pH-dependent equilibria of yeast Met80Ala-iso-1-cytochrome *c* probed by NMR spectroscopy: a comparison with the wild-type protein

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Background: Cytochrome *c* has five distinct pH-dependent conformational states, including two alkaline forms of unknown structure. It is believed that in both of the alkaline forms a Lys residue is ligated to the heme, but the identity of the Lys residue is different. Exchange between these forms would require extensive structural rearrangement. Mutation of the heme axial ligand (Met80) to Ala in *Saccharomyces cerevisiae* iso-1-cytochrome *c* yields a protein (Ala80cyt *c*) capable of binding exogenous ligands such as dioxygen and cyanide. We have analyzed the ^{1}H NMR spectra of this mutant at various pH values in the hope of gaining insight into the structure of the acidic and alkaline forms of native cytochrome *c*.

Results: The pH dependence of the ^{1}H NMR spectrum of ferriAla80cyt *c* is consistent with the high-spin/low-spin transition (pK_a = 6.5) observed by absorption spectroscopy. The T_1 values for the low-spin form are consistent with OH^- ligation, as inferred previously from absorption and electron paramagnetic resonance spectroscopic results. The pH-dependent equilibria of ferriAla80cyt *c* differ from those of the wild-type protein. Both Ala80 and wild-type ferricyt *c* appear to have the same iron coordination at low pH (≈ 2), while only one alkaline form of Ala80cyt *c* (versus two for WTcyt *c*) was detected.

Conclusions: The differences between the pH dependence of the ^{1}H NMR spectra of Ala80cyt *c* and those of the wild-type protein demonstrate that the heme axial ligands influence the relative energies of the conformational states of cytochrome *c*. The results are consistent with the notion that a large rearrangement is required to switch between the two alkaline forms.

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Key words: alkaline transition, axial-ligand mutant, cytochrome *c*, paramagnetic NMR, pH-dependent equilibria

Introduction

The substitution of the axial methionine ligand (Met80) with an alanine residue in *Saccharomyces cerevisiae* iso-1-cytochrome *c* produces a protein (Ala80cyt *c*) that has spectroscopic properties similar to globins and peroxidases [1,2]. The resulting heme cavity within Ala80cyt *c* has remarkable properties. Dioxygen forms an adduct with ferroAla80cyt *c* with a higher binding constant and lower autoxidation rate than myoglobin [1]. The solution NMR structure of ferriAla80cyt *c*–CN^- indicates that the distal residue Tyr67 is positioned to hydrogen bond with small ligands bound to the heme, and may be important in dioxygen stabilization (Fig. 1) (L.B., I.B., K.L.B., H.B.G., P.T. and P. Sompornpisut, unpublished data). Tyr67 may modulate the ligand-binding properties of ferriAla80cyt *c* as well. Hydroxide serves as an axial ligand at pH 7 in the oxidized protein; the pK_a of the heme-bound water (6.5) [1,2] is three units lower than that of myoglobin [3] but is comparable to the pK_a measured for the heme-bound water in some cyt *c* peroxidase mutants [4–6]. Interactions with Tyr67 or the influence of the hydrophobic heme cavity may be responsible for the low pK_a in Ala80cyt *c*.

Solution pH has been shown to affect overall protein structure as well as the active-site properties of cyt *c*. Ferricyt *c* is known to have five major pH-dependent conformational states, four of which are distinguished by their heme ligation [7–9]. At low pH (< 2.5), the cyt *c* crevice opens and one or both axial ligands are detached from iron coordination and possibly replaced by water [7–9]. The alkaline transition has stimulated particular interest [8,9]. In the alkaline (pH > 9) form, the axial Met80 is replaced with another strong-field ligand to maintain a six-coordinate low-spin heme. An ^{1}H NMR study of native and mutant yeast cyt *c* indicates that two isoforms of alkaline cyt *c* exist, one of which has Lys79 as an axial ligand [10].

We have investigated the pH and temperature dependences of the ^{1}H NMR spectra of paramagnetic ferriAla80cyt *c* and ferriWTcyt *c* to probe how the ligand at position 80 affects the pH-dependent conformational equilibria of cyt *c*. Analysis of the results in low and high pH regions has shed light on the structures of the acid and alkaline forms of the wild-type protein (WTcyt *c*). A pK_a of ~6 has been determined for the heme high-spin/low-spin transition in the mutant, in agreement with

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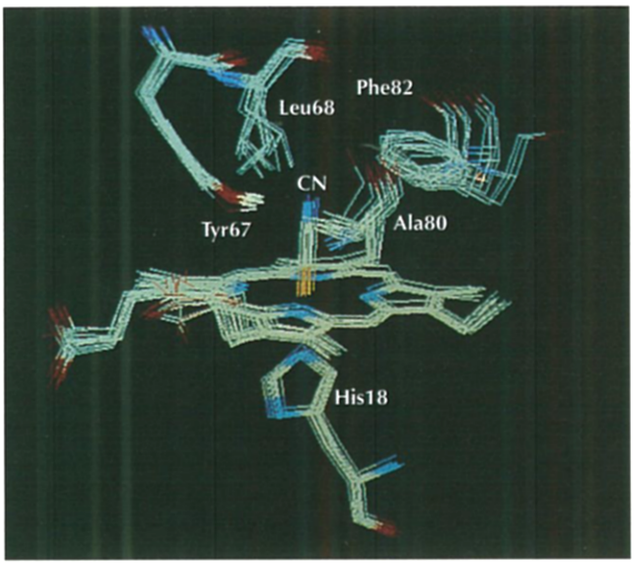

Fig. 1. Family of structures of the active site of ferriAla80cyt *c*–CN^- obtained from NMR spectroscopy (L.B., I.B., K.L.B., H.B.G., P.T. and P. Sompornpisut, unpublished data). The picture shows the hydrogen bond between the Tyr67 hydroxyl proton and the cyanide nitrogen.

other spectroscopic techniques [1,2]. OH^- ligation occurs in the distal site of the low-spin form. Although the cyt *c* fold at pH 7 is not perturbed by the Met80Ala mutation (L.B., I.B., K.L.B., H.B.G., P.T. and P. Sompornpisut, unpublished data), the pH-dependent equilibria are altered, illustrating that the heme ligands have a significant role in the conformational energetics of the protein.

Results and discussion

Spectra at intermediate pH (4–8)

The ^1H NMR spectrum of ferriAla80cyt *c* undergoes dramatic changes (Fig. 2a–c) as pH values are increased from 4 to 8. The most significant change is a transition from high-spin (species HS3, see Table 1) to low-spin (LS3), which is slow on the 200 MHz ^1H NMR timescale. Evaluation of the NMR signal intensities in this pH range gives a pK_a of ~6 for this transition, which correlates well with the value of 6.5 obtained from the pH dependence of the electronic absorption spectra [1,2]. The HS3 species is characterized by broad resonances over a wide spectral range (in particular, around 60 ppm), as expected for heme methyls coupled to a high-spin iron (S = 5/2) [11–13]. The spectrum of the HS3 species, which is dominant between pH 4 and 5, undergoes minor changes with pH (Fig. 2b,c). Species LS3 is dominant between pH 7 and 8; it is characterized by hyperfine-shifted signals in the 30–5 ppm region (Fig. 2a).

The nonselective T_1 values of the heme methyls are reported in Table 2. When electron–nuclear coupling dominates nuclear relaxation, the experimental magnetization recovery is expected to be exponential [14–16]. This is the case for the T_1 values reported here. The experimental T_1 values of species LS3 are shorter than

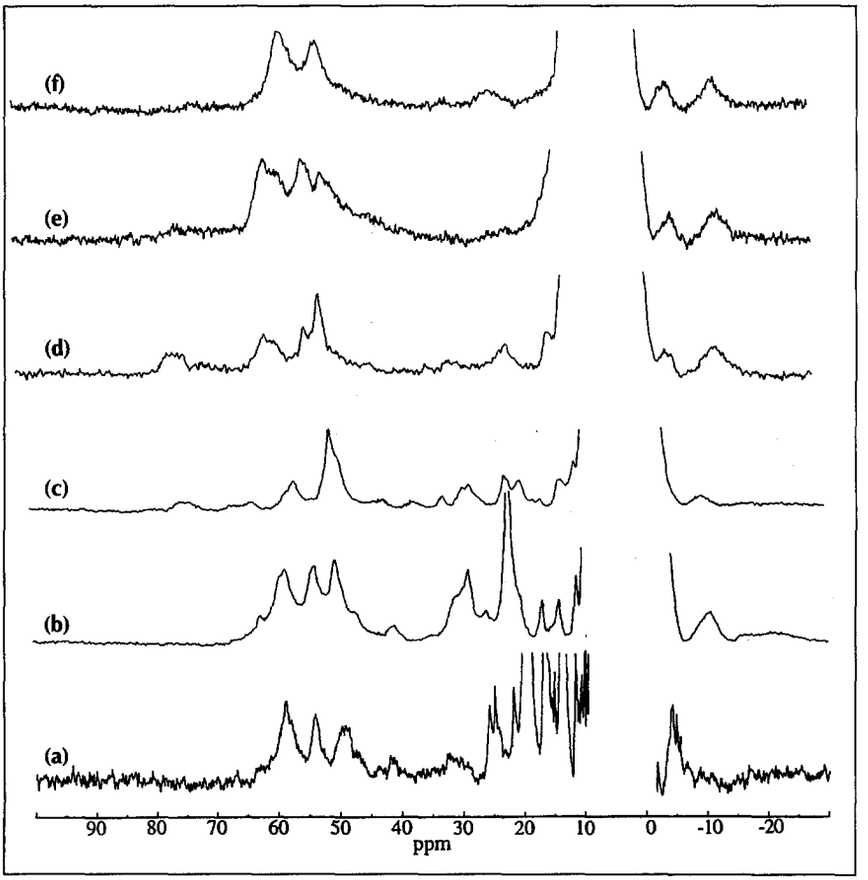

Fig. 2. 200 MHz ^1H NMR spectra of Ala80cyt c. The spectra were recorded in D_2O solutions (50 mM phosphate) at **(a)** pH = 7.0, T = 296 K; **(b)** pH = 5.0, T = 296 K; **(c)** pH = 4.0, T = 296 K; **(d)** pH = 3.7, T = 296 K; **(e)** pH = 2.2, T = 296 K; **(f)** pH = 2.2, T = 306 K. The vertical scale of (a) is eight times that of (b); the (b)–(f) scales are the same.

Table 1. WTcyt *c* and Ala80cyt *c* species at different pH values[a].

	WTcyt *c*		Ala80cyt *c*	
pH	**heme methyl shift range (ppm)**	**species**	**heme methyl shift range (ppm)**	**species**
2.2	60–45	HS1	60–45	HS1
3.0	80–40	HS2	80–45	HS2
4.0	35–30	LS1	80–45	HS2
6.0	35–30	LS1	70–40 + 35–13	HS3 + LS3
7.0	35–30	LS1	20–13	LS3
9.0	35–30 + 25–10	LS1 + LS2(a+b)	20–13	LS3
11.0	25–10	LS2(a+b)	25–10	LS2(a)

[a]HS, high-spin; LS, low-spin.

10 ms (Table 2), that is, they are dominated by the coupling with the unpaired electron. Under these circumstances, the T_1 values depend on the sixth power of the proton–iron distance, and on the electron correlation time τ_s. The τ_s value, in turn, is related to the iron ligand field. A comparison of T_1 values of heme methyls within related heme proteins therefore provides meaningful information, because the distances between the methyls and the iron in the heme are constant and the only variable is τ_s. We have measured the T_1 values of the metmyoglobin, ferriWTcyt *c*, and ferriAla80cyt *c* heme methyls at different pH values, as well as those of ferriAla80cyt *c*–CN^- (Table 2). The LS3 species of Ala80cyt *c* has T_1 values of the same order of magnitude as the alkaline form of metmyoglobin, which is believed to contain a Fe–OH^- species [3]. It is possible that the weak OH^- ligand causes strong axial distortions and long electronic relaxation times [14,17,18]. Note that the T_1 values of LS3 are much shorter than those of hemes with two strong-field ligands, such as cyanide–histidine and histidine–methionine ligated species (Table 2) [19–21], consistent with hydroxide ligation. These results support the assignment of the axial His–OH^- ligation for Ala80cyt *c* at pH 7 previously made on the basis of absorption and electron paramagnetic resonance (EPR) spectroscopies [1,2]. The binding of hydroxide to the heme at pH 7 rather than water, as observed in myoglobin, may be due to stabilization of the Ala80cyt *c* hydroxide species through hydrogen bonding with the distal Tyr67. In addition, the hydrophobic cytochrome heme pocket is expected to lower the pK_a to reduce the charge at the heme.

Table 2. 200 MHz T_1 values (ms) for heme methyl resonances in proteins with different heme coordination.

WT, pH 7, LS1	45–50 ms
WT, pH 11, LS2(a+b)	50–85 ms
Ala80, pH 7, LS3	5.5–9 ms
Ala80, pH 11, LS2a	55–95 ms
Mb–OH^- [a]	1–2 ms
Ala80–CN	73–115 ms
Pxs–CN^- [b]	40–90 ms

[a]Data collected on 0.5 mM sperm whale myoglobin in 0.1 M phosphate buffer, pH 10.4.
[b]Pxs denotes peroxidases. Data on Pxs–CN^- are from references [19–21].

The ^1H NMR spectra of ferriWTcyt *c* between pH 4–8 indicate the presence of a single low-spin iron(III) species (LS1). The spectra (Fig. 3a–c) show two downfield-shifted resonances of intensity 3 that have previously been assigned to heme methyls [22,23]. The single-proton signals in the 25–8 ppm region are due to protons of other heme substituents and to the axial ligands [22,23]. Resonances of the axial Met and His protons are also present upfield in the –23 to –32 ppm range [22,23]. The shifts and T_1 values (Table 2) are consistent with the values expected for a system containing a six-coordinate low-spin iron(III), where both axial ligands are protein side chains [11,12]. The small pH dependence of the shifts of the hyperfine-shifted resonances in the 4–8 pH range is also consistent with that previously reported [24].

Spectra at low pH

As the pH is decreased from 5 to 4, new broad resonances appear in the ^1H NMR spectrum of Ala80cyt *c* in the 80–40 ppm range (species HS2) while the signals of species HS3 decrease in intensity (Fig. 2b–d). Species HS2 and HS3 are in slow exchange on the 200 MHz ^1H NMR timescale. At pH 2.2 and 296 K (Fig. 2e), the NMR spectrum of the mutant indicates another high-spin species (HS1) with four signals in the 60–45 ppm region. This species is also in slow exchange with HS2 and HS3. It appears that there are at least three high-spin species in equilibrium, with one, HS2, appearing and disappearing within three pH units. Its existence therefore depends on more than one ionization. Ionizable groups that may be involved in these low pH transitions are the heme propionates and the coordinated proximal histidine. We also observe a strong influence of temperature on the distribution of the species (Fig. 2e,f).

When the pH of ferriWTcyt *c* is lowered below 4, the signals broaden and other resonances, characterized by larger linewidths, appear in the 80–40 ppm region (Fig. 3c,d). This behavior suggests quasi-slow exchange (on the 200 MHz ^1H NMR timescale) between the neutral

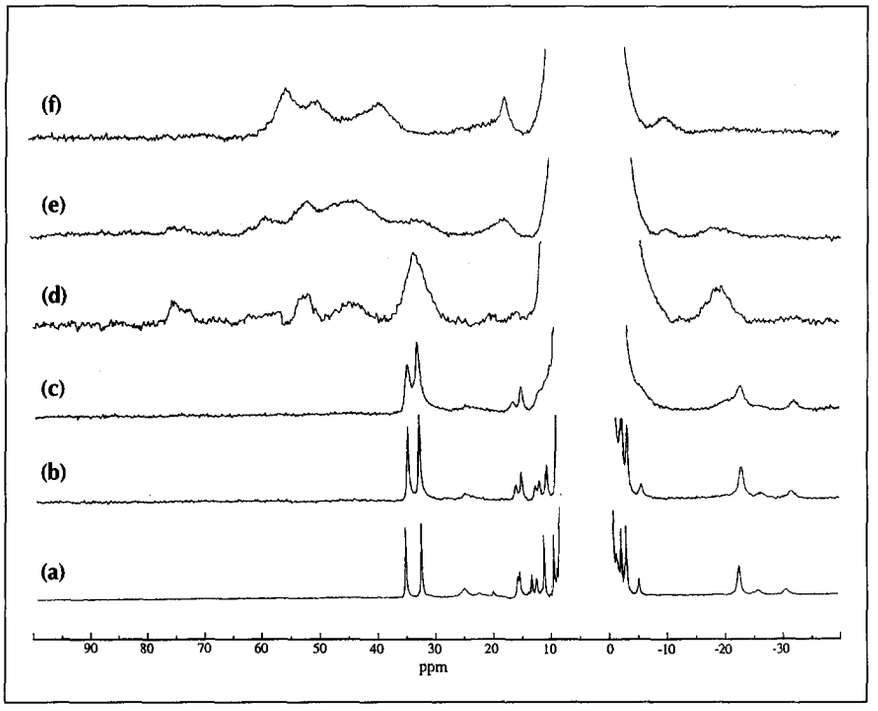

Fig. 3. 200 MHz ^{1}H NMR spectra of WTcyt *c*. The spectra were recorded in D_2O solutions (50 mM phosphate) at **(a)** pH = 8.2, T = 296 K; **(b)** pH = 4.5, T = 296 K; **(c)** pH = 4.0, T = 296 K; **(d)** pH = 3.0, T = 296 K; **(e)** pH = 2.2 T = 296 K; **(f)** pH = 2.2, T = 306 K.

low-spin species (LS1) and a high-spin one (HS2). From the shift differences between the resonances of these two species, it is possible to estimate that the exchange rate is on the order of 2–4 x 10^4 s^{-1}, which is within the minimum and maximum difference in chemical shifts between methyl signals of the two species. The ^{1}H NMR spectrum of this species is very similar to that of the mutant HS2 species. The formation of HS2 could be due to bond breaking between the iron and one or both axial ligands, producing a high-spin iron(III) form. A further decrease in pH (pH 2.2, Fig. 3e) produces another species (HS1) in slow exchange with HS2. HS1 is characterized by broad resonances in the 60–50 ppm region, and is very similar to the analogous species seen for the mutant. Temperature affects both the shifts of the hyperfine-shifted signals (a decrease in temperature producing an increase in shift) and the distribution of the species (Fig. 3e,f).

Spectroscopic studies have shown that the acid denaturation of WTcyt *c* [7–9,25] involves at least two forms (molten globule and unfolded) [25]. In essentially the same pH range we observe two high-spin species (HS2 and HS1) in both variants. The high-spin nature of the low pH forms of WTcyt *c* (HS1 and HS2) indicates that at least one of the two protein axial ligands is not coordinated to the iron. The strong similarities between the spectra of WTcyt *c* and those of Ala80cyt *c* recorded at low pH suggest that these proteins have the same heme coordination under these conditions, and therefore Met80 is not coordinated at low pH in WTcyt *c*. It is possible that the HS2 species of the two proteins, which appear as the pH is lowered below pH 4, still have the His coordinated to the iron and that its detachment is associated with the HS2 → HS1 transition around pH 2. For the HS2 species, the shifts in the diamagnetic region of the NMR spectra show dispersion consistent with the presence of some secondary structure.

Spectra in the alkaline region (pH 8–11)

The alkaline transition of WTcyt *c* is manifested in the NMR spectrum by new resonances that appear in the 25–10 ppm region [10,24]. Consistent with the pK_a of 8.5 for this equilibrium determined by electronic spectroscopy [26], the alkaline transition is almost complete at pH 10 (Fig. 4a–c). The T_1 values of these resonances are only slightly longer than those of the neutral pH form LS1 (Table 2). When the pH is increased to 11, the intensities of some of these signals (indicated by x in Fig. 4c,d) in the 25–10 ppm range decrease, suggesting the existence of two species very similar to each other in terms of chemical shift and T_1 values (designated LS2(a+b)). The equilibrium between these two alkaline species is sensitive to temperature, with an increase in temperature mimicking a decrease in pH (Fig. 4c–e). The shift data at high pH presented here are consistent with literature values [10]. The T_1 values have not previously been reported.

The spectrum of Ala80cyt *c* is essentially pH insensitive between pH 7 and 9 (Fig. 5a,b), but changes drastically above pH 9, showing a pK_a of ≈ 10, as determined from the pH dependence of the intensity of the hyperfine-shifted signals. The spectrum obtained at pH 11 (Fig. 5d) closely resembles that of the dominant species of WTcyt *c* at the same pH (Fig. 4d). In addition, the heme methyls of both proteins exhibit the same T_1 values (Table 2), and these values are also very similar to those of the other low-spin form (LS1) of WTcyt *c*. The T_1 values of the heme methyls of Ala80cyt *c* LS2 are significantly longer than those of Ala80cyt *c* at pH 7, suggesting that a stronger-field ligand is coordinated at high pH. In the

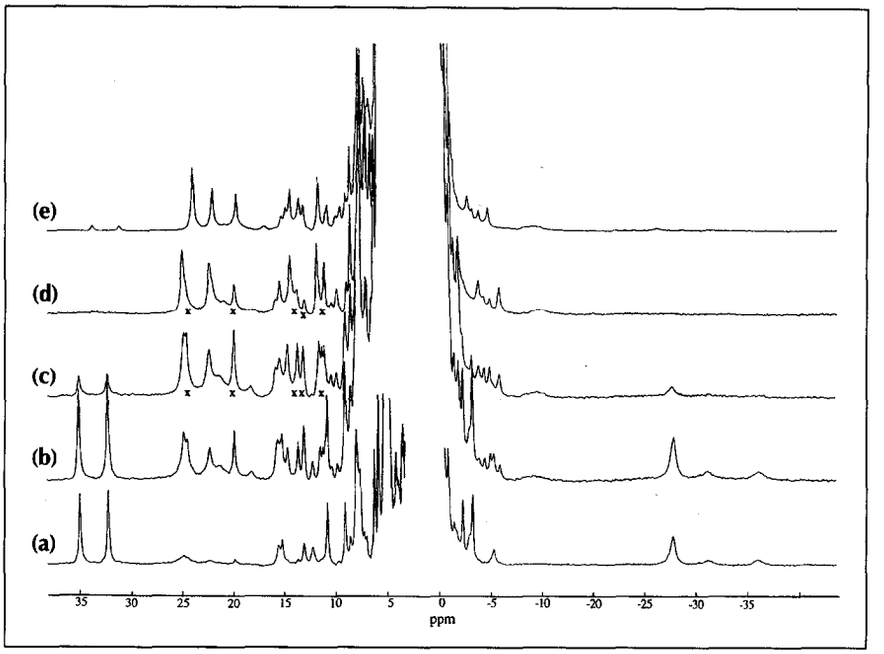

Fig. 4. 200 MHz ^{1}H NMR spectra of WTcyt *c*. The spectra were recorded in D_2O solutions (50 mM phosphate) at **(a)** pH = 8.2, T = 296 K; **(b)** pH = 9.1, T = 296 K; **(c)** pH = 10.0, T =296 K; **(d)** pH = 11.0, T = 296 K; **(e)** pH = 10.0, T = 306 K. An x indicates a signal that decreases in intensity as pH is raised above 10.

mutant, temperature affects the value of the pK_a for the alkaline transition, indicating that a conformational change is involved.

The two alkaline forms of WTcyt *c* observed by ^{1}H NMR have been attributed to ligation of two different lysine side chains, one of which is Lys79 [10]. The detection by ^{1}H NMR of a single alkaline species for the Lys79Ala mutant was the basis for the assignment of Lys79 as the sixth ligand for one of the two forms; the very similar shifts and linewidths of the second species suggested ligation of another Lys residue [10]. (This, however, would require an extensive structural rearrangement, as no other Lys is close to the heme pocket.) The existence of a single alkaline species in Ala80cyt *c* demonstrates that the creation of a less hindered distal pocket can preferentially stabilize one of the alkaline conformations. We propose that the Lys coordinated in the alkaline form of Ala80cyt *c* is not Lys79, but rather one whose coordination would require a significant conformational change, since no other Lys is near the heme pocket. It seems unlikely that Lys79, which does not coordinate to Ala80cyt *c* at neutral pH, would compete with hydroxide as a ligand for iron as the pH is raised. It is more reasonable that hydroxide displacement by a Lys would occur if accompanied by a large conformational rearrangement.

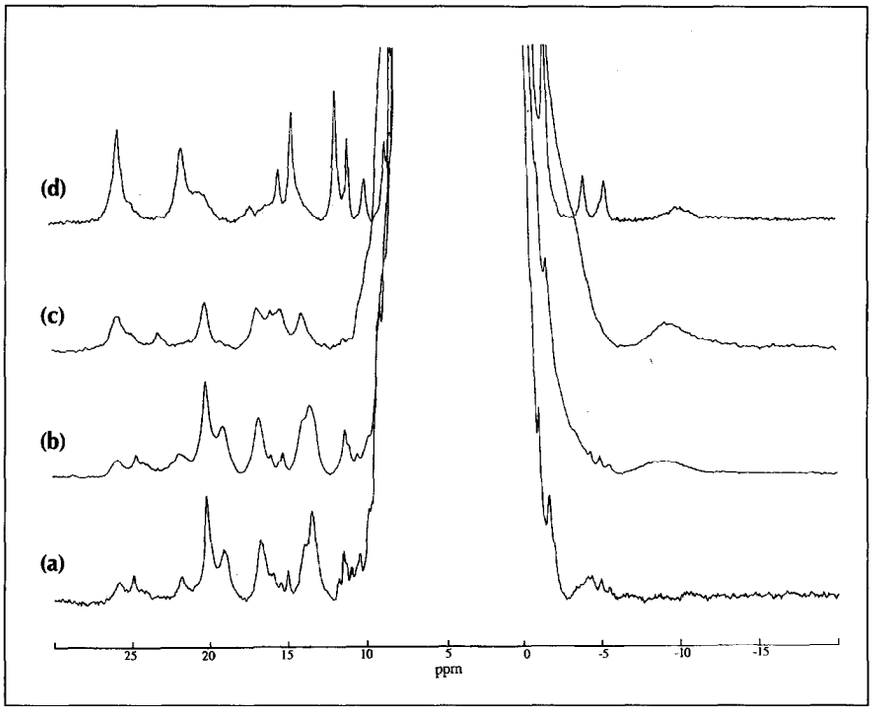

Fig. 5. 200 MHz ^{1}H NMR spectra of Ala80cyt *c*. The spectra were recorded in D_2O solutions (50 mM phosphate) at 296 K and **(a)** pH = 7.0, **(b)** pH = 9.0, **(c)** pH = 10.0, **(d)** pH = 11.0.

Significance

The active site of the electron-transfer protein cytochrome *c* consists of a heme group with two protein-donated axial ligands, the imidazole of His18 and the thioether of Met80. The mutation of a single residue, Met80, to Ala opens a site at the heme capable of binding exogenous ligands, providing a system for probing how axial ligation affects the pH-dependent conformational changes of the protein.

By investigating the pH dependence of the ^{1}H NMR spectra of paramagnetic ferriAla80cyt *c* and wild-type ferricyt *c*, we have determined that the Ala80 protein undergoes a transition from low spin to high spin as the pH is lowered below 7, consistent with the result obtained from absorption spectroscopy. At the lowest pH values examined, the spectra are similar for both proteins, indicating a single protein axial ligand for the wild-type protein. The short T_1 values of the heme methyl resonances at pH 7 support the hypothesis that hydroxide is a heme ligand at this pH. At pH 11, the spectrum of Ala80cyt *c* resembles that of one of the two alkaline forms of the wild-type protein.

The structure and folding of cytochrome *c* have long been subjects of investigation. The alkaline (pH > 9) forms of the protein have been studied for 50 years, but, remarkably, their structures are still a mystery. The preferential stabilization of one of the two alkaline isoforms by mutation of Met80 to Ala is consistent with the hypothesis that formation of one of them requires a significant conformational change. Our findings provide an example of the influence that metal–ligand interactions can have on the conformational energetics of metalloproteins.

Materials and methods

Sample preparation

WTcyt *c* and Ala80cyt *c* were expressed in *S. cerevisiae* and purified as previously reported [2]. The ^{1}H NMR samples were prepared by dissolving lyophilized protein in 50 mM phosphate buffer at pH 7 to give solutions 1 mM in protein. 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 (uncorrected for the isotope effect) with an Orion model 720 pH meter and a Microelectrodes Inc. model MI-410 microcombination pH probe.

Collection and analysis of ^{1}H NMR spectra

The ^{1}H NMR spectra were recorded on a Bruker MSL200 spectrometer. The 200 MHz ^{1}H NMR spectra were recorded using a superWEFT (water-eliminated Fourier transform) pulse sequence [27] with a recycle delay of 200 to 250 ms.

Nonselective T_1 measurements were taken at 200 MHz with the inversion recovery pulse sequence [28] with delay times between subsequent pulses from 2048 to 0.05 ms and a recycle delay of 2048 ms. The recovery of magnetization after a nonselective excitation is expected to be essentially exponential in the case of large paramagnetic contributions (with respect to cross relaxation) [14–16] as is observed in the present case. When the contribution to nuclear relaxation due to the nucleus–electron coupling is large, selective and nonselective nuclear T_1 values tend to be equal. Proton–proton cross relaxation effects are not observed in this case and the magnetization recovery is an exponential process. When the paramagnetic contribution is sizable but not dominant, as in experiments reported here, only the initial part of the magnetization recovery of a nonselective experiment is expected to fit a single exponential. However, within the limitations of this experiment and for the protein derivatives studied, the same T_1 values are given by fitting either the initial part of the magnetization recovery or the whole magnetization recovery curve to a single exponential.

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