The Conformational Flexibility of Oxidized Cytochrome c Studied through Its Interaction with NH_3 and at High Temperatures

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The binding of ammonia to oxidized horse heart cytochrome c has been studied by ^{1}H-NMR, EPR, and CD spectroscopy at pH = 8.0. The affinity constant of the ligand is in the range 1.5−4 M^{-1}. The ^{1}H-NMR spectra of the heme group have been found to be similar to those of the high-pH forms, high-temperature forms, and cyanide adduct of the Met80Ala mutant of *S. cerevisiae* iso-1-cytochrome c. The assignment of a number of signals has led to the determination of the values of the magnetic anisotropy and of the orientation of its axes. The latter are similar to those of the Met80Ala cyanide derivative. The assignment of the high-temperature species has been further pursued during this research. The analysis of the NMR data of the NH_3 adduct leads to the conclusion that substitution of Met80 at high pH or high temperature occurs through a ligand with cylindrical symmetry. This supports the suggestion that Met80 is substituted by a lysine at high pH.

The structure of the native cytochromes c has been extensively characterized by both X-ray crystallography[1][2][3][4][5][6][7] and NMR spectroscopy[8][9][10][11][12]. Several partially unfolded states of the oxidized species have also been detected spectroscopically as a function of temperature, pH, and denaturating agents[13−26] but the structural characterization for these protein forms is not available up to now. One important feature of cytochrome c folding/unfolding processes concerns the involvement of the covalently attached heme group and its axial ligands. The coordination of His and Met stabilizes the native state. Indeed, one of the first steps of ferricytochrome c unfolding upon increasing pH and/or temperature involves the detachment of Met80 from iron coordination[13−26]. In this respect NMR spectroscopy turns out to be a powerful technique for the detection of changes in the iron coordination in ferricytochrome c because the hyperfine shifted resonances are heavily affected by changes in the axial coordination of heme iron[27][28][29][30][31][32][33]. Indeed, through NMR spectroscopy, Hong and Dixon detected two forms of the alkaline isomerized species in slow exchange with the native form on the NMR time scale[34][35]. At neutral pH and temperature above 42°C, Ångstrom et al.[36] observed new species that closely resemble the alkaline isomerized forms. All these species contain low-spin iron(III). Recently, Taler et al. have further characterized the high-temperature species of horse cytochrome c and identified the ^{1}H-NMR resonances of the ε-CH_3 of Met80 in the high-temperature species at typical diamagnetic shift values[14].

It has been proposed that in oxidized cytochrome c at high pH values or at high temperatures the Nζ of a Lys residue substitutes Met80 in binding iron with a pK_a of 8.5−9.5[17][34][35][37][38][39][40][41][42][43][44][45][46][47][48][49]. It is known that pyridine and imidazole bind to iron by replacing Met80[50][51]. We report here an investigation on NH_3 binding to horse heart cytochrome. The results are compared with those related to the high-temperature species, whose characterization has been further extended during this research, with those on the cyanide adduct of the Met80Ala mutant of *S. cerevisiae* cytochrome *c*, and with those on the alkaline forms.

Results

NH_3 Adduct

The formation of the NH_3 adduct of horse heart cytochrome c (hh cyt c, hereafter) upon addition of increasing amounts of a solution of NH_4Br was monitored by ^{1}H-NMR spectroscopy at pH = 8.0 and at 293 K. At this pH this protein is largely in its physiologically relevant conformation, as shown by its ^{1}H-NMR spectrum (Figure 1, A).

Small traces of the alkaline forms are revealed by the peaks marked with asterisks. Upon addition of NH_4Br new resonances appear at δ = 25.2, 23.5, 15.7, 15.5, 11.6, 10.9 whereas the signals of native hh cyt c decrease in intensity (Figure 1, B−D). A broad feature is observed upfield at δ = −11.9. All the above-mentioned resonances are due to non-exchangeable protons because they remain when the spectra are recorded in D_2O.

Analogous titration was performed by circular dichroism spectroscopy (CD) in the Soret region. In the native form, the CD spectrum (Figure 2, A) is characterized by a negative absorption at 417 nm and a positive band at 400 nm, as already reported[40].

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Figure 1. 600-MHz ^{1}H-NMR spectra of hh cyt c recorded in H_2O solution (100 mM phosphate buffer, pH = 8.0, T = 293 K) at increasing amounts of NH_4Br; (A) [NH_4Br] = 0 M; (B) [NH_4Br] = 0.5 M; (C) [NH_4Br] = 0.9 M; (D) [NH_4Br] = 2.7 M

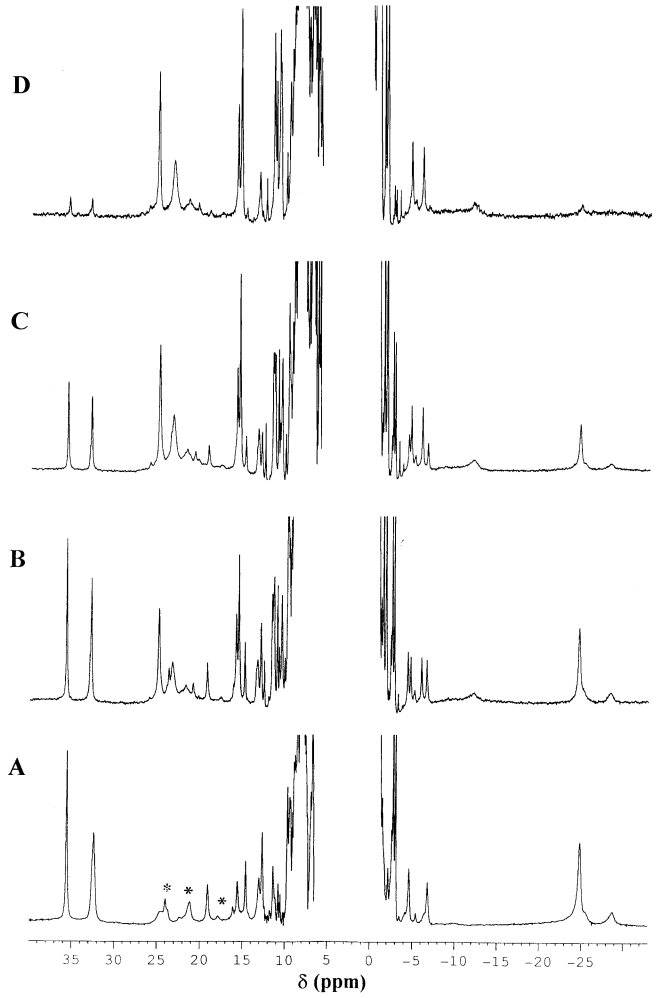


Figure 2. CD spectra on the native hh cyt c (A) and on the NH_3 adduct ([NH_4Br] = 2.7 M) recorded in 100 mM phosphate buffer (pH = 8.0, room temperature)

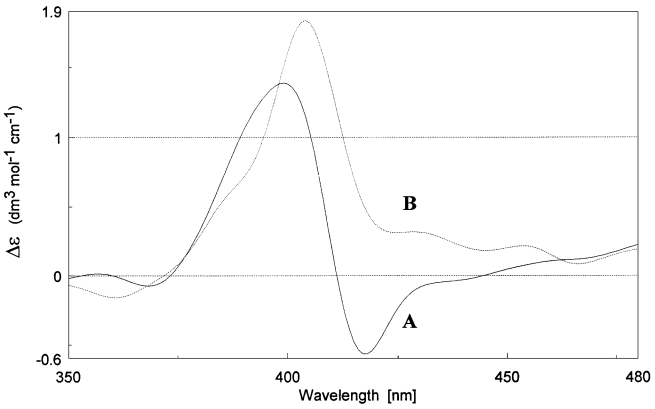
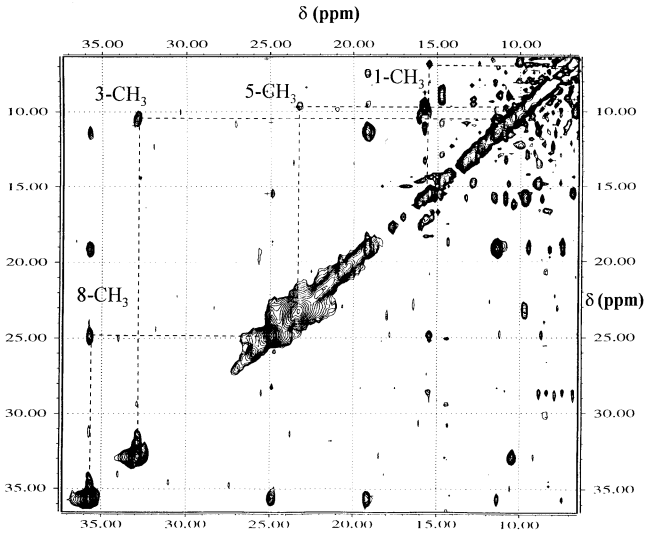


Figure 3. Downfield region of the 600-MHz ^{1}H-NMR EXSY spectrum recorded in H_2O solution (100 mM phosphate buffer, pH = 8.0, T = 293 K) on a sample with a ratio between native and NH_3-bound protein of about 1:1; broken lines indicate EXSY cross peaks between the heme methyl groups in the two protein forms



As the ligand concentration increases, the negative band becomes smaller and the positive absorption gains in intensity and moves towards higher wavelenghts. The final spectrum shows an intense positive band with maximum at 407 nm (Figure 2, B). A similar behavior has been reported as a function of an increase in pH and explained with the hypothesis that the negative 417-nm CD band is related to the ligation of the heme iron by Met80[40]. This idea is supported by chemical modification studies on cytochrome c[52].

From the circular dichroism and the ^{1}H-NMR titration, an apparent affinity constant in the range of 1.5−4 M^{-1} can be estimated.

The ^{1}H-NMR spectrum of the NH_3 derivative of hh cyt c was assigned by means of EXSY and NOESY spectroscopy. An EXSY cross peak is clearly detected between 8-CH_3 of the native form and the resonance at δ = 25.2 (Figure 3).

Other EXSY cross peaks have been observed at δ = 11.0 for 3-CH_3, δ = 15.5 for 1-CH_3, and δ = 23.5 for 5-CH_3. The δ-*meso* proton was assigned to the peak at δ = −6.0, thanks to its NOESY connectivities to both 8-CH_3 and 1-CH_3 of the NH_3-bound form. The 7-Hα proton was assigned at δ = 1.8 based on its EXSY connectivity with the 7-Hα of the native protein and on a strong NOESY connectivity with the 8-CH_3 resonance of the NH_3-bound form. The ε-CH_3 of Met80 gives an EXSY cross peak with a signal at δ = −3.3 (data not shown) which is then the corresponding signal in the NH_3 adduct, thus indicating that, in the presence of ammonia, Met80 is detached from iron and moves towards a position characterized by diamagnetic shift values. The β-CH_2 protons of His18 were assigned in the NH_3 adduct at δ = 15.7 and δ = 9.6. Indeed, an exchange cross peak is observed between one β-CH_2 signal at δ = 14.7 in the native form and that at δ = 15.7 in the NH_3 adduct. The resonances of the other β-CH_2 proton in the NH_3-bound and unbound forms are too close to each other to resolve the EXSY connectivity. The resonance was as-

signed by an NOESY experiment. All the above assignments are summarized in Table 1.

The shift pattern for the heme methyl groups is 8-CH_3 > 5-CH_3 > 1-CH_3 > 3-CH_3. The shift spreading is 14.2 ppm and the mean shift value is 18.5 ppm. At the same temperature the mean shift value for the four heme methyl groups of the native species is 21.3 ppm and the shift spreading is 28.9 ppm. At 2.7 M ligand concentrations, the native species is only about 10% of the NH_3-bound form. NOESY and TOCSY experiments were performed to further assign the spectrum of the adduct. The NOESY connectivities observed within the heme moiety are summarized in Scheme 1 (A).

The NOESY experiment allowed us to assign the Hα1 and Hα2 protons of Gly29 from their connectivities with 5-CH_3. The chemical shift of these two protons in the NH_3 adduct is very similar to that of the native form and therefore the EXSY cross peaks are too close to the diagonal to be clearly resolved. Other spin patterns were clearly identified in the TOCSY spectrum recorded in D_2O solution and are reported in Table 2.

Table 1. Shift values for the hyperfine shifted signals of NH_3 adduct of hh cyt c (pH = 8.0, T = 293 K), alkaline yeast Iso-1-cyt *c*, high-temperature forms of hh cyt *c*, (pH = 7.0, T = 335 K) and cyanide adduct of Met80Ala cyt c (T = 303 K)

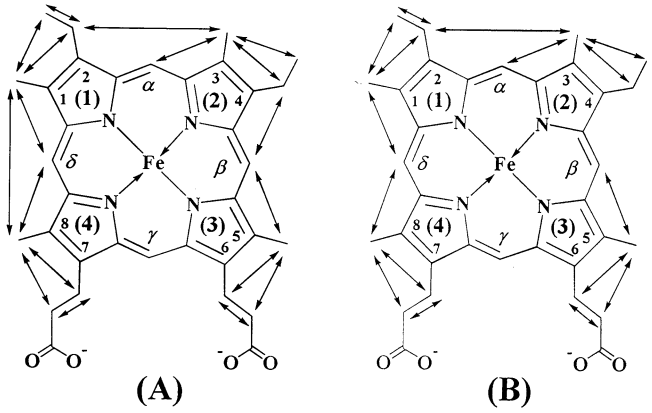
Assignment	hh cyt c-NH_3 293 K (δ, ppm)	Alkaline yeast iso-1-cyt *c* 298 K (δ, ppm)[a]	hh cyt c 335 K (δ, ppm)	Various forms[b]	Met80Ala *c*-CN^- 303 K (δ, ppm)[d]
		Heme			
		24.8	22.3	A	
8-CH_3	25.2	24.0	22.0	B	22.5
			20.7	C	
		14.0	12.4	A	
3-CH_3	11.0	12.0	13.6	B	11.3
			11.3	C	
		21.5	20.6	A	
5-CH_3	23.5	19.0	18.9	B	19.5
			20.2	C	
			12.7	A	
1-CH_3	15.5		12.4	B	15.4
			13.4	C	
			4.0	A	
7-Hα	1.8		3.9	B	6.3
			3.7	C	
			0.8	A	
7-Hα′	−0.5		0.9	B	1.4
			0.4	C	
			−0.2	A	
7-Hβ	−1.5		0.1	B	0.6
			−0.4	C	
			1.1	A	
7-Hβ′	0.8		1.4	B	0.8
			1.7	C	
6-Hα	2.2		2.6		1.8
6-Hα′	−1.6		1.6		1.6
6-Hβ	−0.7		0.3		0.2
6-Hβ′	2.9		5.0		—[e]
2-Hα	0.8		−0.3	A	6.3
			−0.2	B	
2-CH_2	2.5		0.6	A	−0.2
			0.5	B	
4-CH_2	0.2		0.8		0.3
4-Hα	2.6		2.2		−0.1
α-*meso*	4.3		6.1		4.1
β-*meso*	1.4		−0.6		−0.8
γ-*meso*	—[c]		—[c]		—[e]
δ-*meso*	−6.0		−2.8	A	−4.3
			−1.9	B	
		His18			
Hα	10.9		—[c]		9.8
Hβ1	9.6		8.5	A	7.7
			9.0	B	
Hβ2	15.7		13.7	A	12.0
			14.1	B	
Hδ1	—[c]		—[c]		16.6
Hδ2	—[c]		18.3	A	16.1
			20.1	B	
Hε1			—[c]		−3.4
		Met80			
ε-CH_3	−3.3		2.6	A	
			2.3	B	

[a] Estimated chemical shifts taken from ref.[14]. — [b] The relative intensity of the EXSY peaks between the native and high-temperature species of hh cyt c follows the order : A > B > C. — [c] Not observed. — [d] Taken from ref.[60]. — [e] Not reported.

Table 2. Shift values for some residues in the NH_3 adduct of horse heart cyt c (100 mM phosphate buffer, pH = 8.0, T = 293 K)

Signal	hh cyt c-NH_3 (δ, ppm)
Gly1	
Hα1	3.5
Hα2	3.8
Gly6	
Hα1	3.2
Hα2	3.9
Phe10	
Hζ	9.3
Hε2	7.8
Hε1	8.9
Hδ2	6.9
Hδ1	7.3
Ala15	
Hα	6.1
β-CH_3	1.7
Cys17	
Hα	6.1
Hβ	6.9
Thr19	
Hα	6.7
Hβ	5.8
γ-CH_3	2.1
Gly23	
Hα1	3.7
Hα2	4.0
Gly29	
Hα1	−1.8
Hα2	−4.7
Asn31	
Hα	7.0
Hβ1	2.7
Hβ2	3.2
Phe36	
Hδ′s	7.4
Hε′s	6.5
Hζ	6.6
Gly45	
Hα1	3.7
Hα2	4.4
Asn54	
Hα	5.7
Hβ1	3.0
Hβ2	3.2
Gly56	
Hα1	3.8
Hα2	4.0
Trp59	
Hζ2	7.1
Hε3	6.8
Hζ3	6.5
Hη2	6.3
Leu64	
Hα	2.1
Hβ2	0.7
Hγ	0.4
δ1-CH_3	−0.3
δ2-CH_3	−0.6
Leu68	
Hα	2.8
Hβ2	−0.1
Hγ	0.6
δ1-CH_3	−0.9
δ2-CH_3	−1.8
Asn70	
Hα	6.0
Hβ1	3.3
Hβ2	3.4
Tyr74	
Hδ′s	8.1
Hε′s	7.1
Phe82	
Hδ′s	6.2
Hε′s	6.1
Hζ	5.9
Leu94	
Hα	2.1
Hβ1	1.4
Hβ2	0.6
Hγ	0.0
δ1-CH_3	−0.4
δ2-CH_3	−0.8
Tyr97	
Hδ2	7.4
Hε2	7.2
Hδ1	6.4
Hε1	5.6

Scheme 1. Schematic representation of a c-type heme; the dipolar connectivities observed between the heme resonances in hh cyt c NH_3 (A) and in the major high-temperature form (B) are also shown



A broad resonance at δ ≈ 280 was also detected in the ^{1}H-NMR spectrum recorded in H_2O, which is probably due to the bound NH_3. No corresponding signal was detected in analogous spectra recorded in D_2O solutions. The broad signal observed at δ = −11.9, which does not exchange in D_2O, remains unassigned. However, its short T_1 value (< 2 ms) supports the idea that it is one of the two not-exchangeable protons of the proximal histidine ring. Non-selective T_1 values were also measured for the heme methyl groups, which are of the order of 70−80 ms.

From the EXSY cross-peak intensity observed between the heme methyl groups and from the above measured T_1 values, an exchange rate of 2−10 s^{-1} can be estimated.

The shift values of the assigned resonances reported in Table 2 allowed us to estimate the magnetic susceptibility tensor parameters for this protein form. The tensor has $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values of 4.2 ± 0.2 × 10^{-32} and −2.2 ± 0.1 × 10^{-32} m^3, respectively. The χ_{xx} and χ_{yy} axes are close, although not exactly alligned, to the directions defined by the *meso* protons (the deviation is about 11°), while the χ_{zz} axis deviates by about 16° from the heme normal.

Figure 4. X-band EPR spectra of the NH_3 adduct ([NH_4Br] = 2.7 M, 100 mM phosphate buffer, pH = 8.0) recorded at 4.2 K using 30 dB microwave power and a gain of 800,000 (A), and 20 dB microwave power and a gain of 500,000 (B)

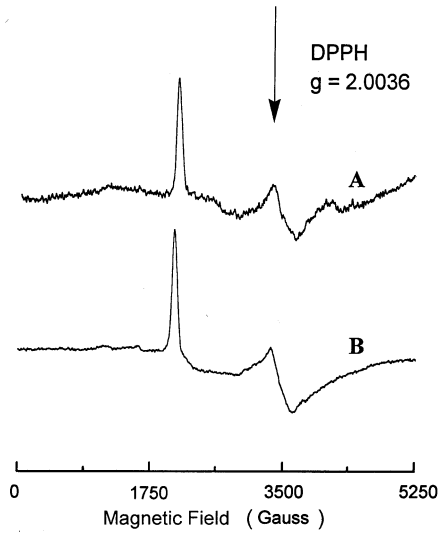
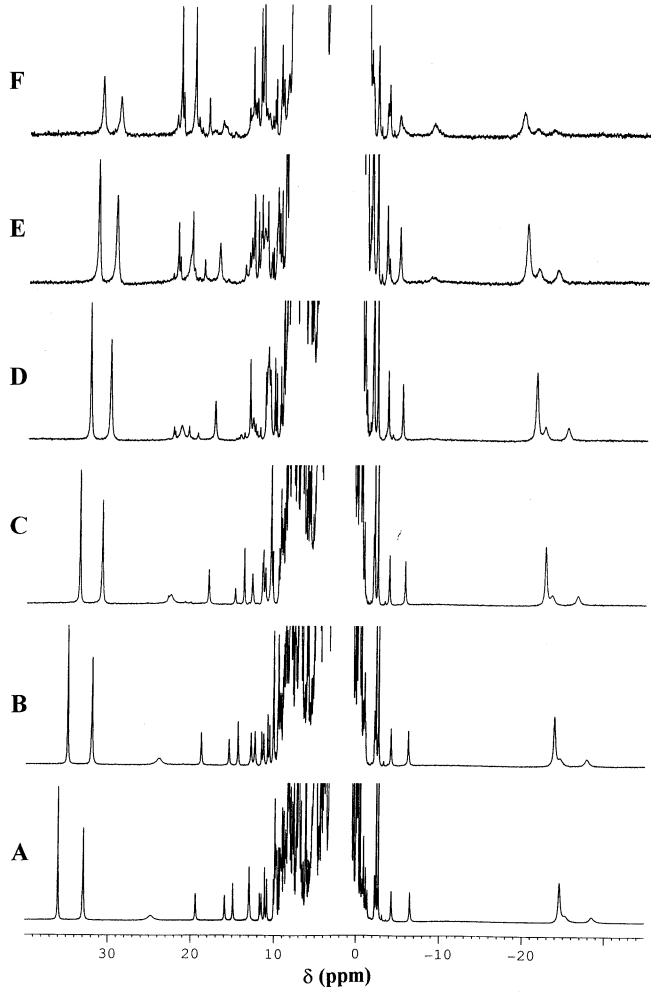


Figure 5. 600-MHz ^{1}H-NMR spectra of native hh cyt c recorded in H_2O solution (100 mM phosphate buffer, pH = 7.0) at various temperatures: (A) T = 293 K; (B) T = 300 K; (C) T = 310 K; (D) T = 320 K; (E) T = 330 K; (F) T = 335 K



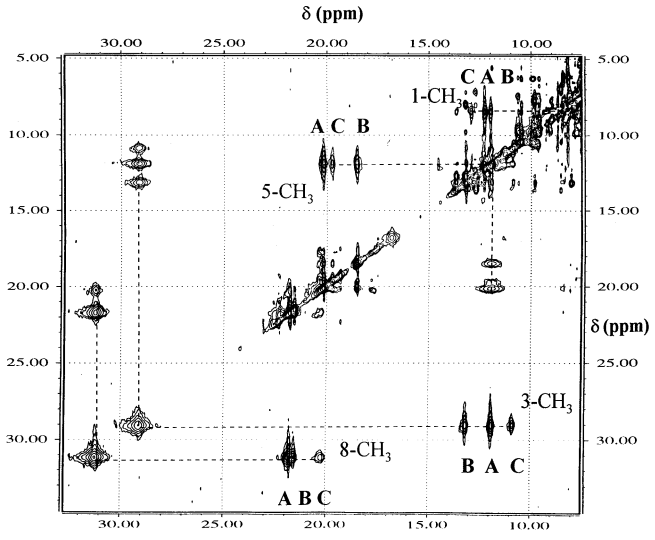
The EPR spectra of the final adduct (2.7 M of NH_4Br) at 4.2 K (Figure 4) show g values of 3.28, 2.01, and 1.66. The g values measured for the NH_3-bound cytochrome c are characteristic for a low-spin iron(III) and are within the range expected for a heme iron coordinated axially by a histidine and an amine group as exemplified by cytochrome f[53].

^{1}H-NMR Spectroscopy on the High-Temperature Species

In Figure 5 the ^{1}H-NMR spectra of oxidized hh cyt c in 100 mM phosphate buffer at pH = 7.0 are shown as a function of temperature in the 293−335 K range. At 293 K only the resonances of the native form are detected[10][54].

Upon increasing the temperature, new hyperfine shifted resonances appear in the 25−20 and 10−13 ppm ranges; a broad signal is also observed at δ = −10. At 335 K the intensity of the signals belonging to the new species is considerably enhanced, being twice as large as the intensity of the native-form resonances. At 335 K, the ^{1}H-NMR resonances due to three out of four heme methyl groups of the native form are well resolved at δ = 31.6, 29.5, and 12.4, while the fourth is at δ = 9.0. Three exchange cross peaks between the native and the high-temperature forms are observed for each methyl group, indicating that three new species are formed (Figure 6).

Figure 6. Downfield region of the 600-MHz ^{1}H-NMR EXSY spectrum of native hh cyt c recorded in H_2O solution (100 mM phosphate buffer, pH = 7.0) at 335 K with a mixing time of 40 ms; broken lines indicate EXSY cross peaks between the heme methyl groups in the native and high-temperature forms; cross peaks due to the three different high-temperature species are labelled accordingly to the concentration of the species: A > B > C



This implies that three different conformations involving the heme and the surrounding residues are present at this temperature. The shifts for these new species are compiled in Table 1. From the peak intensity, the A species is the major one, while the C species has the lowest concentration. The shift range for these resonances corresponds to that reported by Taler et al.[14]. From the EXSY cross-peak intensities, an exchange rate between the native and the

high-temperature forms of the order of 10 s^{-1} can be estimated. EXSY peaks are also observed between the corresponding resonances of the three forms A, B, and C.

EXSY experiments allowed us to identify the 7-propionate Hαs. The 7-propionate Hβs have been assigned on the basis of their NOE connectivities with the 7-Hαs of the same species. Dipolar connectivities were also observed between the 8-CH_3 resonances of the high-temperature forms and the corresponding 7-propionate protons. NOESY cross peaks have been also used to identify the other resonances reported in Table 1. Cross peaks between 5-CH_3 and the α-protons of 6-propionate are detected only for the major of the new species. All the dipolar connectivities between heme resonances observed at 335 K for form A are summarized in Scheme 1 (B).

As already observed by Taler et al.[14] through monodimensional saturation-transfer experiments, two exchange cross peaks were detected in the EXSY experiments between the ε-CH_3 of Met80 of the native form and two resonances at δ = 2.3 and 2.6 of the high-temperature species. Upon saturation of the Hγ proton of Met80 of the native species, located at δ = −22.7, saturation transfer is observed at δ = 3.2 and 2.7. From these assignments, it is clear that all the shift values observed for Met80 in the high-temperature species are typical of a "diamagnetic" methionine and therefore they constitute an additional demonstration that Met80 is detached from the heme iron under these experimental conditions.

By EXSY experiments also some of the His18 resonances were assigned, as reported in Table 1. Only two high-temperature species are detected for this residue.

For the native cytochrome c the heme methyl shift pattern is 8-CH_3 > 3-CH_3 > 5-CH_3 > 1-CH_3. At 335 K the mean shift value for the four heme methyl groups of the native species is 20.6 ppm while the spreading is 22.6 ppm. For the three high-temperature species the observed mean shift values are 17.0, 16.8, and 16.4 ppm for species A, B, and C, respectively. The spreading for the three species is 9.8, 9.6, and 9.4 ppm , in that order. The shift pattern is 8-CH_3 > 5-CH_3 > 1-CH_3 ≈ 3-CH_3.

Discussion

The present data show that NH_3 binds to iron(III) in oxidized hh cyt c by displacing Met80. The system has been characterized by EPR, CD and ^{1}H-NMR spectra. The ^{1}H-NMR spectra of the NH_3 adduct are different from that of the native hh cyt c because Met80 is not bound anymore, but they are, however, very similar to those of the high-temperature forms which experience the detachment of Met80 as well, the binding of another ligand to keep the low-spin nature of the iron. In fact, a heme with only a histidine bound in the fifth position gives rise to a high-spin species as observed in peroxidases[55][56][57][58][59]. Unfortunately, the previous[14] and the present data do not provide evidence for the nature of the sixth ligand. The comparison of the spectra of the NH_3 adduct and of the high-temperature species on one hand, and the spectra of the cyanide derivative of the Met80Ala yeast cytochrome c mutant[60] and of the high-pH species[34] on the other, may be instructive with respect to the conformational and unfolding properties[61][62] of cyt c. It is known[34] that cyt c undergoes a pH-dependent transition with a pK_a of 9.5 at 293 K. Interestingly, the pK_a decreases with increasing temperature, indicating that both pH and temperature cause similar effects. Indeed, Met80 is detached also at high pH[26]. As it results from Table 1, the NMR signals of the high-temperature species are close to those of the alkaline species. The ^{1}H-NMR spectra at high pH and high temperature have been reported also for the cyanide-free Met80Ala yeast cytochrome c[63], where no methionine is bound to the iron. They closely resemble those observed for the native protein under the same experimental conditions, although only one of the two alkaline forms in the mutant has been detected[63].

The ^{1}H-NMR heme shift pattern observed for the high-temperature forms, for the alkaline form, and for the NH_3 adduct closely resembles that of the cyanide adduct of Met80Ala mutant of yeast iso-1-cytochrome c (last column of Table 1)[60]. For the latter protein (at 303 K) the heme methyl resonances order is 8-CH_3 > 5-CH_3 > 1-CH_3 > 3-CH_3, the mean shift value is 17.2 ppm, and the heme methyl signal spreading is 11.2 ppm.

The cyanide adduct of Met80Ala mutant of yeast iso-1-cytochrome c has been extensively characterized by NMR both in terms of structure[60][64] and electronic properties[65][66]. The magnetic susceptibility tensor axes and anisotropies have been evaluated[65] and the contact contribution to the shift for the heme and the axial ligand has been separated from the pseudocontact one (Table 3). The pattern of the contact and pseudocontact contributions to the shift of the porphyrin methyl protons are determined by the orientation of the axial ligands[67−78]. In native cytochrome c both the His and the Met are contributing, whereas in the cyanide adduct of Met80Ala cytochrome c the orientation of the p_π nitrogen orbital of the His ring defines the shift pattern. In the latter system this orbital lies approximately parallel to the β/δ-*meso* direction and the smallest spin density is found for the 3-CH_3 (second column of Table 3)[65]. In native cytochrome c the contact shift pattern is completely different due to the contribution of Met80 which imparts a pairwise pattern to the heme methyl groups on opposite pyrrole rings, the 8-CH_3 and 3-CH_3 having significantly larger shifts than 1-CH_3 and 5-CH_3 (Table 3, third and fourth columns).

Table 3. Contact shift values (ppm) calculated for the NH_3 adduct of hh cyt *c*, the cyanide adduct of Met80Ala *S. cerevisiae* iso-1-cytochrome c (Met80Ala-CN^-)[65], native *S. cerevisiae* cytochrome c (yeast cyt c)[9], hh cyt c[10]

	hh cyt c-NH_3	Met80Ala-CN^-	yeast cyt c	hh cyt c
1-CH_3	24.0	16.9	4.5	3.4
3-CH_3	11.5	10.5	27.1	29.0
5-CH_3	32.0	20.8	7.4	6.1
8-CH_3	27.8	24.6	32.4	33.5

The orientation of the axial ligand determines the in-plane orientation of the magnetic susceptibility tensor axes[72][73][74][75][76][77][78] and then contributes to differentiate the shifts of the methyl signals. The directions of the tensor determined for the NH_3 adduct compare well with those of the cyanide adduct of Met80Ala for which the in-plane axes are essentially aligned with the *meso* proton directions and the *z* direction forms an angle of 6° with the direction of the perpendicular to the heme plane. The contact shift values for the heme methyl groups in the NH_3 adduct are also listed in Table 3 for comparison purposes. Analogously to the cyanide adduct of Met80Ala, the 3-CH_3 experiences the smallest shift.

Unfortunately, the NMR spectrum of the high-temperature species is complicated by the coexistence of four species at the same time. (At higher temperatures the protein denaturates). This prevented us from further extending the assignment of the A, B, and C forms and from calculating the magnetic susceptibility parameters. However, the comparison of the shift patterns of the high-temperature cytochrome c with the NH_3 adduct and with that of the cyanide adduct of the Met80Ala mutant, clearly supports the idea that the sixth axial ligand is axially symmetric. The same indication comes for the high-pH species.

Concluding Remarks

The assignment of the protons of the iron ligands reported in Table 1 for the NH_3 adduct and for the high-temperature species indicates a meaningful similarity between the two species. The similarity extends to the high-pH form of cytochrome c and the cyanide adduct of the Met80Ala mutant. The directions of the magnetic susceptibility anisotropy tensor for the NH_3 adduct are similar to those found in the cyanide adduct of the Met80Ala mutant. All of this has been rationalized in terms of the orientation of the axial histidine being the only factor determining the heme proton shift pattern. This suggests that the other axial ligand in the high-pH and the high-temperature forms has cylindrical symmetry. The results presented here are consistent with the hypothesis of a lysine substituting Met80 at high pH. They also shed further light on the conformational flexibility of the protein under various conditions.

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Experimental Section

Sample Preparation: Horse heart cytochrome c (Type VI) was purchased from Sigma Chemical Co. and used without further purification. The ^{1}H-NMR samples were prepared by dissolving the lyophilized protein in 100 mM phosphate buffer to give 2−4 mM solutions. The pH of the NMR samples was adjusted by addition of small volumes of concentrated solutions of NaOH or H_3PO_4. The pH was measured with an Orion Model 720 pH meter and a Microelectrodes, Inc., Model MI-410 microcombination pH probe. The NH_3 adduct was prepared by adding increasing amounts of a concentrated solution of NH_4Br at pH = 8.0. The same sample was used for EPR measurements. Circular dichroism spectra were obtained with a Jasco J500 dicrograph by titrating with NH_4Br a 8.0 μM ferricytochrome c solution (pH = 8.0, 100 mM phosphate buffer).

NMR Spectroscopy: The ^{1}H-NMR spectra were recorded with Bruker DRX 500 and AMX 600 spectrometers. T_1 measurements at 500 MHz were performed with the inversion-recovery method[79]. The 1D saturation-transfer experiments were performed with the superWEFT pulse sequence[80] and were collected using the standard methodology used for nuclear Overhauser effect (NOE) experiments of paramagnetic macromolecules[81]. All other spectra were acquired using presaturation during relaxation delay. EXSY experiments with the native cytochrome c were performed over the full spectral width with mixing times of 25, 40, and 60 ms at different temperatures (300, 310, 320, 330, 335 K). NOESY[82] and TOCSY[83] maps on a smaller spectral width (21 ppm) were collected in H_2O solutions at 335 K with a mixing time of 100 ms and a spin-lock time of 90 ms, respectively. EXSY experiments with a sample containing about 50% of the NH_3 adduct and 50% of the unbound form of cytochrome c were acquired with a mixing time of 40 ms at 293 K. NOESY and TOCSY maps of the completely NH_3-bound species were collected over a smaller spectral width (21 ppm) in H_2O and D_2O solutions at 293 K with a mixing time of 100 ms and a spin-lock time of 90 ms, respectively. − The 2D maps consisted of 4 K or 2 K data points in the F2 dimension. From 512 to 1024 experiments were recorded in the F1 dimension, using 64−512 scans per experiment. Raw data were multiplied in both dimensions by a pure cosine-squared and Fourier transformed to obtain 2048 × 2048 real data points. A polynomial base-line correction was applied in both directions. Data processing was performed using the standard Bruker software package. The 2D maps were analyzed by means of IBM RISC 6000 computers with the aid of the program XEASY (ETH, Zürich)[84].

EPR Spectroscopy: EPR experiments were performed at about 4.2 K with a Bruker ER200 spectrometer, operating at 9.6 GHz, equipped with an Oxford cryostat. The *g* values were calibrated by taking the 2,2-diphenyl-1-pycrylhydrazyl (DPPH) peak as reference (g = 2.0036). Spectra have been recorded using 30 dB microwave power and a gain of 800,000 or 20 dB microwave power and a gain of 500,000.

Magnetic Susceptibility Tensor Parameters: The hyperfine shift, i.e. the difference in shift of a proton in a paramagnetic system from that in an analogous diamagnetic system, includes contact and pseudocontact contributions[85][86][87][88]. The contact coupling is due to the presence of unpaired spin density on the resonating nucleus and vanishes a few chemical bonds away from the metal center. The pseudocontact contribution (δ_{pc}) arises from the magnetic susceptibility anisotropy and depends on the nuclear position with respect to the principal axes of the magnetic susceptibility tensor. Within the metal-centered point-dipole point-dipole approximation, the following equation holds[89]:

$$\delta_{pc} = \frac{1}{12\pi r_i^3}\left[\Delta\chi_{ax}(3n_i^2 - 1) + \frac{3}{2}\Delta\chi_{rh}\left(l_i^2 - m_i^2\right)\right] \quad (1)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and the rhombic anisotropies of the magnetic susceptibility induced by the paramagnetic ion, r_i is the distance of the nucleus *i* from the metal ion, and l_i, m_i, and n_i are the direction cosines of the position vector of atom *i* (r_i) with respect to the orthogonal reference system formed by the principal axes of the magnetic susceptibility tensor. − Pseudocontact shifts were determined by subtracting the chemical shifts measured in the reduced species of hh cyt c[90] from those of the NH_3-bound oxidized form of the same protein. The resonances of cysteines 14 and

17, of histidine 18, and of methionine 80 would experience non-negligible contact shifts and therefore were not included in the calculations. The 5 independent parameters (i.e. $\Delta\chi_{ax}$, $\Delta\chi_{rh}$, and three independent direction cosines which define the principal directions of the χ tensor with respect to a metal-centered axis system) were determined by finding the best fit of eq. 1 to a set of pseudocontact shifts[65][66], using the solution structure of horse heart cytochrome c[10].

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