Cytochrome c Structure and Redox Function in Mixed Solvents Are Determined by the Dielectric Constant

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Abstract: Cyclic voltammetry (CV), UV−visible (UV−vis), circular dichroism (CD), and resonance Raman (λ_{ex} = 406.7, 413.1 nm) spectroscopy have been used to probe the structure and redox function of horse cytochrome c (cyt c) in aqueous mixtures of three water-miscible organic solvents, specifically, acetonitrile (ACN), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) containing 100−60% water. As the concentration of the organic solvent is increased, significant changes are observed in the spectroscopy of ferricytochrome c (ferricyt c) with the greatest structural changes observed for ferricyt c in mixed solvent media with the lowest dielectric constant (30% ACN in this work). In the UV−visible spectrum, the Soret band blue shifts (1 nm) and the intensity of the 695 nm band decreases. UV CD (185−240 nm) suggest that changes in the protein secondary structure on going from aqueous to nonaqueous media are relatively small and that the protein structure remains largely intact in nonaqueous media. In the visible CD spectrum, the negative 417 nm CD signal disappears, signaling significant changes in heme−polypeptide interactions. Shifts in the vibrational frequencies and changes in the relative intensities of bands in both the marker band and low frequency spectral regions of cyt c in mixed media have been attributed to mixtures of the type IVa, IVb, Va, and Vb alkaline conformers of ferricyt c in mixed media. The change in the composition of these mixtures as the dielectric constant decreases parallels closely that reported by Dopner et al.[1] for yeast iso-1-cytochrome c in aqueous solution as the aqueous solution pH is raised from pH 7 to pH 10. The spectroscopic characteristics of the reduced form of cyt c in mixed solvents are very similar to those of native ferrous cyt c (ferrocyt c) in aqueous solution, reflecting minimal change in heme active site structure in the reduced state. Cyt c in mixed solvents exhibits a quasireversible, one-electron response at 4,4′-dipyridyl disulfide-modified Au electrodes between 5 and 200 mV/s. The redox potential for cyt c in mixed media (199−274 mV vs SHE) and the rate of heterogeneous electron transfer ($0.4-3.5 \times 10^{-3}$ cm/s) decrease as the organic solvent content of the solvent medium increases. Our data support the conclusion that the internal dielectric constant within the heme crevice plays the key role in determining the reduction potential of cyt c and suggest that the effects of axial ligation (Met vs Lys) may be less significant than previously believed.

Introduction

Recently our research group has begun to investigate the use of solvent engineering as a means of probing the structure and function relationship in biomolecules and in particular metalloproteins. We have demonstrated that highly reactive enzyme intermediates such as horseradish peroxidase compound II can be generated and stabilized toward spectroscopic study through the use of nonaqueous solvents.[2] More recently, we have shown that there are significant changes in the structure of biomolecules in nonaqueous media that are directly responsible for the remarkable changes in reactivity observed in unconventional media.[3] With an interest in electrogenerating oxyferryl enzyme intermediates, we have demonstrated proof-of-concept for the direct electrochemical oxidation/reduction of metalloproteins in nonaqueous media. In the process, we have shown that direct electron transfer is persistent and facile at a wide range of naked electrode substrates such as glassy carbon, gold, and platinum, in nonaqueous media, and that heterogeneous electron-transfer rates in organic media are competitive with those in aqueous solution.[4,5] We now report on the utility of a mixed solvent system solvent engineering approach in structure−function studies of metalloproteins, specifically, horse heart cytochrome c.

Horse heart cytochrome c (cyt c), a class I c-type cytochrome involved in electron transfer in the mitochondrial respiratory chain, represents an excellent model biomolecule with which to investigate bioelectrochemistry in mixed media. Cyt c is one of the most thoroughly physicochemically characterized metalloproteins.[6,7] The ferric, ferrous, and alkaline forms of cyt c have been extensively studied by a wide range of optical spectroscopic techniques including UV−vis, CD, and resonance Raman spectroscopy.[6,7] The protein consists of a single polypeptide chain containing 104 amino acid residues that is covalently anchored by two thioether bonds at Cys14 and Cys17 to a heme-binding iron. The polypeptide chain is organized into a series of five α-helices and six β-turns.[8] The heme active site in cyt c consists of a 6-coordinate low-spin heme-binding His18 and

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Met80 in the axial ligands. The heme group, which is located in a groove and almost completely buried inside the protein, is nonplanar and is somewhat distorted into a saddle-shape geometry.[9] Significant changes occur in the heme active site upon change in redox state.[9,10] The reduced protein, ferrocytochrome c (ferrocyt c), is relatively compact and very stable.[11] The stability can be attributed to the fact that the heme group in ferrocyt c is neutral. In the oxidized protein, ferricytochrome c (ferricyt c), the heme group has a net +1 charge (the porphyrin is a dianion) and is surrounded by a relatively hydrophobic pocket.

As the aqueous solution pH is raised, ferricyt c is converted into another conformational form called alkaline cyt c. The alkaline form is much more difficult to reduce than native ferricyt c and is characterized by a redox potential approximately 0.5 V lower than that of native cyt c.[12] This large change in redox potential has been taken as evidence that the alkaline transition may play a useful functional role in the control of the cyt c electron transfer pathway even though this species is not believed to be directly involved in the redox process.[12] Consequently, structural study of this species has recently attracted considerable attention.[1,12−16]

Alkaline cyt c has long been known to be a mixture of several low spin hexacoordinate species, often referred to as types IV and V, in which the axial Met80 heme iron ligand has been replaced.[17] Recent spectroscopic study demonstrated that the type IV alkaline forms of cyt c consist of two lysine-ligated conformers referred to as states IVa and IVb.[16] Until very recently, the identity of the new axial ligand was unknown though it was long suspected to be one of the many nearby surface lysine residues. Lys73 and Lys79, a conserved residue in cyt c that lies near the heme iron, have recently been identified to replace Met80 as the sixth axial heme active site ligand in these two alkaline conformers.[14,15] At very high pH, another alkaline pH state designated V is formed. Hydroxide is generally believed to be the ligand replacement for Lys as the sixth heme active site ligand in this high pH form of cyt c.[18]

Cyclic voltammetry has emerged in the past decade as an extremely useful probe of cytochrome c redox function. Indeed successful efforts in aqueous bioelectrochemistry have largely focused on the study of heme peptides[19−25] and redox proteins, most notably, horse cytochrome c.[12,24,26−34] Conditions favoring facile electron transfer and high quasireversible heterogeneous electron-transfer rates ($\approx 10^{-3}$ cm/s) have been reported for cyt c in aqueous solution at a wide range of solid electrode substrates.[30,31] Efforts have even been directed to probe the direct electrochemistry of cytochrome c in nonaqueous media, specifically, methanol,[35] where spectroscopic and electrochemical measurements indicated the presence of several non-native forms of cytochrome c including the alkaline form in anhydrous methanolic solution.

Spectroscopic studies[3,36−40] of enzymes in nonaqueous media have shown that the structure of enzymes in nonaqueous media is remarkably similar to that of enzymes in aqueous solution suggesting that the structural changes that occur in organic solvents are minor. The most significant effects have been attributed to dehydration and changes in H-bonding usually involving bound water or bound solvent molecules. These findings appear to be generally applicable to enzymes in nonaqueous media and independent of the nature of the enzyme or solution (heterogeneous/homogeneous).

In view of the aforementioned utility of solvent engineering in structure−function studies of metalloproteins and the continued interest in understanding the redox nature of cytochrome c and the extent of its physicochemical characterization, we now report the detailed spectroscopic (UV−vis, CD, resonance Raman (RR)) and electrochemical (CV) characterization of the ferric−ferrous redox couple of horse heart cytochrome c in several mixed solvent systems, specifically, in aqueous mixtures of three water-miscible organic solvents, specifically, acetonitrile (ACN), DMF, and DMSO, containing 100−60% water, with pH adjusted to 7.16 ± 0.16.[41]

Experimental Section

Materials. Horse heart cytochrome c, type VI (Lot No. 67H7190), was purchased from Sigma Chemical Co. and was used as received. The following materials were purchased commercially as ACS certified

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grade and were used as received: 4,4′-dipyridyl disulfide (BPD; Aldrich), sodium perchlorate (Aldrich), fenchone (Aldrich), sodium phosphate monobasic monohydrate (Fisher), sodium phosphate dibasic heptahydrate (Fisher), sodium citrate dihydrate (Sigma), citric acid monohydrate (Sigma), acetonitrile (Fisher), dimethyl sulfoxide (Fisher), and dimethyl formamide (Fisher).

Circular Dichroism Measurement. Circular dichroism spectra (185−800 nm) were obtained at room temperature using a 0.1 cm path length cuvette for the UV CD studies and a 1 cm path length cuvette for the visible CD studies on a JASCO J-715 spectropolarimeter. The CD spectra were processed with Grams/32 Spectral Notebase software (Galactic Industries Corp.).

Cyclic Voltammetry. Cyclic voltammetry measurements were made with a BAS 100B electrochemical analyzer. A single compartment electrochemical cell containing three electrodes was used for the electrochemical studies. A single junction Ag/AgCl (3.0 M NaCl, BAS) reference electrode, a 1.2 mm Au disk working electrode (BAS), and a 1 cm^2 Pt-gauze counter electrode were used for all experiments. To relate redox potentials obtained using different mixed solvent media to each other in the absence of information on the liquid junction potential in each solution, the one-electron oxidation of ferrocene (Fc^+/Fc) (0.400 ± 0.005 V vs SHE) has been used as a voltammetric standard throughout this work.[42,43] Potentials have been derived from measurements made at 20 mV/s scan rates where potential measurements have been shown to be essentially independent of the nature of the electrode and electrolyte used.[42,43] The reliability of potentials measured using the single junction reference electrode was tested and confirmed using a double junction Ag/AgCl reference. Potentials obtained using single and double junction Ag/AgCl reference electrodes were within ±5 mV of each other. Before each experiment, the working electrode was carefully polished with 15 μm, 10 μm, and 1 μm diamond polish (BAS). The electrode was rinsed with methanol after each polishing step. After the final step, the electrode was polished with 0.5 μm alumina (BAS) and rinsed with distilled water. All cyclic voltammograms were recorded in nitrogen-purged quiescent solution, in the presence of 4,4′-dipyridyl disulfide. The solution was saturated with BPD in order to obtain a stable limiting current[44] and to prevent dissolution of adsorbed BPD into the mixed solvent. Sodium perchlorate **(**0.1 M) was used as supporting electrolyte. All measured potentials have been converted to the standard hydrogen electrode (SHE) reference potential. Background subtracted cyclic voltammograms (the total current for all electroactive species minus the current measured for mixed solvent and electrolyte alone) were used in all quantitative analyses of the cyclic voltammetry data. All quantitative analyses of CV data are based on the average of three independent measurements made for three freshly prepared mixed-solvent solutions of cyt c.

Resonance Raman Spectroscopy (RR). Resonance Raman measurements were recorded on a home-built spectrometer, which has a Coherent INNOVA 302 Kr^+ ion laser (6 W all lines) as its excitation source, liquid nitrogen-cooled CCD detector (Princeton Instruments), and a computer-controlled SPEX-1877 triple spectrometer (0.6 m, f/6.3). Samples, contained in 5 mm NMR tubes (Wilmad), were spun with a Raman 101 spinner (Princeton Photonics Instruments) and were typically excited with <50 mW of 406.7 nm light and were sampled with a backscattering configuration. Ferrous cytochrome c was excited using <50 mW of 413.1 nm light. The resolution of the spectrometer is ca. 3 cm^{-1}. All the RR spectra were recorded at room temperature. Absorption spectra (250−800 nm) were measured immediately prior to and following each RR experiment. No degradation was detected under the conditions used in this study. RR spectra were calibrated using fenchone[45] (500−1700 cm^{-1}) or toluene (200−1700 cm^{-1}) as external frequency standards. The vibrational features of the organic solvent were subtracted from all mixed solvent spectra. All spectral data were processed with Grams/32 Spectral Notebase software (Galactic Industries Corp.). None of the Raman spectra were smoothed.

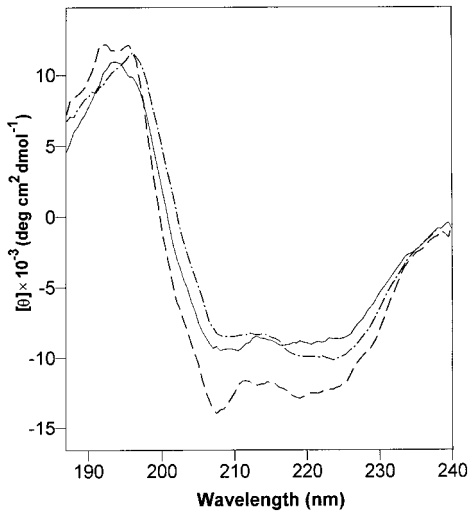

Figure 1. UV circular dichroism spectra (185−240 nm) of 4 μM ferricyt c in (—) 30% ACN solution, (-·-·-) aqueous solution at pH 7, (- - -) aqueous solution at pH 10. All CD spectra shown were smoothed using an 9-point Savitsky−Golay algorithm.

Results

CD Spectroscopy. The secondary structure of the protein matrix in ferricyt c has been probed using UV circular dichroism spectroscopy. Figure 1 shows the UV circular dichroism spectra for native ferricyt c at pH 7, alkaline cyt c at pH 10 and ferricyt c in 30% ACN solution. All three spectra show a relatively intense negative CD feature near 210 nm that is attributable to the characteristically high α-helical content of cyt c.[46,47] Quantitative deconvolution of the UV CD spectrum (185−240 nm) for aqueous alkaline cyt c, pH 10 using a singular value decomposition approach[48] and ridge regression[47] indicates that the α-helical content of alkaline cyt c is approximately 5% lower than that of native ferricyt c. While quantitative deconvolution of the secondary structure of cyt c in mixed solvents is strictly prohibited due to the lack of quantitative information for the CD signatures of the various partial secondary structures in nonpolar media, we have deconvoluted the UV−CD of ferricyt c in 30% ACN and found that the α-helical content is 8% lower in 30%ACN as compared to aqueous solution. The overall similarity between the UV CD spectra for native ferricyt c in aqueous and 30% ACN solution suggests that the protein secondary structure is not significantly perturbed in mixed solvent media and that the secondary structure of ferricyt c in 30% ACN is slightly different from that of native ferricyt c and that of aqueous alkaline cyt c, pH 10.

Figures 2 and 10[41] show the effect of mixed media on the visible CD spectrum for ferricyt c. As shown in Figure 2, when the concentration of ACN increases, the CD spectrum for ferricyt c in the mixed solvent is characterized by a marked decrease in the intensity of the intense negative Cotton effect at 417 nm and a concomitant increase in the intensity of the positive Cotton effect at 398 nm. In DMF or DMSO (Figure 10),[41] the visible CD spectrum of ferricyt c retains the characteristic dichroic signature of ferricyt c in aqueous solution until the organic solvent content reaches approximately 25%. These changes indicate a change in heme polypeptide interactions in the vicinity of the heme active site. All of the spectral changes observed in the visible CD of cyt c in mixed solvents are consistent with

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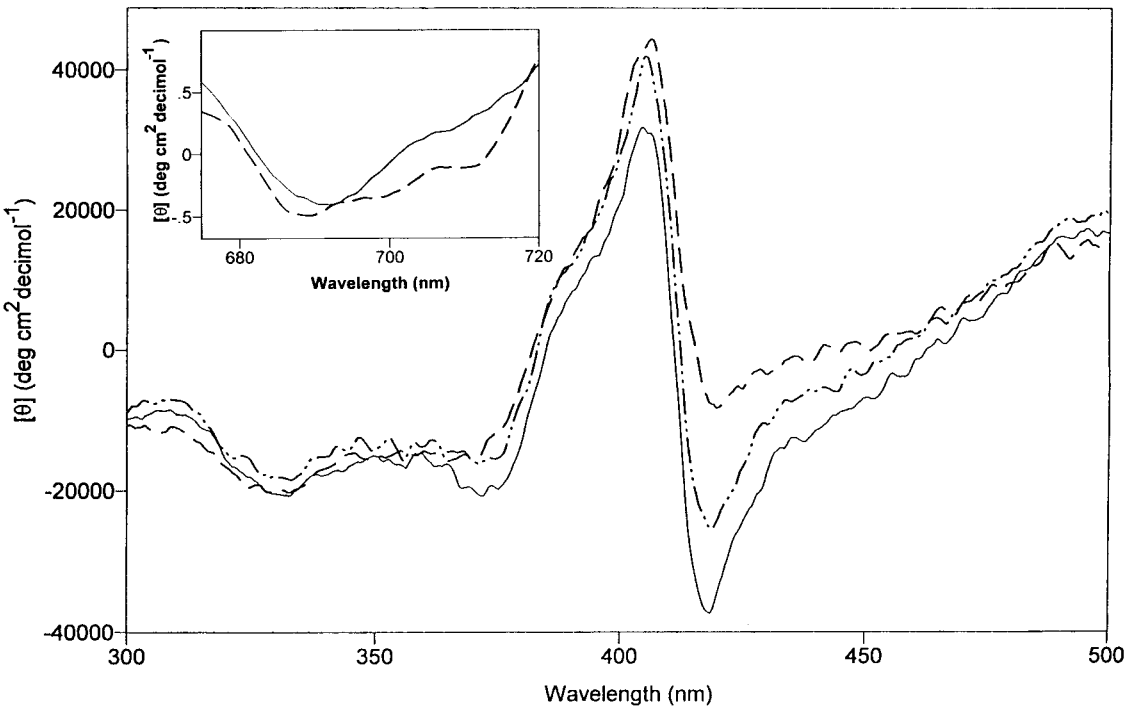

Figure 2. Circular dichroism spectra (300−500 nm) of 10 μM ferricyt c in (—) 10%, (-···-···-) 15%, and (- - -) 25% ACN solutions. The inset shows the 695 nm band of cyt c in aqueous and 30% ACN solution. All CD spectra shown were smoothed using an 11-point Savitsky−Golay algorithm. Inset shows circular dichroism spectra (675−720 nm) of 0.6 mM ferricyt c in (—) aqueous and (- - -) 30% ACN solutions. Both the CD spectra were smoothed using 31-point Savitsky−Golay algorithm.

an increase in the planarity of the ferric heme moiety in mixed solvent media.

The CD properties of the 695 nm band were also investigated. Senn and Wuthrich[49] have correlated the sign and magnitude of the CD signal for the 695 nm band with the orientation in which Met80 binds to the heme iron. As shown in the inset to Figure 2, the sign of the CD signal for the 695 nm band remains the same (negative) in ACN as that for native ferricyt c. The intensity of the 695 nm CD signal decreases in a manner consistent with the decrease in the intensity of this feature in the UV−vis spectrum (vide supra). This observation suggests that the mode of binding for the axial Met80 ligand and the local protein secondary structure are unperturbed by the solvent medium.

Resonance Raman. Ferricytochrome c. The mode assignments for cyt c have been exhaustively investigated and have been published elsewhere.[50,51] The frequencies of the relatively intense bands in the region between 1300 and 1700 cm^{-1} in heme proteins provide information on the oxidation state, spin state, and coordination number for the active site heme iron as well as geometry information for the heme porphyrin. Frequencies for bands in the low-frequency region between 200 and 800 cm^{-1} provide useful information regarding the interaction of the heme with the surrounding protein matrix (the protein pocket).

As shown in Figures 3 and 4, the RR spectra of ferricytochrome c in mixed solvents show some distinct changes in frequency and intensity in both the high-frequency marker band region (1300−1700 cm^{-1}) and in the low-frequency region (200−800 cm^{-1}), respectively. The Raman spectra of cyt c in DMF and DMSO systems (0−40%) are fairly similar to that of

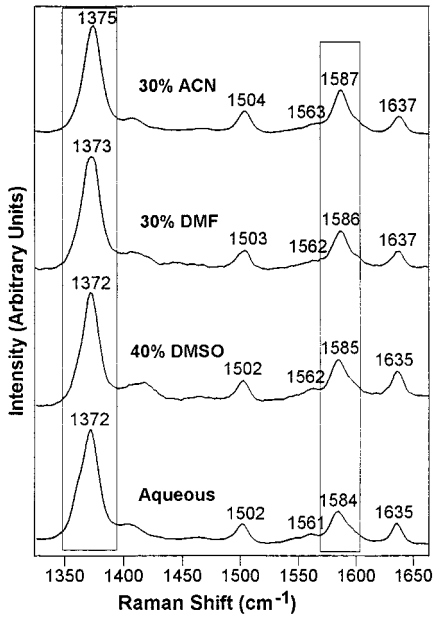

Figure 3. Resonance Raman spectra of 100 μM ferricyt c obtained using 406.7 nm excitation in different solvent media (from top to bottom: 30% ACN, 30% DMF, 40% DMSO, and aqueous), in the marker band region (1300−1700 cm^{-1}), recorded using a spinning sample tube with backscattering geometry. Each spectrum was obtained by averaging 300 10-s transients.

cytochrome c in aqueous solution, pH 7.0. The frequencies of several key marker bands, specifically, ν_4 (1372 cm^{-1}), ν_2 (1585 cm^{-1}), ν_3 (1502 cm^{-1}), and ν_{10} (1635 cm^{-1}), are all consistent with a six-coordinate low-spin ferric heme as in native ferricyt c, pH 7.[51]

However, significant differences in intensity and frequency are observed for these key vibrational marker bands for ferricytochrome c in ACN (>20%) solution. Indeed, frequency

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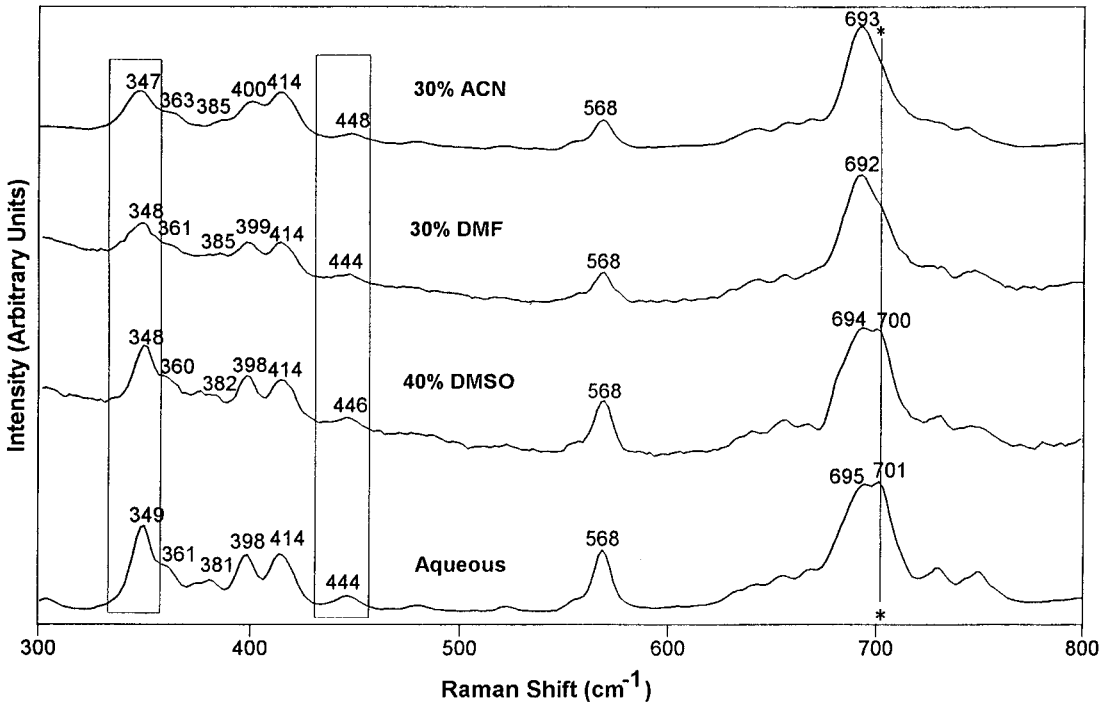

Figure 4. Resonance Raman spectra of 100 μM ferricyt c obtained using 406.7 nm excitation in different solvent media (from top to bottom: 30% ACN, 30% DMF, 40% DMSO, and aqueous), in the low-frequency region (300−800 cm^{-1}), recorded using a spinning sample tube with backscattering geometry. Each spectrum was obtained by averaging 300 10-s transients.

shifts are observed for all of the high-frequency skeletal modes. The oxidation state marker band, ν_4, shifts from 1372 cm^{-1} to 1375 cm^{-1} in 30% ACN. ν_3, ν_2, and ν_{10} all upshift by approximately 3 cm^{-1}. These changes are consistent with a significant contraction of the porphyrin core size and a change in Fe → porphyrin back-bonding. In addition, the intensities of ν_3 and ν_{10} decrease relative to the intensity of ν_2. These changes in intensity and frequency of the marker bands are very similar to those previously reported for alkaline cyt c.[1]

In the low-frequency region as shown in Figure 4, ν_7 (701 cm^{-1}) disappears. The symmetric pyrrole folding mode, γ_{21}, at 568 cm^{-1}, a thioether bending vibration, $\delta(C_\beta C_\alpha S)$, at 398 cm^{-1}, γ_{22}, the out-of-plane pyrrole swivel mode at 444 cm^{-1}, and ν_8 at 349 cm^{-1} decrease markedly in intensity. In addition, γ_{22}, the out-of-plane pyrrole swivel mode, and ν_{50} at 361 cm^{-1} upshift by 4 and 2 cm^{-1}, respectively. The intensity of γ_{21}, a pyrrole folding mode, is believed to be activated by nonplanar distortion of the porphyrin and has been proposed to be a useful monitor of the geometry of the heme.[51] Finally, a new mode is observed at 385 cm^{-1}, which has been attributed to the Fe−N(Lys) stretch in the alkaline form of cyt c.[1] Overall, the spectral changes are consistent with a change in axial ligation and a significant decrease in porphyrin doming accompanied by significant rearrangement of the thioether linkages at the heme periphery in ACN solution.

The vibrational frequencies and relative intensities in both the marker band and low frequency spectral regions are remarkably similar to those recently attributed to the alkaline conformers of cyt c by Dopner et al.[1] Therefore, we have simulated the low-frequency Raman spectra we obtained for cyt c in mixed ACN solutions using Dopner et al.'s[1] component Raman spectra assuming a mixture of native ferricyt c (III), and alkaline conformers (IVa, IVb, Va, and Vb). The results of our analysis are summarized in Figure 5. Figure 5a shows the relative contributions of the native and alkaline conformational species in solutions containing 0 (top), 15% (middle), and 30% (bottom) acetonitrile. Since different mixed solvent compositions correspond to media of different dielectric constant, we have examined the correlation between the native and alkaline conformers with the dielectric constant of the mixed solvent media. Figure 5b shows the relative contributions of the native and alkaline forms as a function of the dielectric constant of the mixed solvent medium. As the dielectric constant of the solvent medium decreases from 76 to 72, the lysine-ligated alkaline conformers IVa and IVb become the dominant species. As the dielectric constant decreases from 72 to 68, the relative contributions from the hydroxide-ligated alkaline conformers Va and Vb begin to increase and become significant. Remarkably, the changes we observe as the dielectric constant decreases parallel closely those reported by Dopner et al.[1] for yeast iso-1-cytochrome c in aqueous solution as the pH is raised from pH 7 to 10 (compare Figure 3 in Dopner et al. [1] and Figure 5b in this paper). The composition of the alkaline mixture of ferricyt c in 30% ACN that reflects the extreme nonpolar limit in our study is comparable to that observed in aqueous solution at pH 10.3.

Ferrocytochrome c.[41] The RR spectrum of ferrous cyt c in the mixed solvents is remarkably similar to the resonance Raman spectrum of ferrous cyt c in aqueous solution at pH 7 (Figures 11 and 12).[41] The similarity of the aqueous and nonaqueous Raman spectra in the marker band region suggests that the heme active site ligation and structure of the ferrous form in mixed solvents is the same as that in aqueous solution at pH 7. This finding was not unexpected since it is well-known that ferrocytochrome c in aqueous solution retains Met80 as the sixth axial ligand in the pH range of 4 to 12.[52]

Cyclic Voltammetry. The electrochemistry of cyt c in aqueous mixtures of 0 to 40% DMSO, DMF, and ACN at BPD-modified Au electrodes was investigated by cyclic voltammetry at scan rates between 5 and 200 mV/s. Figure 6 shows the

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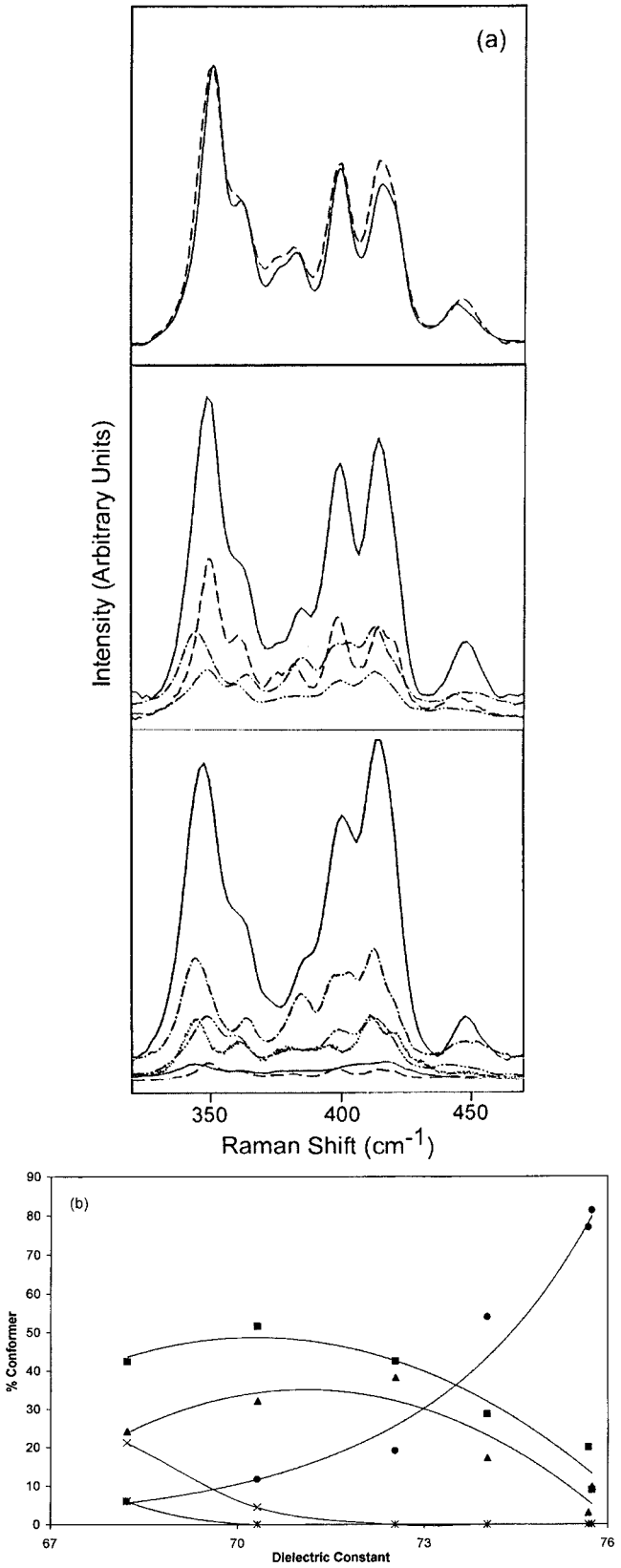

Figure 5. (a) Resonance Raman spectra of 100 μM ferricyt c in 0 (top), 15% (middle), and 30% (bottom) mixed ACN solution showing the relative contributions of the different contributing conformers, specifically, III (– – –), IVa (–• –•), IVb (–•• –••), Va (•••), and Vb (—). The solid line indicates the experimental spectrum. (b) Dependence of the conformational equilibria of ferricyt c in mixed ACN solution on the dielectric constant of the medium. Relative concentrations of the various conformations are represented by the following symbols: III (●), IVa (■), IVb (▲), Va (×), and Vb (*).

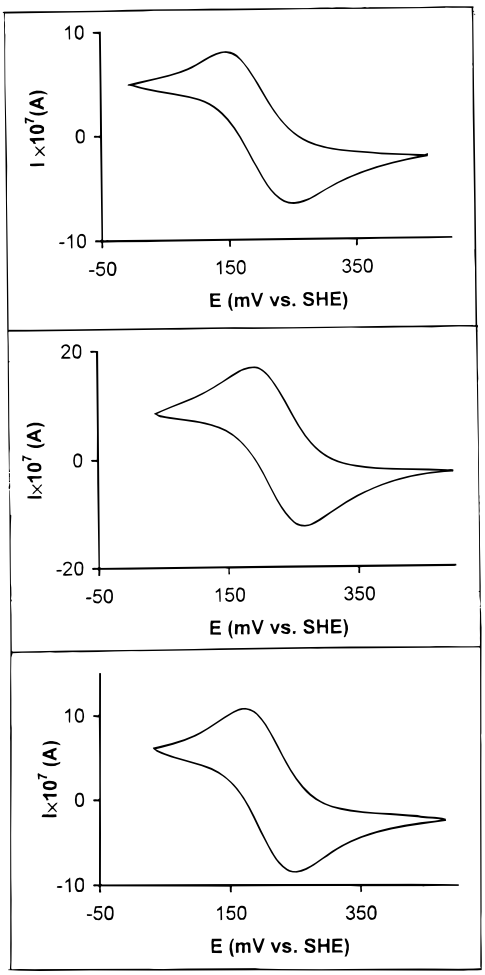

Figure 6. Cyclic voltammograms of (top) 3.2 mM cyt c in 40% DMSO; (middle) 2.4 mM cyt c in 30% ACN; (bottom) 2.9 mM cyt c in 30% DMF. Experimental conditions: BPD-modified gold disk electrode with 1.2 mm diameter; 20 mV/s, scan rate; 295 K, temperature; 0.1 M $NaClO_4$, supporting electrolyte. Potentials are referenced to SHE.

representative cyclic voltammetry for cyt c in 40% DMSO, 30% ACN, and 30% DMF solutions. These solutions represent the extrema studied. In each case, the voltammograms show one well-defined redox wave. Voltammograms (−800 to +600 mV vs Ag/AgCl) obtained in 30% ACN solutions at naked Au and peak currents for both the anodic and cathodic redox processes from those obtained at BPD-modified Au surfaces scan rates between 5 mV/s and 2 V/s (not shown) were indistinguishable in terms of peak potentials at BPD-modified Au surfaces.

Resonance Raman was used to probe for structural changes in ferricyt c in 30% ACN due to the addition of BPD. Raman spectra (300−1700 cm^{-1}) showed no significant change in the intensity or frequency of any vibrational feature. Together both the voltammetry and resonance Raman data indicate that the voltammetry at BPD-modified Au is not affected by the use of a promoter-modified electrode and that BPD does not directly interact with the heme active site of cyt c.

Pyrolytic graphite was also investigated as an electrode substrate. However, when naked pyrolytic graphite (PG) was used, nonaqueous solutions exhibited an irreversible reduction at ≈ -300 mV vs SHE. This response was observed to persist at PG after the electrode had been rinsed and replaced in fresh solvent containing only supporting electrolyte. This observation suggests that the irreversible low potential reduction wave at PG is due to the presence of a strongly adsorbed species.

Table 1. Electrochemical Characteristics of Horse Cytochrome c in Selected Mixed Solvents

solvent system	$E°$, V vs SHE	ΔE_p, V @ 20 mV/s	I_c/I_a	$D_0 \times 10^6$, cm^2/s	$k_s' \times 10^3$, cm/s
aqueous	0.274 ± 0.002	0.075	1.0	1.28 ± 0.03	3.54 ± 0.05
40% DMSO	0.199 ± 0.004	0.100	0.9	0.22 ± 0.05	0.44 ± 0.16
30% ACN	0.223 ± 0.004	0.074	0.9	1.26 ± 0.30	2.76 ± 0.60
30% DMF	0.210 ± 0.002	0.077	1.0	0.39 ± 0.05	1.40 ± 0.17

The voltammograms in mixed media at BPD-modified Au exhibited no significant variation in ΔE_p or I_p with continuous cycling over a 2-h time period. The ratio of the anodic and cathodic peak currents (I_{pa}/I_{pc}) was close to 1.0 (see Table 1) for all the mixed solvent systems. These characteristics are consistent with a chemically reversible electrochemical process. The peak-to-peak potential separations of the anodic and cathodic waves (ΔE_p) for cyt c in the mixed solvent system investigated were dependent on scan rate (70−148 mV) and increased as the scan rate increased from 5 mV/s to 200 mV/s in 40% DMSO. Similar behavior was observed in the other mixed solvent systems studied. Taken together, these data are consistent with a one-electron quasireversible electron-transfer process.

The anodic and cathodic peak currents were linearly related to $\nu^{1/2}$ ($r^2 \geq 0.997$) at sweep rates between 5 mV/s and 20 mV/s, as expected for a diffusion-controlled redox process. The average diffusion coefficient (D_o) for cyt c, for each concentration of organic solvent, was calculated from the slope of these plots, using the Randles−Sevcik equation. As shown at the top of Figure 9, the diffusion coefficient was solvent dependent and decreased as the concentration of organic solvent increased. The decrease was most marked in the case of DMSO which is a relatively viscous solvent and is in line with our expectations for DMSO derived from previous study of the voltammetry of MP-11[4,5] and N-Ac-MP-8[53] in DMSO (<1% water).

For each mixed solvent system, the heterogeneous electron-transfer rate constant, k_s, was calculated from ΔE_p at 20 mV/s using the calculated diffusion coefficient by Nicholson's method,[54] assuming $n = 1$, $\alpha = 0.5$, and $T = 25$ °C (Table 1). The heterogeneous rate constant of cyt c in aqueous solution was found to be $(3.54 \pm 0.05) \times 10^{-3}$ cm/s, which is similar to the value previously reported by Taniguchi (ca. 5×10^{-3} cm/s) at BPD-modified Au electrodes in aqueous solution.[26,28]

As shown at the bottom of Figure 7, the heterogeneous electron-transfer rate constant in mixed solvents decreases as the concentration of the organic solvent increases. In the case of ACN−water system, the rate constant decreases at lower concentrations of organic solvent (<20%), but increases sharply as the concentration of the organic solvent is increased.

Exposure to organic solvents appears to modulate the redox potential of cytochrome c. However, the redox potential remains high close to the value of cyt c in aqueous solution at pH 7 (0.237 ± 0.053 V vs SHE). In the range of organic solvent concentrations of interest, a linear correlation ($r^2 > 0.937$) was observed to exist between the organic solvent concentration and the redox potential of cyt c as shown in Figure 8. Remarkably the correlation is independent of the identity of the specific organic solvent. This observation supports the generally held view that the redox potential of cytochrome c is largely determined by the heme ligation set and the surrounding relatively hydrophobic protein matrix. However, the direction of the shift in the redox potential is not as expected—the redox potential of cyt c decreases as the polarity of the solvent medium decreases.

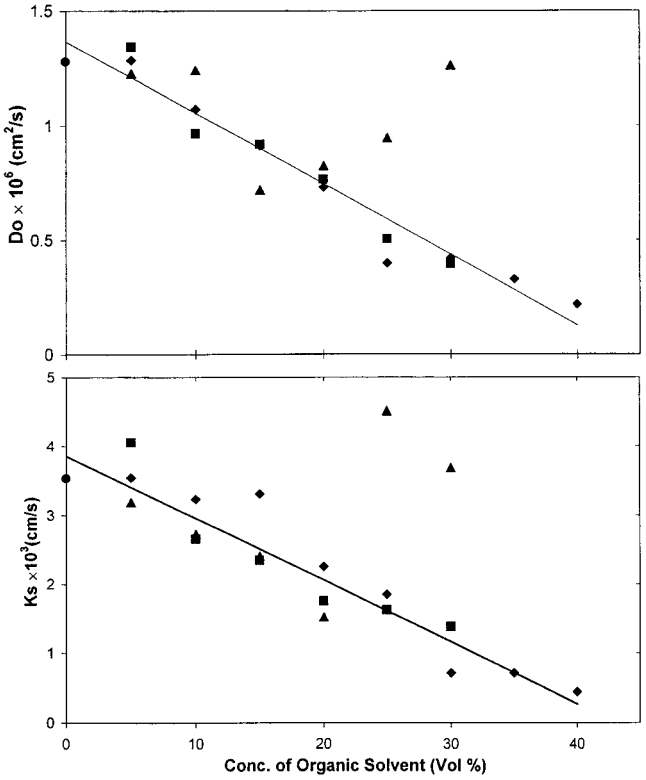

Figure 7. Plot of (top) calculated diffusion coefficient and (bottom) measured heterogeneous rate constant for cyt c vs the concentration of organic solvent in vol %. in (●) aqueous, (◆) DMSO, (■) DMF, and (▲) ACN solution. k_s' calculated from ΔE_p at 20 mV/s. k_s' values represent the average of three independent determinations.

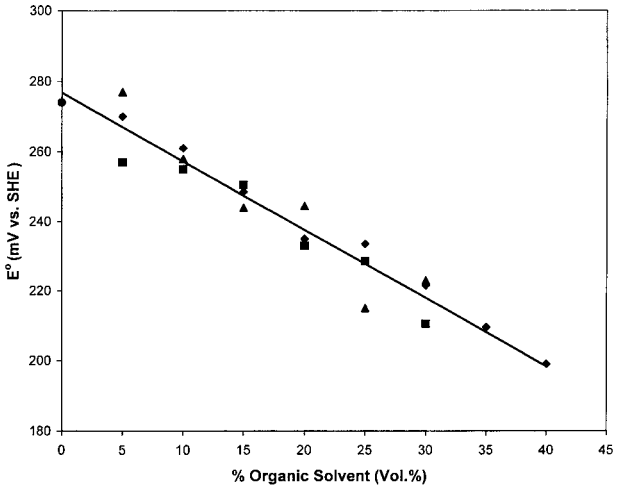

Figure 8. Plot of $E°$ for cyt c vs the vol % organic solvent of the solvent medium for (●) aqueous, (◆) DMSO, (■) DMF, and (▲) ACN solutions. Potentials in mV are referenced to SHE.

Discussion

Similar spectroscopic and electrochemical characteristics have been observed for $Fe^{2+/3+}$ cyt c in the three mixed solvent systems studied: DMSO ($\epsilon = 46.3$), ACN ($\epsilon = 36.0$), and DMF ($\epsilon = 37.0$).[55] The spectroscopic and electrochemical changes

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observed as the organic solvent concentration increases are gradual and appear to be dependent primarily on the dielectric constant of the mixed solvent medium. This conclusion is supported by the fact that mixed solvent systems with comparable dielectric constants show similar spectroscopic and electrochemical characteristics. For example, the UV−vis, CD, and RR spectra for cyt c in 40% DMSO ($\epsilon = 75.7$) are similar to those of cyt c in 10% ACN ($\epsilon = 75.7$). Aqueous mixtures of ACN, which has the lowest dielectric constant of the three organic solvents studied, exhibit the largest spectroscopic and electrochemical differences as compared to aqueous solution. Thus, the reactivity, spectroscopic, and electrochemical characteristics of ferricyt c in 30% ACN are significant in that they reflect the extreme nonpolar solvent limit investigated in the present study.

The spectroscopic characteristics of ferricyt c in 30% ACN are consistent with the presence of a mixture of conformers with active site structure similar to that of the alkaline type IV and V cyt c's. For example, in the UV−vis, ferricyt c in 30% ACN exhibits a blue shifted Soret band and a relatively weak 695 nm band (see Figure 9[41] and Table 2,[41]). In the resonance Raman spectrum, both the frequencies and relative intensities for the vibrations in the low-frequency region have been successfully modeled using the pure IVa, IVb, Va, and Vb conformer spectra recently reported by Dopner et al.[1] for yeast 1-isocytochrome c. Overall the structural changes reflected in the resonance Raman spectra in mixed media are consistent with a decrease in heme doming, significant structural rearrangement at the heme periphery affecting the thioether linkages and one or both of the propionates, and a concomitant change in axial heme ligation from Met80 to Lys73, Lys79, or OH^-.

It is clear, however, that while the electronic structure and redox properties of the ferricyt c in mixed media are very similar in some respects to that of aqueous type IV and V alkaline ferricyt c, there are also significant differences between the structure and function of ferricyt c at alkaline pH and ferricyt c in mixed solvents. For example, on the basis of the UV CD signature for ferricyt c in 30% ACN (see Figure 1), the secondary structure of cyt c in mixed media exhibits a ~3% less α-helical structure than that of alkaline cyt c.

Differences are also apparent in the redox properties of cyt c at alkaline pH and in mixed media. The redox potential in mixed media (0.249 ± 0.036 V vs SHE: 0−30% ACN), though dependent on the organic solvent concentration, is significantly higher than the redox potential previously reported for horse cyt c at pH 10 (+0.11 V vs SHE)[56] or that for aqueous alkaline yeast iso-1-cyt c, which was recently determined to be −0.205 V vs SHE at PG at pH 10.[12]

The spectroscopic changes we have observed for ferricyt c in mixed media make it clear that a change in axial ligation involving Met80 occurs and that Met80 is retained as the axial ligand in the reduced form of cyt. Consequently, oxidation and reduction of cyt c in mixed media must necessarily be accompanied by a significant structural change involving Met80 ligation. Since the voltammetric response at Au in mixed media is quasireversible, the conformational change associated with the change in axial ligation must occur fairly rapidly. This suggests that the replacement ligand must be nearby and that the conformational change must be limited to a short segment of the protein chain in the vicinity of Met80. The UV CD data for ferricyt c support a limited local conformational change for ferricyt c in mixed media that is different from that of alkaline cyt c.

One possible trigger for the structural change in mixed media involves rupture of the hydrogen-bonding network. The hydrogen-bonding network is known to be important in determining the redox potential and stability in cytochrome c.[57] In the hydrogen-bonding network Tyr67, a highly conserved residue, plays a central role.[8] It is in the vicinity of the heme and is bound to a water molecule (WAT166), that is hydrogen-bonded to Met-80, the proximal ligand. This hydrogen-bond is broken in the reduced protein.[58] Movement of this water is believed to be important in the redox transition.[10] Loss of this hydrogen-bonding interaction due to the formation of OH^- has been proposed to be important in the formation of alkaline cyt c at basic pH.[59]

Mutations of Tyr67 that abolish this hydrogen-bonding interaction produce a 30−50 mV decrease in the redox potential.[60,61] Moreover, there is a decrease in the stability of the Fe−Met80 ligation, which is apparent by their tendency to form alkaline-like species.[60] These characteristics are very similar to those we have observed for cyt c in mixed solvents; specifically, cyt c shows a 30−75 mV decrease in the redox potential in the mixed media. The formation of alkaline-like species at relatively high concentrations of organic solvent (20−40%) (see Figure 5b) implies a decrease in the stability of the Fe−Met80 bond. The similarity of these characteristics to those of the Tyr67 mutants suggests that a rupture in the hydrogen-bonding network may also be responsible for structural changes observed for cyt c in mixed solvents. The limited loss of α-helical content in mixed solvents suggests that any conformational change must be highly localized. Rupture in the hydrogen-bonding network involving Tyr67 would likely affect nearby residues and therefore the α-helix involving residues 70−75. Additional support for this proposal is provided by UV−RR studies of partially folded ferricyt c at an acidic pH have shown a disruption of the hydrogen-bonding network involving Tyr67 and Tyr74 signaling flexibility in this segment of the protein.[62]

The differences in the electrochemistry of cyt c in mixed media and at alkaline pH may be due to differences in the heme crevice and local structural flexibility near the heme crevice for cyt c in mixed media. Limited loss of organized secondary structure, as indicated by UV−CD, may translate into greater flexibility for the polypeptide segment involved. Increased flexibility would be required to facilitate the rapid change in axial ligation from Fe−N(Lys) or Fe−OH for ferricyt c to Fe−S(Met) in ferrocyt c that is reflected in the quasireversible voltammetry which characterizes the redox change of cyt c in mixed media.

Implications for Biological Electron Transfer. Electrochemically, we see no evidence that the type IV and type V alkaline forms have different redox potentials even though spectroscopically we know that more than one form is present (see Figure 5b). The full-width-at-half-maximum of the oxidation and reduction waves at Au was constant and unaffected by the concentration of organic solvent. The constancy of the width of the redox waves with increasing concentration of acetonitrile suggests that alkaline conformers IV and V must

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have very similar redox potentials. Since the axial ligation varies significantly in going from native (Met80) to Type IV (Lys79, Lys73) to Type V (hydroxide), the constancy of the redox potential in going from aqueous to 30% ACN solution suggests that axial ligation is not a major factor determining the redox potential of cyt c.

So, what determines the redox potential of cyt c? Our study suggests that at least for cyt c Kassner was correct.[63] The internal dielectric constant of the protein and the dielectric constant of the protein solvent largely determine the redox potential of cyt c. Solvent affects the redox potential directly only to the extent that the solvent affects protein structure and therefore the internal dielectric constant of the protein.

Does solvent affect the electron-transfer rate? If we use k_s', the heterogeneous electron transfer rate constant, as a measure of the redox activity of cytochrome c in mixed media, clearly the redox activity of cyt c is not deleteriously affected by organic media in the concentration range investigated in this work (0−40%). In fact, the rate for heterogeneous electron transfer involving horse cyt c at Au in 30% ACN is as high as that for native cyt c in aqueous solution at pH 7 (see Table 1). Therefore, the heterogeneous electron transfer rate is not significantly affected by the polarity of the medium.

Are our findings in mixed media relevant to the function of cyt c within the inner mitochondrial membrane? Studies by Ashcroft et al.[64,65] suggest that the effective dielectric constant of the mitochondrial membrane decreases sharply from about 30−60 at the outside of the membrane to around 2.1 on the inside where cyt c is found. The redox potential of membrane-bound cyt c is ∼30−40 mV lower than that in free solution.[66] Our study indicates that the structure of cyt c has been designed in such a way as to maintain a relatively high, constant redox potential even in low dielectric constant media, like the inner mitochondrial membrane. Furthermore, the similarities we have observed between ferricyt c in mixed media and alkaline cyt c suggest that alkaline transition may have greater relevance than previously thought in biological electron transport.

Implications for Nonaqueous Enzymology and Bioelectrochemistry. In nonaqueous enzymology, the most useful solvents have generally been found to be those that are relatively hydrophobic and nonpolar. In contrast, thus far in nonaqueous bioelectrochemistry polar solvents have produced the best results. The results of our study suggest that nonaqueous bioelectrochemistry is most likely to be successful when solvents such as DMSO with dielectric constants similar to that of water are used since these solvents are likely to induce less structural change. In fact, we have recently demonstrated proof-of-concept for the direct electrochemical oxidation/reduction of myoglobin in DMSO.[67] If solvents such as ACN with much lower dielectric constants are to be investigated, our study suggests that the role of water and hydrogen-bonding in the structure and function of the biomolecule must first be assessed.

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Supporting Information Available: Experimental details for pH adjustments, introduction of cyt c into mixed solvents, optical absorption measurements; results of UV−vis spectroscopy, resonance Raman study of ferrocyt c and plots of UV−vis spectra (Figure 9), visible CD spectra of cyt c in aqueous, DMF, and DMSO solution (Figure 10), resonance Raman spectra of ferrocyt c in low frequency (Figure 11) and high frequency (Figure 12), and the UV−vis characteristics of cyt c in mixed solvents (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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