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# Forum

## Zinc-porphyrin Solvation in Folded and Unfolded States of Zn-cytochrome *c*

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After a brief review of the use of photochemical triggers and heme metal substitution to probe the folding dynamics of cytochrome *c*, we present new results on the photophysics and photochemistry of folded and unfolded states of the zinc-substituted protein (Zn-cyt *c*). Our measurements of Zn-cyt *c* triplet state decay kinetics reveal a systematic isotope effect on lifetimes: the decay in the folded protein ($\tau_{H_2O} \sim 10$ ms) is only modestly affected by isotopically substituted buffers ($k_{H_2O}/k_{D_2O} = 1.2$), whereas a reduced triplet lifetime (~1.3 ms) and greater isotope effect (1.4) were found for the chemically denatured, fully unfolded protein. The shortest lifetime (0.1–0.4 ms) and greatest isotope effect (1.5) were found for a fully exposed model compound, zinc-substituted *N*-acetyl-microperoxidase-8 (ZnAcMP8), implying that the unfolded protein provides some protection to the Zn-porphyrin group even under fully denaturing conditions. Further evidence for partial structure in unfolded Zn-cyt *c* comes from bimolecular quenching experiments using $Ru(NH_3)_6^{3+}$ as an external Zn-porphyrin triplet state quencher. In the presence of quencher, partially unfolded protein at midpoint guanidinium chloride (GdmCl) and urea concentrations exhibits biphasic triplet decay kinetics, a fast component corresponding to an extended, solvent-exposed state ($6.6 \times 10^8$ $M^{-1}$ $s^{-1}$ in GdmCl, $6.3 \times 10^8$ $M^{-1}$ $s^{-1}$ in urea) and a slow component attributable to a compact, relatively solvent-inaccessible, state ($5.9 \times 10^7$ $M^{-1}$ $s^{-1}$ in GdmCl, $8.6 \times 10^6$ $M^{-1}$ $s^{-1}$ in urea). The variation in Zn-porphyrin solvation for the compact states in the two denaturants reveals that the cofactor in the partially unfolded protein is better protected in urea solutions.

### Introduction

Solvent-mediated interactions often play key roles in protein folding reactions. Water expulsion and inclusion are crucial for native structures,[1] protein−protein recognition,[2] and folding pathways.[3−6] Both folded and unfolded protein structures are associated with solvent molecules,[7,8] and chemical denaturant activity is attributed to changes in water hydrogen bonding networks as well as direct solvation of peptide bonds and hydrophobic residues.[9,10] Molecular dynamics simulations[3,4] suggest that rapidly formed, collapsed structures trap a significant number of water molecules that may expedite hydrogen bond formation along the backbone chain as well as facilitate structural transitions. While these and other computational studies have aimed to elucidate the role of water in protein structure and folding, very few experiments have probed the dynamics of solvation during a folding reaction.

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Cofactors in metalloproteins introduce interesting features into the folding landscape. Cytochromes, like many metalloproteins, adopt discrete structures only when the heme cofactor is present.[11] Since the heme provides crucial contacts in the native protein, it is not surprising that this hydrophobic prosthetic group plays an important role in the folding mechnism. As such, the heme remains a useful probe for elucidating intramolecular changes during folding reactions.[12−15]

**Optical Triggers.** Photoinduced ligand dissociation can be employed to examine the early events in folding reactions.[16] Since CO-ligated, reduced cytochrome *c* (cyt *c*) is less stable toward denaturants than the native protein, Fe(II)-cyt *c* folding can be triggered by laser-initiated carbon monoxide dissociation from the heme despite the fact that CO rebinding prevents complete formation of the native conformation. Rapid ligation of the heme by Met80 and Met65 ($\sim$40 $\mu$s)[16,17] is an early folding event, in contrast to Fe(III)-cyt *c* folding, where the much slower substitution of non-native His33 by Met80 is rate limiting.[18] This difference in pathways and rates may be a consequence of the lack of misligation in unfolded Fe(II)CO-cyt *c*.

The reduction potentials of hemes depend strongly on their outer sphere environments. Hydrophobic burial raises the potentials, as shown by the large difference in the folding free energies of oxidized and reduced horse heart cyt *c* ($\Delta\Delta G_f^\circ \equiv \Delta G_{f,ox}^\circ - \Delta G_{f,red}^\circ$, $\sim$34 kJ/mol), with unfolding midpoints occurring at guanidinium concentrations of 2.8 and 5.3 M, respectively.[19,20] As a result, there is a range of denaturant concentrations in which electron injection into the ferriheme of the unfolded protein will initiate folding; similarly, electron removal from the reduced folded protein will trigger unfolding.

As with Fe(III)-cyt *c*, transient absorption experiments demonstrate that HisX (X = 26 or 33) ligation at neutral pH inhibits Fe(II)-cyt *c* folding, although the situation is different at low pH, where formation of the native Fe−S(Met80) bond is rate limiting.[13] Binding of the native ligand is preceded by more global structural changes, identified by transient luminescence experiments[20] as collapse to nonnative compact states which, according to time-resolved circular dichroism data, feature localized helical structures that are formed on submicrosecond time scales.[21] Although ligand substitution steps are central to the formation of native Fe(II)-cyt *c*, it is apparent from the data that these reactions must be coupled to rearrangements of the polypeptide backbone.

**Metal Substitution**. Replacement of the iron center with cobalt(III) or zinc(II) alters the protein folding landscape of cyt *c*. Remarkably, neither the protein structure nor the folding thermodynamics is perturbed by either of these metal ion substitutions, as shown by spectroscopic and structural probes.[22−24] Owing to an extremely high ligand substitution barrier, Co(III)-substituted cyt *c* (Co-cyt *c*) is a kinetically stabilized system in which early folding intermediates can be examined over a period of hours. Both optical and NMR spectroscopic measurements reveal parallel folding pathways involving misligated Co-cyt *c* species that persist for extended periods of time.[24] Combined with fluorescence energy transfer kinetics data, these results confirm that rapidly equilibrating populations of compact and extended polypeptide conformations are present prior to formation of the native structure.[24]

In contrast to Co(III), Zn(II) is normally five-coordinate. As a result, there is little or no barrier to ligand binding in unfolded zinc-substituted cytochrome *c* (Zn-cyt *c*), whose folding rate is much higher than that of Fe(III)-cyt *c*.[14,25] Despite this difference in rates, however, a heterogeneous population of rapidly exchanging, roughly degenerate extended and compact non-native structures persists throughout the Zn-cyt *c* folding reaction.[23] In addition to unveiling features of cyt *c* folding in the absence of misligation, Zn-cyt *c* facilitates study of the denatured state. Denatured proteins are dynamic structures in which intrachain diffusion produces transient tertiary contacts, and the shortest time in which such contacts form is an estimate of the folding speed limit.[26] Measurements of rates of ligand binding to denatured cyt *c* reveal that loops of 45−60 residues form in $\sim$40 $\mu$s, suggesting a speed limit of about 1 $\mu$s.[17] Our work on the kinetics of intramolecular electron transfer in ruthenium-modified Zn-cyt *c* shows that a 16-residue loop in the denatured protein forms in 250 ns,[27] and theory suggests that a 10-residue loop would be formed even faster, with a folding speed limit near 100 ns.[28,29]

**Zn-porphyrin Solvation.** Zn-cyt *c* is ideally suited to monitor cofactor solvation in folded and unfolded protein


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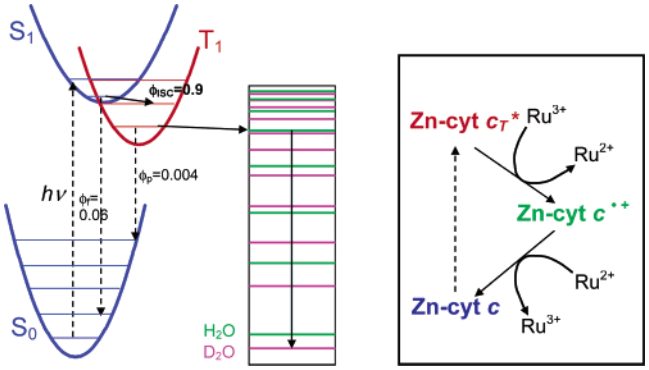


**Figure 1.** Two pathways for deactivation of the Zn-porphyrin triplet state: (1) vibronic coupling to a high-frequency water stretch mode (left); and (2) electron transfer to an external quencher (right). Dashed lines indicate optical transitions, and solid lines denote radiationless events. $\phi_f$ and $\phi_p$ are the fluorescence and phosphorescence quantum yields, respectively, and $\phi_{ISC}$ is the singlet ($S_1$) to triplet ($T_1$) intersystem crossing quantum yield.[30] Abbreviations: Zn-cyt *c* porphyrin triplet excited state (Zn-cyt $c_T$*); Zn-cyt *c* porphyrin radical cation state (Zn-cyt $c^{\bullet+}$).

states. Upon photon absorption, rapid and efficient (quantum yield ~0.9)[30] intersystem crossing creates a long-lived porphyrin triplet state whose deactivation depends strongly on outer sphere solvation (Figure 1). Strong coupling to vibrational modes of the porphyrin and the first solvent shell efficiently converts the >13 000 $cm^{-1}$ of electronic energy to vibrational energy, enhancing the nonradiative triplet decay rate.[31−33] Significant solvent deuterium isotope effects on decay rates implicate high-frequency O−H stretches as efficient acceptor modes.[31,32] Another channel for triplet state deactivation involves electron transfer from the powerfully reducing ($E° = -0.8$ V vs NHE) triplet state to an external redox reagent, such as $Ru(NH_3)_6^{3+}$ ($E° = 0.06$ V vs NHE).[34] Here, quenching of the triplet state proceeds with high driving force ($-\Delta G° = 0.9$ eV), creating a Zn-porphyrin cation radical and $Ru(NH_3)_6^{2+}$ that subsequently react to re-form ground-state Zn-porphyrin and $Ru(NH_3)_6^{3+}$.[34] Such bimolecular electron-transfer experiments on Zn-cyt *c* have been reported using a variety of quenchers.[35] The degree to which there is an isotope effect or efficient triplet decay in the presence of redox quenchers directly reflects the extent of hydration and solvent accessibility of the Zn-porphyrin, and hence, each of these properties can report on the solvation of various conformational states of cytochrome *c*.

Zn-cyt *c* is structurally similar to the native protein (Fe-cyt *c*), which has been studied in great detail.[14,25,36] NMR and crystal structures of Zn-cyt *c*, Fe(II)-cyt *c*, and Fe(III)-cyt *c* reveal only minor differences in backbone positions, identical His18 and Met80 axial ligands, and unchanged porphyrin environments upon Zn(II) substitution.[22,37] The

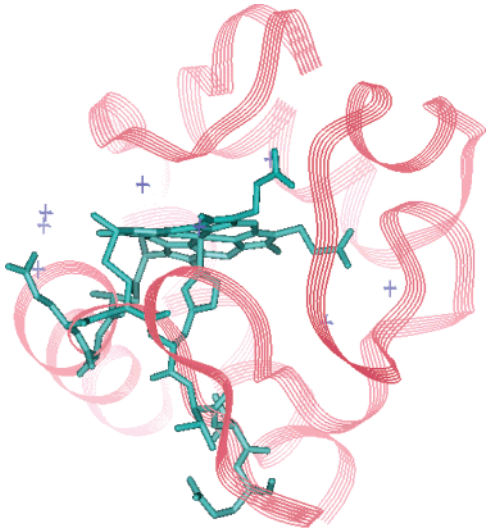

**Figure 2.** Structure of MP8, which consists of the heme group plus residues 14−21, is shown in green in horse heart cytochrome *c* (PDB file 1HRC).[42] Water molecules within 5 Å of the heme are indicated by blue symbols.

stability of folded Zn-cyt *c* ($\Delta G_f° = -35$ kJ/mol) is comparable to that of the Fe(III) form ($\Delta G_f° = -40$ kJ/mol), but both are less stable than Fe(II)-cyt *c* ($\Delta G_f° = -74$ kJ/mol).[19,23] The structural and thermodynamic similarities to Fe-cyt *c*, the existence of a long-lived, solvent-sensitive triplet state, and the lack of axial ligand traps make Zn-cyt *c* an ideal system for investigations of the role of hydration in folding.

We have probed the Zn-porphyrin environment in folded as well as the compact and extended unfolded states of Zn-cyt *c* in urea and guanidinium chloride (GdmCl) denaturants. Zinc-substituted *N*-acetyl-microperoxidase-8, ZnAcMP8, was employed to distinguish the effects of solvent coupling from intramolecular interactions on Zn-cyt *c* triplet lifetimes. MP8 is a heme octapeptide derived from enzymatic cleavage of cyt *c* (Figure 2); the heme + loop region 14−18 comprises an active site whose structure is virtually the same as that in the holoprotein, including axial ligation by His18.[38] Because the tryptic fragment is fully exposed to the aqueous environment, it is reasonable to assume that triplet state decay rates in this case will be dominated by solvent effects. Our combined studies of Zn-cyt *c* and ZnAcMP8 shed light on the immediate solvent environment of the cofactor in equilibrium folded and unfolded states and on unfolded structures resulting from two different chemical denaturants; they also provide a foundation for future studies of solvation during a protein folding reaction.

## Results

We have measured transient absorption spectra of the Zn-porphyrin triplet in ZnAcMP8 and Zn-cyt *c*; the change in absorbance from 420 to 500 nm due to formation of triplet state in ZnAcMP8 is displayed in Figure 3 along with the ground-state absorption spectrum. Our work shows that triplet ZnAcMP8 exhibits an absorption maximum near 440 nm,

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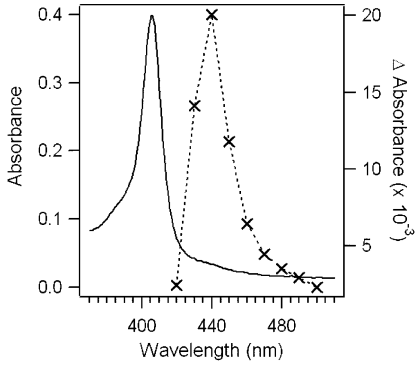


**Figure 3.** Ground-state (—, left axis) and triplet transient (- - -×- - -, right axis) absorption spectra of ~2 $\mu$M ZnAcMP8 in 20 mM phosphate + 0.1% TFA solution.

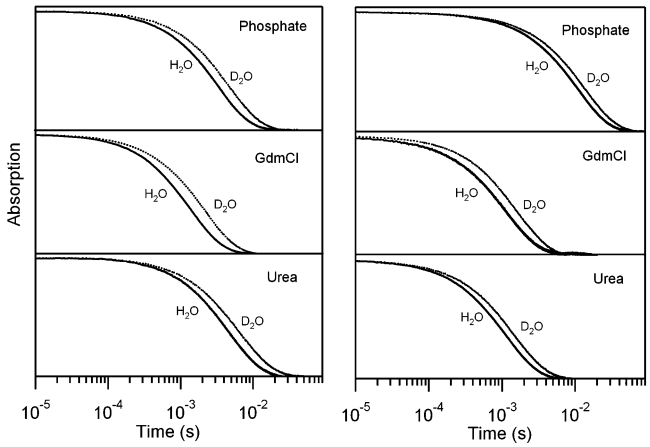


**Figure 4.** Transient absorption decay kinetics of triplet ZnAcMP8 (left) and Zn-cyt *c* (right) in protonated and deuterated solutions. Buffers were 20 mM phosphate (top) with ~5 M GdmCl (middle) or ~9 M urea (bottom). For ZnAcMP8, 0.1% TFA was added to the buffer; for Zn-cyt *c*, solutions were pH = pD = 7.4.

whereas triplet Zn-cyt *c* absorbs strongly near 460 nm, consistent with previous results.[34,39] Comparison with the ground-state Zn-cyt *c* bleach reveals an increase in molar extinction coefficient of 30−40 $mM^{-1}$ $cm^{-1}$ at 450 nm upon formation of triplet Zn-cyt *c*. Thus, 450 nm is an excellent probe wavelength for the Zn-porphyrin triplet state, since ground-state absorption, cation radical absorption ($\lambda_{max}$ ~675 nm),[34] and triplet luminescence ($\lambda_{max}$ ~736 nm) are minimal at this wavelength.[34,40]

Decay kinetics of the ZnAcMP8 and Zn-cyt *c* triplet states in protonated and deuterated phosphate buffers, GdmCl, and urea are shown in Figure 4. Triplet decays in phosphate buffer can be described by monoexponential kinetics, while decays in urea and GdmCl exhibit biphasic behavior, with major (>80%) and minor (<20%) components similar to those described previously.[35] The major components of the decay rates are set out in Table 1. The triplet state of ZnAcMP8 decays faster than those of both folded and unfolded Zn-cyt *c*, consistent with maximum solvent exposure of the cofactor in the tryptic fragment. Comparison of ZnAcMP8 photophysics in the two denaturants reveals longer

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**Table 1.** Rate Constants[a] and Isotope Effects for ZnAcMP8 and Zn-cyt *c* Triplet States

| | $k_{H_2O}$ ($s^{-1}$) | $k_{D_2O}$ ($s^{-1}$) | $k_{H_2O}/k_{D_2O}$ |
|---|---|---|---|
| ZnAcMP8 in phosphate | 3030 | 1990 | 1.5 |
| GdmCl | 6980 | 4955 | 1.4 |
| urea | 2325 | 1580 | 1.5 |
| Zn-cyt *c* in phosphate | 105 | 90 | 1.2 |
| GdmCl | 810 | 600 | 1.4 |
| urea | 720 | 525 | 1.4 |

[a] Errors for rate constants are ±15%.

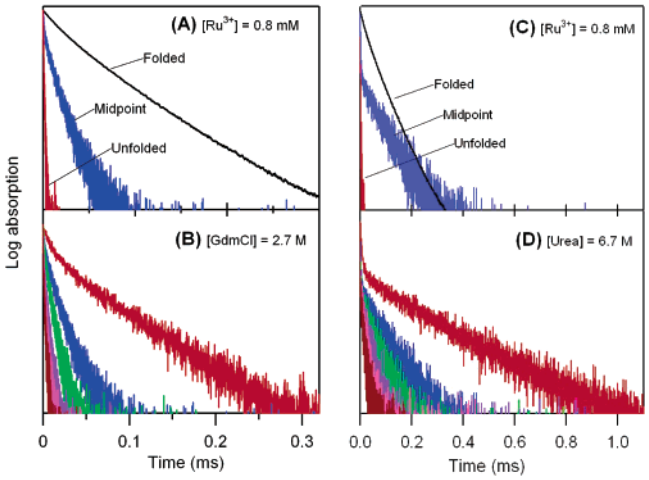


**Figure 5.** Transient absorption decay kinetics of triplet Zn-cyt *c* in 20 mM phosphate + GdmCl (left) and urea (right) solutions: (A) [GdmCl] = 0 M (black), 2.7 M (blue), and 5.6 M (red) with [$Ru^{3+}$] = 0.8 mM; (B) [$Ru^{3+}$] = 0.3 mM (red), 0.8 mM (blue), 1.3 mM (green), 2.4 mM (purple), 3.3 mM (pink), and 4.5 mM (brown) with [GdmCl] = 2.7 M; (C) [urea] = 0 M (black), 6.7 M (blue), and 8.6 M (red) with [$Ru^{3+}$] = 0.8 mM; and (D) [$Ru^{3+}$] = 0.3 mM (red), 0.8 mM (blue), 1.4 mM (green), 2.4 mM (purple), 3.7 mM (pink), and 4.9 mM (brown) with [urea] = 6.7 M. Note different time scales.

lifetimes in urea relative to GdmCl. Since the vibrational spectra of the two denaturants are similar in the high frequency region, the difference in decay rates may reflect the role of ionic strength in triplet deactivation for the fully exposed Zn-porphyrin. The triplet lifetimes of folded and unfolded Zn-cyt *c* are substantially longer than those of ZnAcMP8. In addition, the solvent deuterium isotope effect ($k_{H_2O}/k_{D_2O}$) is smaller in the protein, reflecting greater cofactor protection. As expected, the folded protein has the least exposed Zn-porphyrin (longest triplet lifetime of ~10 ms and smallest isotope effect of 1.2). The cofactor in the denatured protein appears to be only partially exposed; although triplet decay is 7−8 times faster than in the folded protein, it is 3−9 times slower than in ZnAcMP8.

The degree of solvent exposure of the Zn-porphyrin was further explored in a series of bimolecular quenching experiments with $Ru(NH_3)_6^{3+}$. Parts A and C of Figure 5 display decay traces of folded and unfolded triplet Zn-cyt *c* in the presence of quencher. When [$Ru^{3+}$] = 0.8 mM, the majority decay component in the folded state is 7630 $s^{-1}$, which is 76 times faster than the triplet decay rate of ~100 $s^{-1}$ in the absence of quencher (a rate similar to one obtained in a previous study).[34] For the fully unfolded protein, triplet decays increase 500−800 times upon addition of quencher from pure decay rates of 810 (GdmCl) and 720 (urea) $s^{-1}$ to 4.4−6.4 × $10^5$ and 4.8 × $10^5$ $s^{-1}$, respectively. This increase

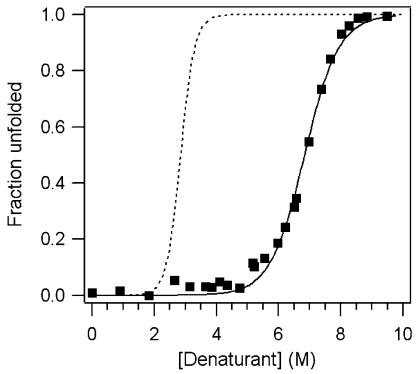


**Figure 6.** Unfolding curve for Zn-cyt *c* in urea (■, solid line) is from absorption measurements; the GdmCl curve is from ref 23 (- - -). Half the population is unfolded when [urea] = 6.9 M ($\Delta G_f^\circ = -31$ kJ/mol) or [GdmCl] = 2.9 M ($\Delta G_f^\circ = -35$ kJ/mol).

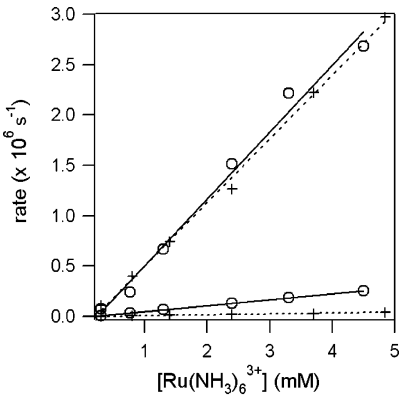


**Figure 7.** Fast and slow kinetics decay components with linear fits of triplet Zn-cyt *c* in 2.7 M GdmCl (○, solid line) and 6.7 M urea (+, dashed line) as a function of quencher concentration.

in quenching rate is primarily due to greater porphyrin accessibility to the quencher in the unfolded protein, and may also reflect the change in ionic strength in the GdmCl case.[35,41] Partially unfolded protein was monitored near the GdmCl and urea midpoints of 2.9 and 6.9 M, respectively (Figure 6). Near denaturant midpoints, the decays are biexponential, with slow (40−70%) and fast (60−30%) components corresponding to compact and extended structures. Parts B and D of Figure 5 illustrate the increase in triplet decay rates upon continual addition of quencher from 0.1 to ∼ 5 mM. Rate constants at denaturant midpoints are displayed in Figure 7, along with linear fits. The second-order rate constants are $6.6 \times 10^8$ (extended) and $5.9 \times 10^7$ (compact) $M^{-1}\ s^{-1}$ in GdmCl, and $6.3 \times 10^8$ (extended) and $8.6 \times 10^6$ (compact) $M^{-1}\ s^{-1}$ in urea. These rate constants are similar to those obtained in our previous work.[23]

## Discussion

**Zn-porphyrin Solvation in Folded Zn-cyt *c*.** The cofactor in folded Zn-cyt *c* is partially hydrated, as indicated by the modest, yet reproducible, isotope effect $k_{H_2O}/k_{D_2O}$ of 1.2 (Table 1 and Figure 4). This isotope effect is smaller than that observed for the fully exposed Zn-porphyrin in ZnAcMP8 ($k_{H_2O}/k_{D_2O} \sim 1.5$), and the triplet lifetime of the folded protein ($\tau \sim 10$ ms) is much longer than that for ZnAcMP8 ($\tau \sim 0.3$ ms). The difference in lifetimes arises because the folded protein restricts solvent access to the cofactor. The observed isotope effect may be explained by the presence of water molecules in the Zn-porphyrin binding pocket of the folded protein combined with partial cofactor exposure to the bulk solvent. Several internal water molecules are present in crystals of horse heart ferro- and ferricytochrome *c*, including two believed to interact with the heme.[42] One of these heme-associated water molecules moves ∼4 Å away from the metal upon upon oxidation of Fe(II) to Fe(III),[43] suggesting that buried structural waters in cytochrome *c* play a role in solvent reorganization associated with electron transfer.[44] The observation that numerous $H_2O$ molecules occupy conserved positions in a variety of cytochromes *c* indicates that these solvent molecules are critical for protein function.[42]

The internal water molecules in folded cytochrome *c* and other proteins are dynamic and exchangeable, as supported by experimental and theoretical studies of water penetration, mobility, and exchange in folded proteins.[8,45−50] A great deal of NMR work has revealed that surface-bound waters exchange rapidly (pico- to nanoseconds), while buried solvent molecules have much longer residence times (micro- to milliseconds).[47−50] In contrast to these ordered molecules, crystallographically invisible, disordered solvent molecules may reside in highly hydrophobic cavities for up to hundreds of microseconds.[49] Several models for exchange of deeply buried molecules in folded proteins have been proposed. In the "penetration" model, transient access to hydrophobic cores is provided by fluxional protein motion, while the "local unfolding" model describes cooperative disruption of hydrogen bonds along parts of the helix chain, thereby allowing solvent exchange in regions where secondary structure is intermittently lost.[51] No experiment thus far has definitively tested these proposals; however, on the basis of our observation of an isotope-sensitive triplet state in Zn-cyt *c*, we suggest that at least some of the water molecules in the cofactor binding pocket are rapidly mobile.

In addition to these internal waters, the porphyrin is hydrated in part by the bulk solvent, as indicated by several lines of evidence. First, our observation of triplet state quenching by $Ru(NH_3)_6^{3+}$ in the folded protein (Figure 5A) confirms that the porphyrin is accessible to bulk solvent. Second, the X-ray crystal structure reveals that ∼8% of the heme group in Fe(III)-cyt *c* is exposed to solvent, and this

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exposed region is encircled by charged residues.[42] Finally, under folding conditions, metal-free cytochrome *c* has exchangeable pyrrolic protons,[30,52] as evidenced by a relatively large isotope effect ($k_{H_2O}/k_{D_2O} = 2.2$) associated with triplet decay.[30] This large isotope effect arises because changes in intramolecular vibrations upon deuteration of the metal-free porphyrin affect the strength of coupling between the triplet and ground states. The upper limit for an isotope effect arising primarily from solvent coupling is 1.5, and this value of $k_{H_2O}/k_{D_2O}$ is similar to that observed for relatively small organometallic molecules in protic solvents.[32] A prior study[30] did not resolve the modest isotope effect we have found for triplet Zn-cyt *c*, presumably due to less sensitive detection. While the relatively long lifetime of the Zn-porphyrin triplet state reported here and elsewhere[30,34,35] is consistent with a buried cofactor in the folded protein, other results and observations (e.g., proton exchange, an isotope effect of 1.2, and partial cofactor exposure in the crystal structure) confirm that at least a some of the porphyrin must be exposed to solvent.

**Zn-porphyrin Solvation in Unfolded Zn-cyt *c*.** Hydration of the cofactor in Zn-cyt *c* increases dramatically upon protein unfolding. The lifetime of the triplet porphyrin changes nearly an order of magnitude, from ~10 ms (folded) to ~1.3 ms (unfolded). This change in lifetime cannot be attributed solely to the change in heme−solvent vibronic coupling in water relative to denaturant; our studies with ZnAcMP8 indicate that a change in cosolvent affects triplet lifetimes only by a factor of ~3. Instead, the decreased lifetime reflects enhanced Zn-porphyrin solvation, and this interpretation is further supported by the larger isotope effect and greater quencher accessibility for unfolded relative to folded protein. An increased number of water molecules surrounding the porphyrin in the unfolded species also has been observed in a small-angle X-ray scattering study,[53] which reported an increase in the radius of gyration attributable to water-induced swelling upon unfolding cytochrome *c*.

The isotope effect observed for unfolded Zn-cyt *c* is less than that for the fully exposed porphyrin in ZnAcMP8, suggesting that there is partial shielding of the cofactor in the unfolded protein. The presence of this shielding supports the notion that unfolded proteins are not random polymers, but instead may adopt partial structure even under fully denaturing conditions.[54−58] This unfolded structure may feature hydrophobic clustering,[55] nativelike topology,[54] or, in the case of heme proteins, well-defined heme ligation geometry.[56] While water penetration into these non-native species is enhanced even before full protein denaturation occurs,[9] unfolded proteins do not appear to achieve complete solvent exposure.[59] The existence of such partially structured unfolded states with locally buried regions is supported by our bimolecular quenching experiments. At denaturant midpoints, the biphasic triplet lifetimes are interpreted in terms of comparable compact (solvent-inaccessible) and extended (solvent-exposed) populations.[12,23,60] This heterogeneity persists even at high denaturant concentrations,[12,60] supporting the picture of dynamic and rapidly interchanging subpopulations of compact and extended unfolded structures upon chemical denaturation.

The finding that five-coordinate Zn-myoglobin and Zn-hemoglobin[61] exhibit triplet lifetimes similar to that of folded six-coordinate Zn-cyt *c* suggests that coordination structure has only a minor effect on the photophysics of porphyrins buried in proteins, and that direct vibrational coupling to solvent molecules plays a more critical role. The dominance of solvent effects is evident by the nearly 10-fold difference in the triplet lifetimes of unfolded Zn-cyt *c* and folded Zn-myoglobin. While absolute cofactor hydration levels may be different for unfolded five-coordinate Zn(II)- and six-coordinate Fe(III)-cyt *c*, we suggest that the isotope effect observed here serves as a reasonable indicator of changes in hydration that accompany unfolding the native protein.

**Denaturant-Dependent Compact States.** A molecular description of the mechanism of chemical denaturation is a goal of much current research.[10,62−64] For Zn-cyt *c*, the free energies of unfolding are comparable in urea ($\Delta G_f^\circ = -31$ kJ/mol) and GdmCl ($\Delta G_f^\circ = -35$ kJ/mol), and the difference in midpoints may be attributable to ionic strength effects.[65] In addition, fully unfolded proteins in high urea and GdmCl concentrations exhibit similar triplet lifetimes, isotope effects, and bimolecular quenching rates, suggesting that these denaturants create nearly identical localized heme environments under these conditions. Unfolded extended structures formed at midpoint urea and GdmCl concentrations also exhibit indistinguishable solvent accessibility, demonstrated by similarly high second-order rate constants. Our observation that these triplet quenching rates for fully unfolded protein in the two denaturants are virtually identical suggests that ionic strength plays a minor role in bimolecular quenching of unfolded relative to folded[35] Zn-cyt *c*.

In contrast to fully denatured protein, the nature of the compact species depends on denaturant (Figure 7). Bimolecular quenching of the triplet Zn-porphyrin in the compact state in urea is ~7 times slower than in GdmCl, and this difference likely reflects greater protection of the porphyrin group from the aqueous urea solvent. An additional contribu-

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tion to this difference may come from electrostatic effects, since it has been shown that bimolecular quenching of the protected cofactor in folded Zn-cyt *c* is sensitive to ionic strength and quencher charge.[35] However, the bimolecular quenching rates observed here are similar to those reported for folded and molten globule states of Zn-cyt *c*,[35] supporting our interpretation that the rate differences are attributable to variations in the equilibrium GdmCl- and urea-induced compact structures. It is possible that these partially unfolded species are similar to the intermediates associated with the burst phase (hydrophobic collapse) of cytochrome *c* folding.[12,14,23,24,66] The compact structure observed here likely represents this partially unfolded intermediate as well, and our finding that the structure of this species is denaturant-dependent supports the notion of non-native cofactor environments for this compact, partially unfolded species.

Our proposal that urea creates a more compact species than GdmCl is supported by preliminary fluorescence energy transfer kinetics experiments on Zn-cyt *c*; the average distance between Trp59 and the Zn-porphyrin is shorter at high urea concentrations than in GdmCl (data not shown). This extra compaction in urea may reflect the relative strengths of these two widely used denaturants. There is general consensus that chemical denaturants act through a combination of direct binding to the peptide backbone and residues, and by altering the hydrogen bonding network of water in a structure-making or structure-breaking manner, thereby diminishing the hydrophobic effect.[9,10] It is well established that the transfer free energy for the peptide group from water to aqueous solutions of urea or GdmCl is favorable, with GdmCl being a better solvent for the peptide backbone than urea.[67] In contrast, side chains show wide variability in transfer free energy to denaturant solutions; however, for a given denaturant concentration, GdmCl is more effective in its ability to solvate both hydrophobic and hydrophilic residues.[68,69] Studies on charge effects suggest, not surprisingly, that denaturation by urea is more sensitive to protein charge and ionic strength than GdmCl.[63−65,70] Collectively, these reports indicate that GdmCl is more effective than urea in its ability to disrupt and solvate hydrophobic pockets of folded proteins, in accord with our finding that the Zn-porphyrin in the compact state of Zn-cyt *c* is more exposed to solvent in GdmCl than in the urea.

## Concluding Remarks

We have demonstrated that Zn-porphyrin triplet lifetimes are sensitive indicators of heme-pocket hydration. Our finding that folded and unfolded species have different isotope effects and bimolecular quenching rates indicates that changes in hydration can be monitored during a folding reaction. Important issues such as the time scales for water expulsion,[71] the nature of dehydrated and hydrated intermediates, and even the effect of denaturant on folding pathways can be addressed by means of triplet lifetime measurements.

## Materials and Methods

**Preparation of ZnAcMP8 and Zn-cyt *c*.** AcMP8 was prepared from horse heart cytochrome *c* (Sigma) following a literature procedure.[72] Lyophilized AcMP8 was treated with anhydrous HF at −78 °C to create metal-free AcMP8 (Beckman Institute Biopolymer Synthesis Facility) which, when dissolved in 100 mM potassium acetate buffer (pH 5.0), displayed a broad Soret absorption at 391 nm and smaller bands at 506, 540, 568, and 620 nm, characteristic of free base porphyrins. Subsequent steps were performed in the dark. Zinc acetate was added to a final concentration of 10 mM, and this solution was stirred in a 55 °C water bath for 2 h. Zinc incorporation was confirmed by a shift in the Soret absorption from 391 nm to a sharp peak at 406 nm. Crude ZnAcMP8 was washed, and the buffer was exchanged to a 6% acetonitrile, 0.1% TFA solution using ultrafiltration. Pure ZnAcMP8 was obtained after purification on a reverse phase column (Pep-RPC 16/10, Amersham Biosciences) using a linear gradient up to 60% acetonitrile. ZnAcMP8 was then washed, concentrated, and stored in aqueous 6% acetonitrile, 0.1% TFA solution at −80 °C.

Zn-cyt *c* was prepared using a modified literature procedure.[73] Zn(II) incorporation was monitored via the Soret red-shift from 404 nm (free base) to 423 nm (Zn-cyt *c*). Crude protein was washed by dialysis into 20 mM potassium phosphate buffer (pH 7.0), loaded onto a cation exchange column (HiTrap CM, Amersham Biosciences) and eluted using a linear gradient up to 0.5 M NaCl. Only samples whose $A_{423}/A_{549}$ value was greater than 15.8 were concentrated into 20 mM phosphate buffer (pH 7.0) and stored at −80 °C.

**Deuterated Buffers**. Urea-$d_4$ (Sigma) was used without further purification. Guanidine-$d_5$ deuteriochloride was made by dissolving guanidine hydrochloride (Sigma) in $D_2O$ (99.9%, Cambridge Isotope Laboratories), followed by repeated recrystallization or solvent evaporation. Isotopic purity was determined by Raman spectroscopy using 514 nm excitation and a spectrograph-CCD detector system. Both protonated and deuterated denaturants had >95% purity.

**Transient Absorption Experiments.** Zn-porphyrin was photolyzed by a 10 Hz, 10 ns Nd:YAG laser coupled to a 355 nm-pumped optical parametric oscillator to form a transient triplet state that was subsequently probed by a continuous-wave 75 W xenon arclamp. The 550 nm, 1.6-mm diameter pump and 450 nm, 0.7-mm diameter probe beams were collinear in a 1-cm quartz cuvette. Probe light was further dispersed in a 10-cm, F/3.5 double monochromator (DH10, Instruments, S.A.) and detected by a PMT. Power dependence experiments verified that, under these conditions, pump (1.0−1.5 mW) and probe (1.8−2.3 mW) powers were well within the linear regime for triplet formation and decay. Reported kinetics curves are the average of 1000 shots, and photodegradation of the degassed 1−4 $\mu$M Zn-cyt *c* solutions (20 mM phosphate, pH = pD = 7.4) monitored by UV−vis absorption spectroscopy was typically <10% after each experiment. For ZnAcMP8 (2−3 $\mu$M in 20 mM phosphate + 0.1% TFA) bimolecular quenching

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experiments with $Ru(NH_3)_6^{3+}$, no photodegradation was detected. Denaturant concentrations were determined after each experiment by index of refraction measurements.

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