triplet state of zinc cytochrome *c* in the folded forms at ionic strengths of 20 mM (F_{low}) and 1.500 M (F_{high}), molten-globule (MG) form, and forms unfolded by acid (U_{acid}) and by urea (U_{urea}) with the four quenchers shown. The solid lines are obtained from linear least-squares regression; their slopes yield the bimolecular rate constants k_{bim}, given in Table 3. Invisible error bars are smaller than the symbols. 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.

lack of a transient signal at 676 nm rules out the presence of $Zncyt^+$, the intermediate that would have arisen in redox quenching of ^{3}Zncyt by H_2cyt. 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 H_2cyt 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 H_2cyt 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 ^{3}Zncyt 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_{low}, F_{high}, MG, U_{acid}, and U_{urea} forms. The probes of ^{3}Zncyt reactivity were the following three inorganic and one protein electron acceptors: anionic $Fe(CN)_6^{3-}$, cationic $Co(phen)_3^{3+}$, electroneutral $Co(acac)_3$, and cationic cyt(III). This native protein was chosen as a probe because 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,

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 (μ)

	conditions		k_{bim} x 10^{-6}, M^{-1} s^{-1} at 25 °C			
form[a]	pH	μ (mM)	$Fe(CN)_6^{3-}$	$Co(phen)_3^{3+}$	$Co(acac)_3$	cyt(III)
F_{low}	7.00	20	10700 ± 100	0.44 ± 0.03	3.7 ± 0.1	0.56 ± 0.07
F_{high}	7.00	1500	543 ± 11	108 ± 4	3.8 ± 0.1	3.9 ± 0.2
MG	2.0	1500	882 ± 17	443 ± 11	39 ± 1	5.5 ± 0.4
U_{acid}	2.0	20	8020 ± 590	205 ± 2	230 ± 11	20 ± 1
U_{urea}	7.00	20	6260 ± 350	591 ± 5	200 ± 9	94 ± 11

[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.

but reproducible, increases occur upon addition of $Co(phen)_3^{3+}$ and cyt(III) at low ionic strength. Evidently, electrostatic repulsions limit collisional quenching between two cationic species: the F_{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_{low} and F_{high} forms, decay of the triplet state, ^{3}Zncyt, remained monoexponential upon addition of all four quenchers. In the MG, U_{acid}, and U_{urea} forms, however, the decay was biexponential. Typically, the faster phase accounted for only ca. 15%−20% of the total amplitude. In the U_{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,[72,74,83] but partially and completely unfolded forms of Zncyt have not yet been fully characterized.[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.[88] Reduction potentials of the F_{high} (+255 mV) and MG (+233 mV) forms of cyt(III) are similar,[89] but that of the forms unfolded by guanidine hydrochloride[90] or urea[91] (ca. −150 mV) is much lower and close to the potential (ca. −200 mV) of bis-(imidazole) iron(III) porphyrin in aqueous solution.[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_{low} (pH 5, low salt concentration) form and five-coordinate (with Met80 detached) in the MG (pH 2.0, 1.00 M $NaClO_4$) form.[86] Reconsideration[74] of NMR spectra[73] suggested that even the F_{low} form of Zncyt is five-coordinate, that is, that Met80 is not coordinated. Similarity of the absorption spectra for the F_{low} 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 U_{acid} form and of the reported Zncyt in the MG form induced by ClO_4^- are similar.[86] In cyt(III), small anions (such as Cl^-, Br^-, and NO_3^-) induce formation of a compact, highly stuctured MG form, restoring the native Fe(III)−Met80 bond and nativelike redox properties. Large anions (such as I^-, ClO_4^-, and CCl_3COO^-) 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 ClO_4^- 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 (U_{acid} and U_{urea}) forms. The metal ion, its oxidation state, and its axial ligand(s) in cytochrome *c* affect the free energy of unfolding in aqueous solution, $\Delta G_U(H_2O)$. 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) toward unfolding.[95] Values of $\Delta G_U(H_2O)$ estimated by urea-induced unfolding follow the trend

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cyt(II) > Zncyt ≈ cyt(III) ≫ H_2cyt.[96] We chose the conditions for the U_{acid} and U_{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_{low}, F_{high}, and MG forms have almost the same conformation.[54,72,74,86] Marked loss of ellipticity at 222 nm for Zncyt in the U_{acid} and U_{urea} forms indicates lack of secondary structure, as with iron cytochrome *c* in U_{acid} and U_{urea} forms.[38,54]

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_{high} < F_{low} < MG < U_{acid} < U_{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.[97] 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,[34,35] this rate constant increases greatly. Because the rate constants for natural decay of ^{3}Zncyt in the U_{acid} and U_{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_{low}, F_{high}, MG, U_{acid}, and U_{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)folded 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.[9] 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_{bim} 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 20-fold at the high ionic strength.

Comparison of ^{3}Zncyt reactivity in the MG (μ = 1500 mM) and U_{acid} (μ = 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_{bim} 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_{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 their 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}Zncyt; indeed, this reaction is independent of ionic strength. Reactivity of ^{3}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_{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

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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} forms (5 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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