Reductive Quenching of the Triplet State of Zinc Cytochrome *c* by the Hexacyanoferrate(II) Anion and by Conjugate Bases of Ethylenediaminetetraacetic Acid

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The long-lived triplet state of zinc cytochrome *c*, designated ^{3}Zn(cyt), has often been used as a reductant, in oxidative-quenching reactions. This article seems to be the first report of the use of ^{3}Zn(cyt) as an oxidant, in two reductive-quenching reactions. Conjugate bases (anions) of ethylenediaminetetraacetic acid (EDTA) quench ^{3}Zn(cyt) at pH 6.5 with the observed rate constant that is 2 times greater than the rate constant for natural decay of this excited state. Electrostatic attraction between these quenchers and Zn(cyt) is a necessary but not sufficient condition for quenching. A transient species observed at 690 nm has the absorbance and the time profile expected of the anion radical Zn(cyt)$^-$. Detection of this species is possible because of the rapid decomposition of EDTA upon oxidation. The complex $[Fe(CN)_6]^{4-}$ quenches ^{3}Zn(cyt) at pH 7.0 with the rate constant of $(1.5 \pm 0.3) \times 10^8$ M^{-1} s^{-1}. This fast quenching is not caused by electrostatic association of ^{3}Zn(cyt) and $[Fe(CN)_6]^{4-}$ nor by energy transfer from the former to the latter. The complex $[Fe(CN)_6]^{4-}$ is not detectably contaminated by the similar complex $[Fe(CN)_6]^{3-}$, which might act as an oxidative quencher. The evidence supports reductive quenching of ^{3}Zn(cyt) by the $[Fe(CN)_6]^{4-}$ ion. The anion radical Zn(cyt)$^-$ is not detected, probably because it is rapidly consumed in the back-reaction with the $[Fe(CN)_6]^{3-}$ ion. To act as reductive quenchers for ^{3}Zn(cyt), chemicals must have favorable electrostatic properties, redox potential, and reactivity. These requirements are discussed so that further studies of this new reaction may be possible.

Introduction

Various metalloproteins act as electron carriers and redox enzymes in photosynthesis, respiration, nitrogen fixation, nonmetal metabolism, DNA biosynthesis, DNA repair, detoxification of various compounds, and other biological processes. To understand biological functions of metalloproteins, one must understand their chemical reactivity. Despite vigorous current research,[1−6] molecular mechanisms of electron-transfer reactions of metalloproteins are only partially understood.

Much of this chemical research has involved heme proteins, such as myoglobins, hemoglobins, and cytochromes *c*. The proteins of the last family are prototypical electron carriers. Their oxidoreduction reactions with various chemical and biochemical agents have been much studied, and their three-dimensional structures are known in detail.[7]

Almost all metals and metalloids form porphyrin complexes. Because heme is a kind of porphyrin, some of this diversity has touched the proteins.[8] Derivatives of myoglobin,[9−16] hemoglobin,[17−25] and cytochrome *c*[25−43] containing various metals in the place of iron have been prepared and characterized. Owing to their interesting spectroscopic, photophysical, and photochemical properties, these so-called reconstituted proteins have proven very useful. Cytochrome *c* noninvasively recon-

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stituted with zinc(II), called simply zinc cytochrome *c* and designated Zn(cyt),[36−39] has been widely used in determination of interprotein distances[41−43] and in kinetic studies of electron-transfer reactions.[44−55] 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 ^{3}Zn(cyt). Because its lifetime is unusually long—between 7 and 15 ms, depending on preparation and experimental conditions[56−60]—electron-transfer reactions of the triplet state can be studied relatively easily.

All of the previous studies known to us have dealt with *oxidative* quenching by various quenchers, designated Q. Among them are organic compounds,[39] transition-metal complexes, and metalloproteins.[44−55] The products of these reactions, represented by eq 1, are zinc cytochrome *c* cation radical,

$$^3\mathrm{Zn(cyt)} + \mathrm{Q} \rightarrow \mathrm{Zn(cyt)}^+ + \mathrm{Q}^- \qquad (1)$$

designated Zn(cyt)$^+$, and the reduced form of the quencher, designated Q$^-$. This study deals with *reductive* quenching, represented by eq 2, products of which are zinc cytochrome *c*

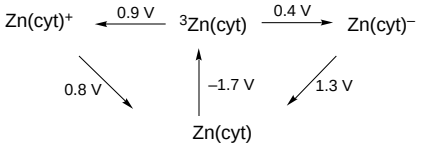
$$^3\mathrm{Zn(cyt)} + \mathrm{Q} \rightarrow \mathrm{Zn(cyt)}^- + \mathrm{Q}^+ \qquad (2)$$

anion radical, designated Zn(cyt)$^-$, and the oxidized form of the quencher, designated Q$^+$. To our knowledge, this is the first investigation of the reaction in eq 2. It is needed for a better understanding of electron-transfer reactions of zinc cytochrome *c*.

Experimental Procedures

Chemicals. Distilled water was further demineralized and purified to a resistance greater than 15 MΩ·cm. Horse-heart cytochrome *c* was obtained from Sigma Chemical Co. Iron was removed, the free-base protein purified, and zinc(II) ions inserted according to published procedures,[36,38] as quickly as possible. Zinc cytochrome *c* was always handled in the dark. Glutathione and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma. Trimethanolamine, trimethylamine, and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate (designated CDTA)[61] were obtained from Aldrich Chemical Co. The complex salt $[Ru(NH_3)_6]Cl_2$ was obtained from Alfa Products, Inc. Disodium salt of ethylenediaminetetraacetic acid (Na_2-EDTA), $K_4[Fe(CN)_6]$, and all the other chemicals were obtained from Fischer Chemical Co. All chemicals were of reagent grade. They were used as received, unless stated otherwise. The compounds [Fe(CDTA)],[61] $K_3[Co(CN)_6]$,[62] and methylviologen monocation radical,[63] designated MV$^+$, were prepared by published procedures.

Scheme 1. Potentials (versus Normal Hydrogen Electrode) for the Redox Half-Reactions (at pH 7.0 and 25 °C) of Zinc Cytochrome *c* That Are Shown by the Arrows



Kinetics. The solvent was always sodium phosphate buffer. Experiments with EDTA were done at pH 6.5, whereas experiments with $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$ were done at pH 7.0. Flash kinetic spectrophotometry, so-called laser flash photolysis, on the microsecond scale was done with a standard apparatus.[53,54] The sample solution in a 10-mm cuvette was thoroughly deaerated by gentle flushing with ultrapure wet argon, obtained from Air Products Co. A Phase-R (now Lumenex) DL1100 laser containing a 50 μM solution of rhodamine 590 in methanol delivered 0.4-μs pulses of excitation light. The monochromatic monitoring beam from a tungsten−halogen lamp was perpendicular to the excitation beam. The absorbance−time curves were analyzed with kinetic software obtained from OLIS, Inc. Each signal was an average of six pulses. Appearance and disappearance of the triplet state, ^{3}Zn(cyt), were monitored at 460 nm, where the transient absorbance is greatest. The concentration of Zn(cyt) was always 10 μM. The concentration of ^{3}Zn(cyt) depended on the excitation power but was always kept well below the concentration of the quencher. The only exceptions were studies of oxidative quenching by the $[Fe(CN)_6]^{3-}$ ion; in these control experiments the concentration of $K_3[Fe(CN)_6]$ was kept below 1.0 μM.

Purity of $K_4[Fe(CN)_6]$. Our method is a modification of the standard one.[64] To a solution of 8.9 g of $K_4[Fe(CN)_6]$ in 100 mL of water were added, in this order, 20 mL of a 0.25 M solution of KI, 2.0 mL of 1.00 M H_2SO_4, 10.0 g of $ZnSO_4 \cdot 7H_2O$, and 2.0 mL of a saturated starch solution; the solvent was always water. Color of the final mixture indicated the purity of the complex salt. Blue color is diagnostic of the $[Fe(CN)_6]^{3-}$ ion.

Results and Discussion

Triplet State, ^{3}Zn(cyt). The excitation by laser of the porphyrin π-system can be considered a promotion of an electron from the HOMO to the LUMO, with the inversion of the electron spin. The natural decay is a return to the ground state. Because the excited (triplet) and the ground (singlet) states differ in spin multiplicity, the lifetime of ^{3}Zn(cyt) is long, i.e., the decay is slow. Our rate constant for the natural decay is 100 ± 10 s^{-1}, consistent with previously published values.[55−60] This rate constant is independent of ionic strength in the entire range studied, from 2.5 mM to 1.00 M.[53]

Redox Potentials and Quenching Modes. Redox potentials for four of the five redox couples shown in Scheme 1 were obtained from the literature or calculated directly from the accepted values in the literature.[39,44] The value of 0.8 V was determined by differential pulse voltammetry.[39] The same value was obtained for zinc tetraphenylporphyrin.[65] This similarity between the heme protein reconstituted with zinc(II) and a

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simple zinc(II) porphyrin[65] shows that the redox potentials are not markedly affected by the protein matter surrounding the zinc(II) porphyrin molecule. The value of 1.7 V in Scheme 1 is the experimental result for the excitation energy.[44] Then the value of 0.9 V follows from the thermodynamic cycle. The value of 1.3 V was determined experimentally for different zinc(II) porphyrins,[65−67] and we used it also for the protein; this method was justified above. Finally, the value of 0.4 V follows from the thermodynamic cycle. All of the values are defined with respect to the normal hydrogen electrode, at pH 7.0 and 25 °C. The number of significant figures reflects our conservative analysis of redox potentials.

By convention, redox potentials should be given as reduction potentials. Consistent adherence to this useful practice would, however, make our discussion confusing. For example, reduction potential of −0.9 V pertains to the half-reaction $Zn(cyt)^+ + e^- \rightarrow {}^3Zn(cyt)$, which we do not study (and which would be difficult to effect in any case). Because we study the reverse of this half-reaction, it is more convenient to use the potential of 0.9 V. *All of the potentials in Scheme 1 correspond to the half-reactions shown by the arrows*, those that we actually study. Because two of them are reductions, their potentials (0.8 and 0.4 V) are true reduction potentials.

The main conclusion from Scheme 1 is that $^3Zn(cyt)$ is both a stronger reductant and a stronger oxidant than Zn(cyt). Electron excitation simultaneously creates a high-lying electron that can be removed relatively easily by an oxidative quencher (as in eq 1) and a low-lying "hole" that can be "filled" relatively easily by a reductive quencher (as in eq 2). The values 0.9 and 0.4 V in Scheme 1 show that, in a thermodynamic sense, $^3Zn(cyt)$ is a stronger reductant than oxidant. This is probably the reason why oxidative quenching of this triplet state is well-known, whereas reductive quenching, to our knowledge, has not been reported.

Optical Absorption of the Anion Radical $Zn(cyt)^-$. We did not find in the literature the optical absorption spectrum of this anion radical and had to infer its likely spectroscopic properties from those of closely related compounds and their ion radicals. This method is validated by the following facts. Ultraviolet−visible absorption spectra of zinc tetraphenylporphyrin and of zinc heme in Zn(cyt) are very similar,[67−69] and the cation radicals of both show an absorption maximum at 675 nm.[70] The triplet states of both zinc 5,10,15,20-tetra(*N*-methylpyridinium-4-yl)porphyrin, designated $[Zn(TMPyP)]^{4+}$, and zinc cytochrome *c* show maximum absorbance at 460 nm.[71a] Zinc porphyrin complexes usually have one axial ligand, i.e., the coordination number of 5. This axial ligand in zinc myoglobin seems to be histidine,[25] as in the native (iron-containing) form of this protein. According to the latest study, by optical and NMR spectroscopic methods, the zinc(II) atom in zinc cytochrome *c* has the coordination number of 6, i.e., two axial ligands—a methionine and a histidine residue.[38] Again, this is the same coordination as in the native (iron-containing) form of cytochrome *c*. Although these two reconstituted heme proteins differ in axial ligation, they have very similar absorption spectra.[13,71b] Evidently, the electronic structure of zinc porphyrin is not grossly perturbed by the substituents in the porphyrin group, the axial ligands, and the surrounding protein matter.

Now we consider the properties of known porphyrin anion radicals. The anion radical of metal-free (so-called free-base) cytochrome *c* was produced by pulse radiolysis; it has a broad absorption band at 700 nm. Photoreduction of zinc porphin by ascorbic acid and photoreduction of zinc tetraphenylporphyrin by benzoin, both in aqueous solution, gave stable species that showed a characteristic absorption band at 620 nm.[68,69] This species, however, is not the porphyrin anion radical but a product of its protonation or of some other chemical transformation.[72] Chemical[68] and electrochemical[67] reductions of zinc tetraphenylporphyrin in nonaqueous solutions produced the anion radical, which showed absorption maxima at 455 and 710 nm. Experiments with pulse radiolysis established that anion radicals of several zinc porphyrins show broad absorption maxima around 700−730 nm.[73−75] Photochemical studies of various zinc porphyrins also showed that the corresponding anion radicals absorb light at 700 nm.[70] For all of these reasons, we expected also the zinc cytochrome *c* anion radical, designated $Zn(cyt)^-$, to show marked absorbance around 700 nm.

Requirements for Reductive Quenching. Because the relevant potential in Scheme 1 is 0.4 V, the reduction potential of the couple Q^+/Q must be lower (i.e., less positive or more negative) than 0.4 V, so that the reaction in eq 2 is spontaneous. Since the anion radical $Zn(cyt)^-$ is a strong reductant—the relevant potential in Scheme 1 is 1.3 V—the back-reaction in eq 3 must be contended with. The condition for its suppression,

$$Zn(cyt)^- + Q^+ \rightarrow Zn(cyt) + Q \qquad (3)$$

that the reductant Q have the reduction potential lower (more negative) than −1.3 V, cannot be met in aqueous solution. Because the undesirable reaction in eq 3 has a large thermodynamic driving force and is likely to be fast, the anion radical $Zn(cyt)^-$ can be detected only as a transient intermediate, if at all.

In addition to the thermodynamic requirements outlined above, there must also exist kinetic requirements for the occurrence and detection of reductive quenching in eq 2. We learned about these requirements in a series of unsuccessful attempts at reductive quenching of $^3Zn(cyt)$. These experiments are worth mentioning, briefly. The parenthetical values that follow are reduction potentials at pH 7.0 for the redox couples, the reductant member of which is given.[76]

Glutathione (−0.23 V) and NADPH (−0.32 V) failed at the concentration of 1.0 mM, presumably because their redox reactions involve transfer of atoms as well as electrons; the rate constant for reductive quenching by both reagents is less than 1×10^5 M^{-1} s^{-1}. The iron(II) complex [Fe(CDTA)] (0.090 V)[61] and the methylviologen monocation radical (−0.40 V), used at the concentration of 10 μM, proved unstable. Even though we observed quenching of $^3Zn(cyt)$ in experiments with these two reagents, we could not be sure that it was due entirely to the reduction, as in eq 2. Even partial autooxidation of these reagents is intolerable, because their oxidized forms could effect the reaction in eq 1. Ferromyoglobin (0.047 V) at the concentration of 50 μM failed to quench, presumably because it is an oxygen carrier and intrinsically a poor redox agent;[16]

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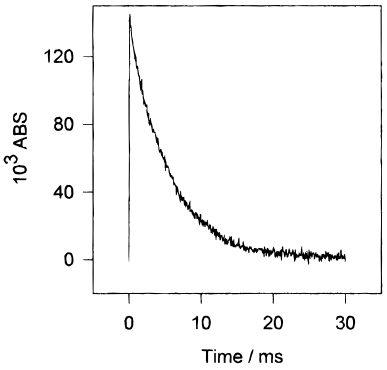

Figure 1. Reductive quenching by EDTA anions of the triplet state of zinc cytochrome *c*, designated ^{3}Zn(cyt), monitored at 460 nm. The solution was made 10 μM in zinc cytochrome *c* and 10 mM in Na_2-EDTA, and the solvent was a sodium phosphate buffer at pH 6.5 and ionic strength of 5.0 mM. The relative concentrations of the ions $HEDTA^{3-}$ and H_2EDTA^{2-} were 7:1.

the rate constant is less than 2×10^6 M^{-1} s^{-1}. The positively-charged complex $[Ru(NH_3)_6]^{2+}$ (0.060 V) failed at the concentration of 20 μM, presumably because it and Zn(cyt) repel each other at the low ionic strength (40 mM) used in these experiments; the rate constant is less than 5×10^6 M^{-1} s^{-1}. When $[Ru(NH_3)_6]^{2+}$ concentration was 20 μM to 1.0 mM, ^{3}Zn(cyt) was quenched, but the cation $Zn(cyt)^+$ was observed as well. This oxidative quenching (eq 1) may be due to the impurity $[Ru(NH_3)_6]^{3+}$, which remained after the recrystallization of $[Ru(NH_3)_6]Cl_2$ from water. Horse-heart ferrocytochrome *c* (0.25 V) failed at the concentration of 50 μM, probably because both it and its zinc derivative bear the net charge of +6 at pH 7.0; indeed, the electron self-exchange reaction of the native (iron) protein is relatively slow.[7] The rate constant for reductive quenching is less than 2×10^6 M^{-1} s^{-1}. The absence of oxidative quenching by ferricytochrome *c* was confirmed in this laboratory.[77] The upper limits mentioned above are conservative, and therefore probably high, estimates. These failed attempts were nevertheless useful, for they taught us how to choose effective reductive quenchers, so that quenching can occur during the lifetime of ^{3}Zn(cyt), which is 10 ms.

Reductive Quenching of ^{3}Zn(cyt) by EDTA. Reductive quenching of zinc porphyrins by EDTA, as in eq 2, is known;[78−81] the rate constant for a particular zinc porphyrin is 1.7×10^5 M^{-1} s^{-1} at pH 5.0 and ionic strength of 50 mM. Our experiments with zinc cytochrome *c* gave positive results. The lifetime of the triplet state, ^{3}Zn(cyt), is shortened in the presence of EDTA; one of many such experiments is shown in Figure 1. The observed rate constant, however, was only 200 ± 20 s^{-1} when the concentration of EDTA (added as its disodium salt) was 10 mM. At lower concentrations of EDTA the quenching was noncompetitive with natural decay, whereas higher concentrations perturbed the pH value and ionic strength of the reaction mixture. After the observed rate constant is corrected for the natural decay (100 ± 10 s^{-1}), the bimolecular rate constant for the reaction in eq 4 can be estimated at 100 s^{-1}/

$$^3\text{Zn(cyt)} + \text{EDTA} \rightarrow \text{Zn(cyt)}^- + \text{EDTA}^+ \qquad (4)$$

0.010 M = 1×10^4 M^{-1} s^{-1}. This is a reasonable value. Zinc

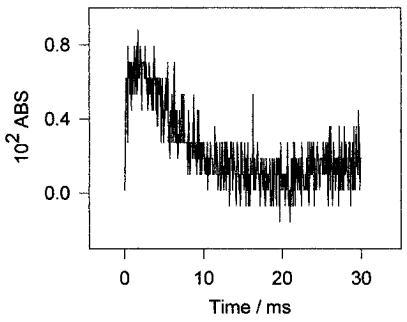

Figure 2. Transient absorption of zinc cytochrome *c* anion radical, designated $Zn(cyt)^-$, monitored at 690 nm. The solution composition is given in the caption to Figure 1.

heme inside cytochrome *c* is approximately ten times less reactive than free zinc porphyrin, mentioned above. The considerable reactivity of zinc heme in the protein may be due to its partial exposure on the protein surface.[38]

Rapid decomposition of the cation radical $EDTA^+$ suppresses the reaction in eq 5, and the anion radical $Zn(cyt)^-$ is expected to be detectable. Although thermal oxidation according to eq

$$\text{Zn(cyt)}^- + \text{EDTA}^+ \rightarrow \text{Zn(cyt)} + \text{EDTA} \qquad (5)$$

5 is not the only reaction that consumes this anion radical, its detection was worth attempting. Indeed, monitoring at 690 nm, a wavelength at which $Zn(cyt)^-$ is expected to absorb, consistently showed a transient species with a correct time profile; for a typical experiment, see Figure 2. Despite repeated attempts we could not record a complete absorption spectrum of the transient anion radical. At wavelengths less than 600 nm all three species—Zn(cyt), ^{3}Zn(cyt), and $Zn(cyt)^-$—have very strong and overlapping absorption bands.

The evidence for reductive quenching so far is positive, but not compelling. We have to rule out nonredox modes of quenching and chemical degradation of zinc cytochrome *c*. Incubation of 10 μM zinc cytochrome *c* and 10 mM EDTA for 24 h did not affect the UV−visible spectrum of the protein. Clearly, even EDTA present in large excess over the protein cannot remove the zinc(II) ions from the heme or otherwise degrade the active site. At pH 6.5, the dominant forms of the quencher are the trianion, $HEDTA^{3-}$, and the dianion, H_2EDTA^{2-}; their relative concentrations are approximately 7:1. Concentration of carboxylate anions in a 50 mM solution of sodium acetate is approximately two times higher than their concentration in a 10 mM solution of EDTA, both at pH 6.5. That addition of 50 mM sodium acetate to the buffered 10 μM solution of zinc cytochrome *c* does not affect the lifetime of the triplet state proves that quenching is not caused by mere binding of EDTA anions to the positively-charged surface of Zn(cyt). Identity of cytochrome *c* conformation in the crystal containing a very high concentration of salt[82] and in solution of low ionic strength,[83] as determined by X-ray diffraction and by NMR spectroscopy, proves that bound counterions are unlikely to cause significant structural changes that would affect the lifetime of the triplet state.

Trimethanolamine and trimethylamine, commonly used as so-called sacrificial reductants in photoinduced electron-transfer reactions, did not affect the lifetime of ^{3}Zn(cyt). The former quenches the triplet state of a zinc porphyrin with a rate constant of 4.0×10^3 M^{-1} s^{-1},[70] and probably so does the latter; the exact value is not available. Because of the shielding by the

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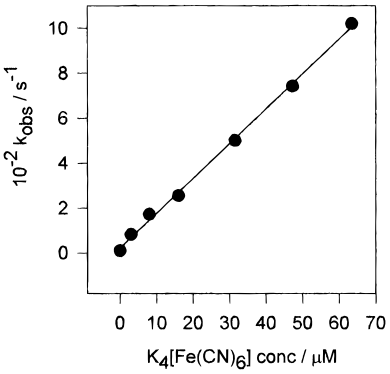

Figure 3. Reductive quenching by the $[Fe(CN)_6]^{4-}$ ion of the triplet state of zinc cytochrome *c*, designated ^{3}Zn(cyt), in a sodium phosphate buffer at pH 7.0 and ionic strength of 40 mM.

protein, the corresponding rate constant for zinc cytochrome *c* must be even lower than that for the free zinc porphyrin. For any quenching to compete with natural decay (100 s^{-1}) of ^{3}Zn(cyt) at the 1.0 μM concentration, the quenching rate constant must be greater than 1×10^4 M^{-1} s^{-1}. These two amines with electroneutral substituents actually exist in solution as ammonium cations. They electrostatically repel zinc cytochrome *c* and cannot quench the triplet state before it decays naturally. These findings show that the negative charge of the EDTA anions is essential for quenching.

Photochemical and pulse-radiolytic studies of various zinc porphyrins found the anion radical to be unstable in aqueous solution.[69,72] Dimerization, disproportionation, and protonation ultimately yield stable zinc dihydroporphyrins.[84] In our experiments, 500 successive laser pulses did not produce any detectable changes in the UV−visible spectrum of zinc cytochrome *c*. The anion radical $Zn(cyt)^-$ seems to be formed, but it is prevented by the protein envelope from dimerizing and ultimately yielding the hydroporphyrin species. We have no evidence for the reaction(s) consuming the small amount of $Zn(cyt)^-$ that may be transiently formed.

Reductive Quenching of ^{3}Zn(cyt) by $[Fe(CN)_6]^{4-}$. The experiments with EDTA described above indicated that negatively-charged quenchers are the correct choice for zinc cytochrome *c*, which has a positively-charged surface patch and bears a net positive charge. The $[Fe(CN)_6]^{4-}$ ion fits this requirement and is thermodynamically capable of reducing ^{3}Zn(cyt)—the reduction potential of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple is 0.35 V. Indeed, we found fast quenching. The slope of the plot in Figure 3 is $(1.5 \pm 0.3) \times 10^8$ M^{-1} s^{-1}. It remained to be determined whether this rate constant corresponds to the reaction in eq 6. Before it, we considered three other conceivable mechanisms of quenching of ^{3}Zn(cyt) in the presence of the $[Fe(CN)_6]^{4-}$ ion.

$$^3Zn(cyt) + [Fe(CN)_6]^{4-} \rightarrow Zn(cyt)^- + [Fe(CN)_6]^{3-} \quad (6)$$

First, the ion $[Fe(CN)_6]^{4-}$ is known to bind electrostatically to the surface of cytochrome *c*.[85] Although there is no evidence that this binding perturbs the protein conformation, we considered the possibility that it may enhance radiationless decay of the triplet state ^{3}Zn(cyt). We used the redox-inactive ion $[Co(CN)_6]^{3-}$, which otherwise resembles the $[Fe(CN)_6]^{4-}$ ion in interactions with cytochrome *c*.[85] The triplet state ^{3}Zn(cyt) decayed with the same rate constant, 100 ± 10 s^{-1}, in the presence and in the absence of the $[Co(CN)_6]^{3-}$ ion. Evidently, quenching in Figure 3 is not due to electrostatic association of the protein and the $[Fe(CN)_6]^{4-}$ ion.

Second, energy transfer requires overlap between the emission spectrum of ^{3}Zn(cyt) (maxima at 590 and 640 nm)[36] and the absorption spectrum of the $[Fe(CN)_6]^{4-}$ ion (a maximum at 330 nm). Because there is no significant overlap, energy transfer can be ruled out as a cause of fast quenching.

Third, the $[Fe(CN)_6]^{3-}$ ion is an efficient oxidative quencher of ^{3}Zn(cyt), as in eq 7. Without attempting a detailed study of this reaction, we examined it under conditions under which it

$$^3Zn(cyt) + [Fe(CN)_6]^{3-} \rightarrow Zn(cyt)^+ + [Fe(CN)_6]^{4-} \quad (7)$$

would occur if $K_4[Fe(CN)_6]$ were contaminated with $K_3[Fe(CN)_6]$. Since under these conditions the $[Fe(CN)_6]^{3-}$ ion would not be present in excess over ^{3}Zn(cyt), we investigated the reaction in eq 7 under approximately equimolar, not the pseudo-first-order, conditions. The rate constant of $(5 \pm 3) \times 10^9$ M^{-1} s^{-1} is an approximate one, but it is adequate for the purpose of these control experiments. For the quenching at $(1.5 \pm 0.3) \times 10^8$ M^{-1} s^{-1}, shown in Figure 3, to be caused by the $[Fe(CN)_6]^{3-}$ ion, this impurity would have to constitute approximately 3% or more of the reagent-grade $K_4[Fe(CN)_6]$, at concentrations of this salt shown in Figure 3. Although improbable, this possibility had to be considered.

The assay for the $[Fe(CN)_6]^{3-}$ ion was negative. Control experiments showed this analytical method to be sensitive to the $[Fe(CN)_6]^{3-}$ concentration that is 0.10% of the $K_4[Fe(CN)_6]$ concentration used in the assay. Quenching of ^{3}Zn(cyt) with recrystallized $K_4[Fe(CN)_6]$ occurs with the rate constant identical, within the error bounds, to the one reported above.

We cautiously conclude that the results in Figure 3 are attributable to reductive quenching, represented by eq 6. This "forward" reaction has a thermodynamic driving force of only ca. 0.1 eV, and the subsequent thermal back-reaction in eq 8

$$Zn(cyt)^- + [Fe(CN)_6]^{3-} \rightarrow Zn(cyt) + [Fe(CN)_6]^{4-} \quad (8)$$

has a driving force of ca. 0.9 eV. This simple consideration suggests that the back-reaction is faster than the forward reaction, and that the intermediate $Zn(cyt)^-$ cannot accumulate. The concentration of this reactive anion radical remains undetectably low, and it could not be observed in transient-absorption experiments at a wavelength around 700 nm. Such detection apparently succeeded—see Figure 2—in reductive quenching of ^{3}Zn(cyt) with EDTA, when the back-reaction was suppressed.

Conclusion

Photoinduced electron-transfer reactions of simple zinc porphyrins proved to be intricate, and ion radicals involved in them were detected with difficulty. In zinc cytochrome *c* the protein envelope modulates the reactivity of the heme group, but the anion radical may be involved, and its detection should be possible when the relative rates of its appearance and disappearance are favorable. In view of the wide and growing use of zinc-substituted heme proteins in kinetic, mechanistic, and spectroscopic studies, their electron-transfer reactions should be studied thoroughly.

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