Insights into the alkaline transformation of ferricytochrome c from ^1H NMR studies in 30% acetonitrile–water

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Abstract

Recently, we found that ferricytochrome c (ferricyt c) undergoes significant structural changes in mixed aqueous–nonaqueous media, resulting in the formation of a mixture of alkaline-like species. The equilibrium composition of this mixture of species is dependent on the dielectric constant of the mixed solvent medium. One-dimensional (1D) and two-dimensional (2D) ^1H nuclear magnetic resonance (NMR) methods have now been used to study these alkaline-like forms in 30% acetonitrile–water solution. A native-like (M80-ligated) III* form, two lysine-ligated forms (IVa* and IVb*), and a hydroxide-ligated form (V*) were observed. Heme proton resonance assignments for these forms were accomplished using 1D ^1H NMR and 2D nuclear Overhauser effect spectroscopy methods at 20°C and 35°C. The chemical exchange between the alkaline forms in 30% acetonitrile solution facilitated heme proton resonance assignments. Based on examination of the heme proton chemical shifts and several highly conserved amino acid residues, the electronic structure, secondary structure, and hydrogen bond network in the vicinity of the heme in the III* form were found to be intact. Similarly, the heme electronic structure of the IVa* form was found to be comparable to that of the IVa form. Differences in the order of the heme methyl resonances in the IVb* form, however, suggest that the heme active site in this form is somewhat different from that observed in aqueous alkaline solution. In addition, resonance assignments for the 8- and 3-methyl heme protons were made for the hydroxide-ligated V* form for the first time. The observation of chemical exchange peaks between all species except IVb* and IVa* or V* was used to propose an exchange pathway between the different forms of ferricyt c in 30% acetonitrile solution. This pathway may be biologically significant because ferricyt c, which resides in the intermembrane space of mitochondria, is exposed to medium of relatively low dielectric constant when it interacts with the mitochondrial membrane.

Keywords: Cytochrome c; alkaline transition; NMR; nonaqueous enzymology; heme protein

Recently, we probed the structure and redox function of horse ferricytochrome c (ferricyt c) and ferrocytochrome c in aqueous mixtures of three different water-miscible organic solvents, specifically, acetonitrile (ACN), dimethyl formamide, and dimethyl sulfoxide, containing 100%–60% water (Sivakolundu and Mabrouk 2000). We found that fer-

ricyt c undergoes significant structural change in mixed solvents, producing a mixture of up to five different forms, depending on the dielectric constant of the solvent media. The concentration profile of these species at decreasing dielectric constant closely parallels that at increasing alkaline pH (Dopner et al. 1998).

In alkaline solution, ferricyt c is known to exist as a mixture of five species distinguished by their axial ligand (Greenwood and Palmer 1965; Moore and Pettigrew 1990; Scott and Mauk 1996). In four of these forms, M80, which is the sixth axial ligand in native ferricyt c at pH 7, is replaced by lysine (IVa and IVb) or hydroxide (Va and Vb).

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Extensive nuclear magnetic resonance (NMR) studies (Ferrer et al. 1993; Banci et al. 1995; Pollock et al. 1998) of ferricyt c and several variants at neutral and alkaline pH, have identified two of the ligands: K73 (IVa form) and K79 (IVb form). Resonance Raman spectroscopic analysis of cytochrome c (cyt c) at alkaline pH has confirmed the presence of two additional species identified (Dopner et al. 1998), which are believed to bind hydroxide as the sixth axial ligand (Va and Vb).

NMR spectroscopy is a powerful technique and well suited for the study of the structure of heme proteins like cyt c (Bax 1984; Wider et al. 1984; Bertini et al. 1993; Croasman and Carlson 1994). Indeed, ferricyt c has been extensively studied by one-dimensional (1D) and two-dimensional (2D) NMR techniques (Redfield and Gupta 1971; Gupta et al. 1972; Feng et al. 1989, 1990; Banci et al. 1997). Due to the presence of paramagnetic iron in ferricyt c, the proton NMR spectrum has an unusually wide chemical shift range (−40 to 30 ppm). The heme protons and the protons from amino acid residues spatially close to the heme are hyperfine shifted outside the usual diamagnetic envelope of 0–10 ppm. The hyperfine shift of these resonances reduces the degree of crowding of resonances in the diamagnetic region and has simplified the assignment of essentially all of the proton resonances for the 104 amino acid residues. This has greatly facilitated determination of the solution structure of ferricyt c using 2D NMR methods (Qi et al. 1994, 1996; Banci et al. 1997). Because of the aforementioned utility of NMR in the past studies of cyt c in aqueous solution, we investigated the application of 1D and 2D ^1H NMR methods

to probe the structure of the alkaline-like forms of ferricyt c in 30% ACN solution.

Results

Resonance assignments

Figure 1A shows the NMR spectrum of ferricyt c in 30% ACN at 20°C. The pattern of resonances is similar to that observed for ferricyt c in aqueous alkaline solution (Fig. 1B). The complex pattern of the resonances observed in the region between 21 and 25 ppm, due to the 8- and 5-methyl heme protons, signifies the presence of lysine-ligated alkaline-like forms in 30% ACN (Gadsby et al. 1987; Santos and Turner 1987; Hong and Dixon 1989; Ferrer et al. 1993; Banci et al. 1995; Rosell et al. 1998). The proton resonances at 36 ppm and 33 ppm, which are due to the 8- and 3-methyl heme protons, are characteristic of the M80-ligated (III) form. The similarity of the chemical shift patterns in the spectra of ferricyt c in aqueous alkaline solution and 30% ACN solution (Fig. 1C) indicates that ferricyt c does indeed exist as a mixture of M80-ligated and lysine-ligated alkaline-like species in 30% ACN solution. This observation is consistent with the findings of our previous resonance Raman study (Sivakolundu and Mabrouk 2000).

Assignments for individual proton heme resonances were made using a three-pronged strategy. Preliminary assignments were made by comparison of the chemical shift and spin-lattice relaxation time (Table 1) with assignments pre-

Fig. 1. One-dimensional ^1H nuclear magnetic resonance (NMR) of 2 mM ferricytochrome (ferricyt) c recorded at 20°C in (A) 30% acetonitrile (ACN) and 70% D_2O (pH^* 6.9), (B) phosphate buffer (pH^* 10.8), and (C) phosphate buffer (pH^* 7). (m) Methyl.

Table 1. Assignment for proton resonances of ferricyt c in 30% acetonitrile solution

Assignments		III* form	IVa* form	IVb* form	V* form
Meso- δ	CH	−2.75	−6.06	−5.68	
Ring-1	CH ₃	7.20 (78)	12.93	13.64	
Ring-3	CH ₃	32.44 (91 ¹)	11.45	11.81	12.10 ^a
Ring-5	CH ₃	9.77 (79)	21.11	21.11	
Ring-8	CH ₃	35.74 (83)	22.87 (127)	21.56 (107)	24.50 ^b (58°)
Propionate-7	C α H ₂ (17-1b)	19.14	−1.02	−0.81	
	(17-1a)	11.61 (101)	0.00		
	C β H ₂ (17-2a)	1.51	2.00		
	(17-2b)	−0.59	0.98		
Propionate-6	C α H ₂ (13-1a)	−1.33			
	(13-1b)	1.95			
Bridge-2	CH ₃	−2.63			
Bridge-4	CH	2.17			
	CH ₃	3.10			
H18	C β H ₂	9.00			
		14.60			
	C5H	24.50 (8)			
W59 ζ -2	CH	7.51			
P30	C α H ₂	−4.43			
		−0.90			
	C ζ H	−2.32			

The temperature is 20°C unless otherwise specified. The T_1 (msec) values, calculated at 500 MHz and 20°C, are given in parentheses for peaks, which were well resolved.

^a At 35°C.

^b Composite peak.

^c At 40°C.

viously made for aqueous alkaline ferricyt c whenever possible. Assignments were then verified and refined by thoughtful examination of the presence or absence of cross peaks in the 2D nuclear Overhauser effect spectroscopy (NOESY) spectra obtained in different solvent mixtures (15% and 30% ACN) as a function of temperature (20–45°C). Figures 2 and 3 show representative 2D NOESY spectra for cyt c in 30% ACN at 20°C and 35°C, respectively. Temperature was used to distinguish between cross peaks due to a pure NOE mechanism versus those due to a chemical exchange mechanism. Because the poise of the conformational equilibrium is sensitive to both the composition of the solvent medium and the temperature (Hong and Dixon 1989; Sivakolundu and Mabrouk 2000), cross peaks due to chemical exchange were distinguished by their temperature dependence. Temperature was expected to increase the intensity of cross peaks due to proton resonances undergoing exchange but to decrease the intensity of cross peaks due to a pure NOE mechanism (Neuhaus and Williamson 1989). To differentiate between ferricyt c forms in alkaline and ACN–water solution, throughout our discussion we represent the ferricyt c forms in ACN–water solution with an asterisk. Specific arguments for the assignment of individual types (heme methyl, propionate, and meso δ) of heme proton resonances follow.

Heme methyl protons of the M80-ligated form

All of the assigned heme protons of the III* form of ferricyt c in 30% ACN (see Table 1) showed chemical shifts similar to those of native cyt c. The order of the 1-, 3-, 5-, and 8-heme methyl proton resonances, which is known to be dependent on the heme electronic structure and sensitive to the orientation of the heme with respect to the protein matrix (Wuthrich 1970, 1976; Senn et al. 1980), is identical to that of native cyt c (Keller and Wuthrich 1978) (8-Me>3-Me>5-Me>1-Me). Furthermore, the T_1 values of the heme methyl protons varied between 78 and 91 ± 11 msec. The range of relaxation times compares favorably with that reported for ferricyt c in neutral aqueous solution (70–95 msec). This indicates that the heme electronic structure is very similar to that of native ferricyt c (Senn and Wuthrich 1985).

To gain insight into the secondary and tertiary structure of the M80-ligated form in 30% ACN, we probed the interactions of several key amino acid residues, specifically W59 and F82, which are known to be sensitive to the changes in the protein secondary structure and hydrogen bonding effects. W59, a highly conserved residue in ferricyt c, believed to increase the hydrophobicity in the vicinity of the heme (Moore and Pettigrew 1990), hydrogen bonds with the

Fig. 2. Nuclear Overhauser effect spectroscopy (NOESY) spectra of ferricyt c in 30% ACN and 70% D₂O (pH* 6.9) at 20°C; a mixing time of 50 msec was used.

nearby oxygen of the heme propionate-7. Because the distance between the ζ -2 proton of W59 and the 17-1b proton of the heme propionate is 2.3 Å, based on the examination of the 1AKK solution structure (aqueous pH 7) these protons were expected to give rise to a significant NOE cross peak in a native-like M80-ligated form (Banci et al. 1997). Indeed, the ζ -2 proton of W59 of the III* form of ferricyt c in 30% ACN solution shows a significant NOE cross peak with the 17-1b proton of the heme propionate at 20°C, which corresponds to an interproton distance of 1.9 ± 0.2 Å, based on the integration of the NOE cross peak volume. This indicates that the relative position of the W59 residue with respect to the propionate-7 moiety of ferricyt c in 30% ACN solution is unchanged, and the hydrogen bond between the ϵ -1 proton of the W59 residue and the oxygen of the propionate remains intact in the M80-ligated form of ferricyt c in 30% ACN.

The proximity of another conserved residue, F82, believed to play an important role in limiting the solvent access to the heme (Louie et al. 1988), with respect to the

3-methyl heme proton in the III* form of ferricyt c in 30% ACN solution was also examined. In neutral aqueous solution, the 3-methyl proton resonance is split. This distinctive splitting has been attributed to the interaction of F82 with the 3-methyl heme protons (Burns and La Mar 1979, 1981; Moench et al. 1991). In 30% ACN solution, the 3-methyl heme proton resonance of the III* form of ferricyt c is observed to split into two peaks at 10°C (Fig. 4A). Observation of this splitting in the III* form of ferricyt c in the 30% ACN solution indicates that the F82 residue remains in close proximity to the 3-methyl heme proton, as it is in native cyt c in aqueous solution. The preservation of the hydrogen bond network (involving the heme propionate-7) and the proximity of the F82 residue to the 3-methyl heme proton indicates that the secondary structure of ferricyt c in the vicinity of the heme remains intact.

Heme methyl protons of the lysine-ligated forms

As shown in Figure 3, three distinct exchange cross peaks with resonances at 22.21, 21.35, and 24.50 ppm for the

Fig. 3. NOESY spectra of ferricyt c in 30% ACN and 70% D₂O (pH* 6.9) at 35°C; a mixing time of 50 msec was used. Inset shows cross peak of 3-methyl (III*) and 3-methyl (V*) at 2× magnification.

8-methyl heme resonance of the M80-ligated ferricyt c (33.93 ppm) are observed in 30% ACN solution. This indicates that there are at least three different NMR-detectable forms of ferricyt c that are in exchange with M80-ligated ferricyt c. The T_1 values of the resonances at 21.35, 22.21, 24.50, and 33.92 ppm are 107, 127, 58 (only detectable at 40°C), and 83 msec, respectively. Because the T_1 values for the heme methyl protons of lysine-ligated ferricyt c (122–130 msec) are usually higher and significantly different from those of M80-ligated ferricyt c (~100 msec) (Banci et al. 1995), the relaxation times for these resonances support the proposal of the presence of three different forms of ferricyt c, each having a different axial ligand in 30% ACN, specifically, lysine- (22.21 ppm and 21.35 ppm), M80- (33.93 ppm), and hydroxide-ligated ferricyt c (24.50 ppm; see following). The presence of cross peaks between the protons of the heme methyl groups of the M80-ligated form

and the lysine-ligated forms in the 2D NOESY spectra of ferricyt c in 30% ACN at 35°C confirms that these species interconvert. No exchange peaks were observed between the heme methyl protons of the lysine-ligated forms, even at 40°C. The absence of detectable cross peaks suggests that if exchange occurs between the lysine-ligated forms, it is very slow even at 40°C.

Assignments of the 1, 3, and 5 heme methyl protons of both the lysine-ligated forms were made as discussed earlier. The difference in the intensities of the 8-methyl proton resonances of the two lysine-ligated forms, which varied with the ACN concentration, was used to differentiate between the sets of heme methyl (1, 3, and 5) resonances for the two lysine-ligated forms.

The chemical shift pattern of the heme methyl resonances of both lysine-ligated forms of ferricyt c (IVa and IVb) in ACN–water is generally comparable to that in alkaline so-

Fig. 4. Ferricyt c in 30% ACN and 70% D₂O (pH* 6.9) at (A) 10°C, (B) 15°C, (C) 20°C, (D) 25°C, (E) 30°C, and (F) 40°C.

lution (Hong and Dixon 1989; Pollock et al. 1998). For example, consider the resonances of the 8-methyl protons at 24.62 ppm in Figure 1B and 22.87 ppm in Figure 1C and the resonances due to the 5-methyl protons at 22.27 ppm in Figure 1B and 21.01 ppm in Figure 1C. The similarity of the chemical shift pattern for the heme methyl protons of the lysine-ligated ferricyt c in the ACN–water solution suggests that the lysine-ligated forms of ferricyt c in ACN–water solution are likely very similar to IVa and IVb forms of alkaline ferricyt c. In the case of lysine-ligated forms of yeast ferricyt c (Pollock et al. 1998), the resonance of the 8-methyl protons of the IVa form appears farther downfield with respect to that for the IVb form. Hence, the lysine-ligated ferricyt c in 30% ACN for which the 8-methyl heme proton resonance appears at 22.87 ppm was assigned to the

IVa* form, and that which appears at 21.56 ppm was assigned to the IVb* form.

The order of the heme methyl chemical shifts of the lysine-ligated IVa* form of ferricyt c in 30% ACN solution (8-Me>5-Me>1-Me>3-Me) is identical to that of the lysine-ligated IVa form of ferricyt c in alkaline aqueous solution (Hong and Dixon 1989; Pollock et al. 1998). This indicates that the heme electronic structure of the IVa* form is likely to be similar to that in the IVa form.

Differences are, however, observed in the case of the IVb* form. The order of 1-methyl and 3-methyl heme resonances is reversed for the IVb* form as compared with that observed for IVb (Pollock et al. 1998) (8-Me>5-Me>3-Me>1-Me). Because the order of these heme resonances is known to be highly sensitive to the nature of the axial ligand

and to the orientation of this axial ligand with respect to the heme plane (Smith and McLendon 1981; Yamamoto 1996; Shokhirev and Walker 1998; Walker 1999, 2000;), the difference in the order of the heme methyl resonances indicates that the IVb* form in 30% ACN is structurally perturbed compared to that of the IVb form observed at alkaline pH.

Extensive studies on heme model complexes have shown that several factors significantly affect the order of the heme methyl resonances. These factors include (1) the dihedral angle between the plane of the projection of H18 imidazole and the plane intersecting the heme N_{II}-Fe-N_{IV} axis (ϕ) (Yamamoto 1996; Shokhirev and Walker 1998; Walker 1999, 2000); (2) the orientation of the axial ligand with respect to the z-axis of the heme (Smith and McLendon 1981); (3) the identity of the substituents in the 2, 4-positions of the heme macrocycle (La Mar et al. 1978; Viscio and La Mar 1978a,b; La Mar 1979); and (4) the nature of the axial ligand (Smith and McLendon 1981). Our resonance Raman study (Sivakolundu and Mabrouk 2000) indicated loss of the M80 induced nonplanarity of the heme and structural perturbation in the region of the thioether linkage of the protein in cyt c in 30% ACN. Thus, it is possible that in ferricyt c in the 30% ACN solution, the inversion in the order of the 1- and 3-methyl heme proton resonances may be due to factors 1 and 2. However, given the information we have at present, it is difficult to estimate the relative importance of these factors in affecting the change in the order of the 1- and 3-heme methyl resonances observed for the IVb* form in 30% ACN solution.

Propionate protons of the IVa and IVb* forms*

The assignment of the propionate proton resonances was accomplished by taking advantage of their proximity to the 8- and 5-methyl heme protons (≤ 3.8 Å). Based on an examination of the heme structure, the 17-1b proton (nomenclature of protons as in Scott and Mauk 1996) is closest to the 8-methyl heme proton. Thus, the resonance producing the most intense cross peak with the 8-methyl protons of forms IVa* (−1.02 ppm) and IVb* (−0.81 ppm) was assigned to the 17-1b proton. Additional support for this assignment for IVa* is provided by the observation of additional cross peaks between the 17-1b resonance of IVa* at −1.02 ppm, the 8-methyl, and the 17-1a, 17-2a, and 17-2b propionate protons. The same strategy was used to affect the assignments of the 17-1a, 17-2a, and 17-2b propionate protons for the IVa* form. In the case of IVb*, only the 17-1b proton could be unambiguously assigned because of spectral crowding in the 1–3 ppm spectral region where the cross peaks for the 17-1b proton were expected.

Meso- δ proton of the IVa and IVb* forms*

Intense cross peaks are observed between the 8- and 1-methyl heme protons of the IVa* form and a peak is

observed at −6.06 ppm. Similarly, the peak at −5.68 ppm shows cross peaks with both the 8-methyl and 1-methyl protons of the IVb* form. The resonances at −6.06 ppm for the IVa* form and −5.68 ppm for the IVb* form could be due to (1) a proton of a residue that is close to the 8- and 1-methyl heme protons, or (2) the meso- δ proton of the heme. The ratio of the intensities of the cross peaks between the peak at −6.06 ppm (−5.68 ppm for the IVb* form) and the resonances of the 8- and 1-methyl protons showed little change ($>10\%$) when the temperature was varied from 20°C to 45°C. This indicates that the ratio of the distance between the proton of these peaks (the −6.06 ppm and −5.68 ppm resonance) and the 8- and 1-methyl protons remains unchanged with temperature. Examination of the solution structure of ferricyt c revealed the absence of proton of any amino acid residues that occur within the 3.5 Å of both 8- and 1-methyl protons and give rise to intense cross peaks for both the 8- and 1-methyl protons. On the other hand, the meso- δ proton is expected to give rise to an intense cross peak with both the 8- and 1-methyl protons owing to its proximity (≈ 2.3 Å) to these protons, in the IVa* and IVb* forms. Hence, the resonance at −6.06 ppm and −5.68 ppm could be due to the meso- δ heme proton of the IVa* and IVb* forms, respectively. Indeed, the chemical shift values for the meso- δ proton in the lysine-ligated alkaline cyt c (−5.4 ppm) (Russell et al. 2000) and the M80A-CN[−] cyt c (−4.3 ppm) (Bren et al. 1995) are also observed in the same chemical shift range. Consequently, the peak at −6.06 ppm that produced intense cross peaks with both the 8-methyl and 1-methyl (IVa*) proton for the IVa* form was assigned to the meso- δ proton of the IVa* form and the peak at −5.68 ppm was assigned to the meso- δ proton of the IVb* form.

V form*

At elevated temperatures ($>35^\circ\text{C}$) in 30% ACN solution, two new resonances were observed at 24.50 ppm and 12.10 ppm (Fig. 4F). As mentioned earlier, the resonance at 24.50 ppm has a relatively low T_1 value (58 msec), indicating that the sixth ligand is not lysine or methionine, which typically have much longer T_1 values. In 30% ACN solution at 35°C, a cross peak is observed between the resonance at 24.50 ppm and the 8-methyl heme proton resonance of M80-ligated ferricyt c (33.93 ppm) in the 2D NOESY spectra (Fig. 3). The second new resonance at 12.10 ppm was not observed directly in the 1D spectrum because of spectral crowding, but was detected indirectly through the observation of a weak cross peak with 3-methyl (M80-ligated) resonance at 30.95 ppm in the 2D NOESY data at 35°C in 30% ACN (see inset to Fig. 3). Because the cross peaks for both resonances cannot be observed unless there is a high acetonitrile concentration ($\geq 20\%$), their presence is consistent with the formation of a new form of cyt c that is not present at lower ACN concentrations. Proton resonances at very

similar chemical shifts (10 and 25 ppm), have been observed in the ^1H NMR of a Lys73Ala/Lys79Ala variant of yeast cyt c at pH 10 and 45°C. There they were attributed to the formation of a low-spin hydroxide-ligated heme iron (Rosell et al. 1998). Because of the similarity in the chemical shift to the low-spin hydroxide-bound cyt c, the relatively low T_1 value of the 24.50 ppm resonance, and the presence of cross peaks between these resonances and M80-ligated cyt c, we tentatively assign the peaks 24.50 ppm and 12.10 ppm to the 8-methyl and the 3-methyl heme protons of the V^* form, in which the hydroxide is the sixth ligand.

Exchange between the different forms of ferricyt c

The intensities of the heme methyl resonances of the different forms of ferricyt c were found to be significantly dependent on temperature. When the temperature was increased from 10°C to 40°C (Fig. 4), there was a decrease in the relative intensities of the 8- and 3-methyl methyl proton resonances (see peaks at 32.44 ppm and 35.74 ppm in Fig. 4A–F) of the M80-ligated III^* form and a concomitant increase in the relative intensities of the 8- and 5-methyl methyl proton resonances of lysine-ligated forms (see peaks at 22.87 ppm, 21.56 ppm, and 21.11 ppm of the IV^* form in Fig. 4A–F). Moreover, the peak corresponding to the 8-methyl proton resonance of the hydroxide-ligated V^* form appears at elevated temperature (35°C) (24.50 ppm in Fig. 4F). These changes in the relative intensities of the heme methyl resonances show significant variation in the concentrations of the different forms of ferricyt c. This behavior indicates that the M80-ligated III^* form undergoes exchange with one or more alkaline forms of ferricyt c in 30% ACN. At lower temperatures, the M80-ligated III^* form predominates, whereas the equilibrium shifts in favor of the alkaline species at higher temperature.

Exchange peaks were observed between the 8-methyl heme proton resonance of the M80-ligated III^* form (33.93 ppm) and all three alkaline forms of ferricyt c (at 22.21 ppm for IVa^* , 21.35 ppm for IVb^* , and 24.50 ppm for V^*) in 30% ACN solution at 35°C (Fig. 3). This indicates that the III^* form of cyt c undergoes exchange with three different forms of ferricyt c. Further support for this is provided by the experimental observation of the reformation of the III^* form when ferricyt c in 30% ACN solution is dialyzed repeatedly against water. The presence of an exchange peak between the 8-methyl proton resonance of the IVa^* form (22.21 ppm) and the 8-methyl proton resonance (24.50 ppm) of the V^* form of cyt c indicates that the K73-ligated IVa^* form is in exchange with the hydroxide-ligated V^* form. However, no exchange peaks were observed between the heme methyl resonances of IVb^* and other alkaline forms of ferricyt c. The absence of the expected exchange peak indicates that either there is no interconversion or that exchange is slow between the K79-ligated IVb^* form and

the hydroxide-ligated V^* form in 30% ACN solution. Figure 5 shows our proposal for the exchange pathway between the different forms of ferricyt c in 30% ACN (III^* , IVa^* , IVb^* , and V^*) derived from these data.

Discussion

As discussed earlier, ferricyt c in 30% ACN solution exists as a mixture of four different NMR-observable forms that differ in their axial ligands, specifically, methionine, lysine (two forms), and hydroxide. The NMR evidence indicates that the heme electronic structure, the protein secondary structure, and the hydrogen bond network of the M80-ligated form of ferricyt c, at least in the vicinity of the heme active site, are preserved intact in 30% ACN solution. However, differences in the order of the 1-Me and 3-Me heme resonances for the IVb^* form indicate that there are some structural differences between the alkaline conformers observed in the aqueous and nonaqueous media. We believe that greater insight into the differences between the IVb^* and IVb forms can be obtained by analyzing the Fermi contact shifts of the heme carbon resonances (Turner 1995) and this will be the subject of future work. The 8- and 3-methyl heme resonances of the hydroxide-ligated V^* form has been assigned for the first time. However, no conclusions regarding the structure of the V^* form could be derived from this limited information.

Biological significance of the exchange process to alkaline ferricyt c

As discussed earlier, an exchange pathway, shown in Figure 5, between the different forms of ferricyt c in 30% ACN has been derived from the NMR data. Dopner et al. (1998) proposed a similar mechanism on the basis of a study of ferricyt c at alkaline pH. Our pathway is slightly different from theirs in that they propose that there is exchange between IVb and V , whereas we do not. No experimental

Fig. 5. Proposed mechanism for the exchange process between III^* , IVa^* , IVb^* , and V^* forms of ferricyt c in 30% ACN at >35°C.

evidence, however, has been reported supporting exchange between IVb and V.

The network of exchange that we have derived on the basis of a study of ferricyt c in 30% ACN may have physiological relevance. Cyt c resides in the intermembrane space (the space between the inner and outer membrane) of the mitochondrion, where it mediates electron transfer between the cyt c oxidase and CoQ cy c oxidoreductase and at times binds to the mitochondrial membrane (Cortese et al. 1998). When cyt c undergoes lateral diffusion and rotation along the inner mitochondrial membrane (Pettigrew and Moore 1987) or binds electrostatically to the membrane (Cortese et al. 1998; Kostrzewa et al. 2000), it experiences a medium with a low dielectric constant estimated to be between 2 and 60 (Ashcroft et al. 1981, 1983). Because the species we observe are produced in response to change in the dielectric constant of the solvent medium ($\epsilon = 68$), and because cyt c resides in the intermembrane space in the proximity of the mitochondrial membrane, a medium characterized by a low dielectric constant (Ashcroft et al. 1981, 1983), the alkaline-like cyt c forms we have observed in our work may represent the predominant forms of ferricyt c and the exchange pathway we have proposed (Fig. 5) may represent nature's preferred method of preventing spurious electron transfer with proteins other than cyt oxidase and oxidoreductase.

In summary, partial heme proton resonance assignments have been made for four different forms (III*, IVa*, IVb*, and V*) of ferricyt c in 30% ACN. The heme electronic structure, the secondary structure of the residues in the vicinity of the heme and hydrogen bond network involving the propionate-7 moiety of the III* form of cyt c in 30% ACN solution, was found to be intact. The lysine-ligated IVa* and IVa forms also appear to have similar heme active site structure. However, the IVb* form appears to be somewhat different from the IVb form observed in aqueous alkaline solution, although the particular differences have not yet been identified. A proposal for the network of exchange processes between the different forms of ferricyt c generated in 30% ACN, a medium characterized by a low dielectric constant ($\epsilon = 68$), was made that may be biologically significant. Study of ferricyt c in mixed media allowed us to make resonance assignments for the 8- and 3-methyl heme protons of the hydroxyl-ligated form (V*) of cyt c. This represents the first direct observation of the hydroxide species by NMR methods. Clearly, the study of other proteins in the mixed solvents by NMR may provide new insight into the structure and function of biomolecules. Consequently, NMR study of ferrocyt c and other enzymes in mixed solvents is currently in progress.

Materials and methods

The following chemicals were purchased commercially and were used as received: Horse heart cyt c (cyt c, Type IV, Sigma),

CD₃CN (<99.7% D, Merck Sharp and Dohme), D₂O (<99.96%, Cambridge Isotopes), DCl (Merck Sharp and Dohme), and NaOD (Aldrich).

Cyt c concentrations of 2–4 mM were used for all of the experiments reported. The protein was exchanged in D₂O at 50°C for 6 h and then concentrated with D₂O using an ultrafiltration (Amicon Corp.) apparatus (YM10) (Patel and Canuel 1976). Cyt c was dissolved in a mixture of CD₃CN and D₂O as described previously (Sivakolundu and Mabrouk 2000). The pH* (measured pH) was adjusted by adding small aliquots of DCl or NaOD and was not corrected for isotope effects.

1D and 2D ¹H NMR measurements were made using a 500-MHz NMR instrument that was homebuilt at the Center for Magnetic Resonance at MIT/Harvard. The 500-MHz NMR system is based on an Oxford wide-bore magnet and a console designed and constructed by the Center. It has four fully equipped RF channels and a temperature controller with 0.1°C resolution. The data were collected using RNMR software (David Ruben, CMR at MIT/Harvard) based on the VMS operating system. The data were then exported and processed in Felix 98 (Molecular Simulations Inc.). Ferricyt c spectra were referenced to TMS ($\delta = 0$ ppm) externally, through the residual HDO peak.

1D NMR

1D ¹H NMR spectra of cyt c were obtained with a spectral width of 80 ppm. Five hundred transients were acquired with 16K complex points. The 1D data were apodized with a 2-Hz exponential line broadening.

*T*₁ measurement

Two independent measurements of *T*₁ values were made by using the nonselective inversion recovery experiment and a time delay of 0.005–1 sec. The *T*₁ values were obtained by fitting the area of the peaks with a nonlinear regression routine in SPSS (v.10). The average error in these measurements was calculated to be 15%.

2D NMR

2D NOESY (Jeener et al. 1979) were recorded with a sweep width of 70 ppm with 2048 points in the *F*₂ dimension and 512 points in the *F*₁ dimension. The 2D data were zero-filled to 4096 × 1024 points (*F*₂ × *F*₁) and were apodized, in the both dimensions, with a sine-squared bell-wave function phase-shifted by $\pi/2$. A fifth-order polynomial baseline correction was done. The mixing time (τ_m) was selected by recording a series of NOESY spectra with different mixing times ranging from 25 to 100 msec, and an optimal value of 50 msec was used as mixing time to observe cross peaks due to the heme protons and protons from residues in close proximity to the heme.

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