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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · FEBRUARY 2005

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EPR and Optical Spectroscopic Studies of Met80X Mutants of Yeast Ferricytochrome *c*. Models for Intermediates in the Alkaline Transition

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Abstract: The ferric forms of Met80X mutants of yeast iso-1-cytochrome *c* (X = Ala, Ser, Asp, and Glu) display EPR and optical spectra that are strongly pH dependent. At low pH values (pH $\approx$ 5) the sixth coordination sites are filled by H_2O that, on elevating the pH, is replaced by OH^- in the cases of Met80Ala and -Ser (p$K \approx$ 5.6 and 5.9, respectively) and by a lysine amino group in the cases of Met80Asp and −Glu (p$K \approx$ 9.3 and 11.6, respectively). The ligand sets and the pK values of the transitions are rationalized in terms of the structure of the heme pocket, and a possible mechanism of the "trigger" in the alkaline transition of the native protein is suggested.

Introduction

Eukaryotic cytochrome *c* is located in the intermembrane space of mitochondria where it functions to transfer electrons from the bc_1 complex to cytochrome *c* oxidase. It is a small water-soluble protein that has been much studied both because of its important function and because it is amenable to study by a large range of structural and spectroscopic techniques. Its important role in apoptosis has recently greatly added to the interest it has attracted.[1,2]

As part of a study of electron transfer initiated by photoactivation, we have prepared a set of cytochrome *c* mutants in which the intrinsic ligand to the central iron atom, methionine-80, has been substituted by a number of other residues (namely, Ala, Ser, Glu, and Asp) that coordinate weakly, or not at all, to the iron.[3−6] In this paper we report the properties of the ferric derivatives of these mutants and compare them to those of the native molecule and of carboxymethyl cytochrome *c* (Cm cyt *c*) in which the methionine residue has been chemically modified.[7,8] Our objective has been to understand the factors that control the nature of the iron ligand set in these mutants and how this is influenced by changes in pH. Of particular interest is the pH-induced conformational change that, in the native ferric protein, has been termed the alkaline transition in which the ligand filling the sixth coordination site switches from a methionine to a lysine residue.[9−16] This transition, although apparently simple, is in fact rather complex involving first the deprotonation of a "trigger" group, or groups, variously identified as a heme propionate, the proximal histidine, or a buried water molecule, and second a conformational equilibrium.[9] As the ligand switch has a dramatic effect on the redox potential of the cytochrome and the mobility of the protein structure around the heme crevice,[17] it remains an interesting speculation that this transition (either in the free protein or when bound to its reductase or oxidase) has functional importance in vivo. Mutants in which the methionine-80 ligand has been substituted can be considered as models for intermediates populated during the methionine-80 to lysine transition and throw light on the mechanism of the trigger.[11,15]

The structures of native cytochrome *c* and of CN^--Met80Ala yeast iso-1-cytochrome *c* have been determined, and the solution structure of the latter is given in Figure 1.[18] In this figure, we indicate the positions of Lys73 and Lys79 that have been

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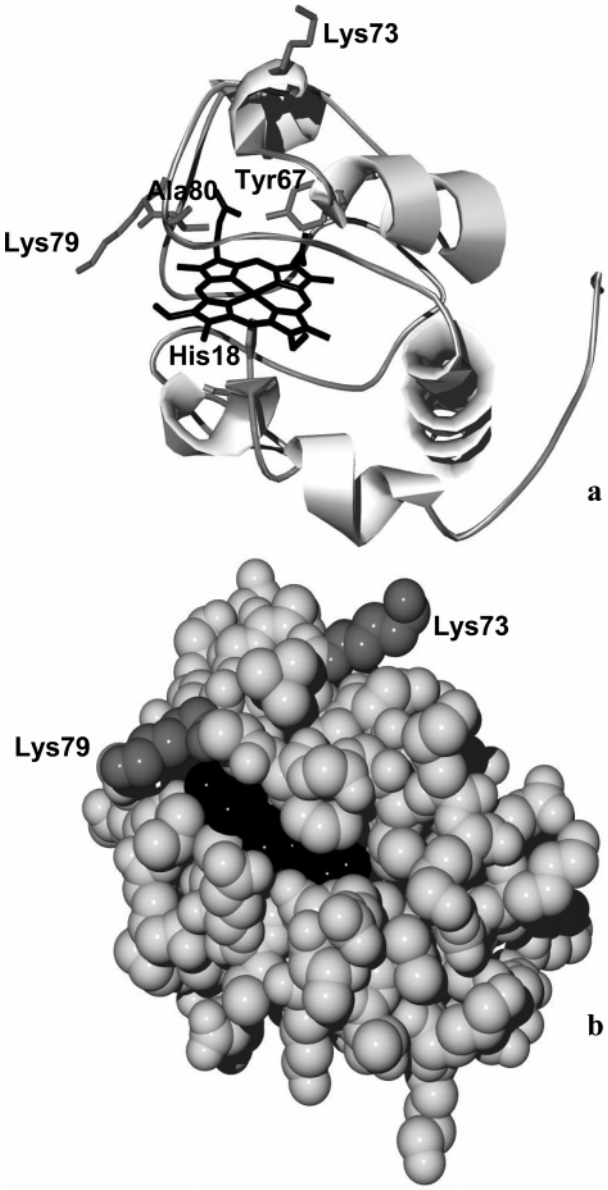

Figure 1. Structures of the ferric form of Met80Ala yeast iso-1-cytochrome *c* (CN^- not shown). (a) Clearly shown is the heme group (black) and the central iron atom bound to four nitrogens of the pyrrole ring and His18 on the proximal side. Some important amino acid residues are shown including lysines-73 and -79 that may bind to the central iron in the ferric forms of some cytochrome *c* molecules on increasing the pH, and Tyr67 suggested as being a hydrogen-bonding partner to the side chains of the engineered acidic 80 mutants Glu and Asp. (b) Space-filling model showing the heme group (black) and lysines-73 and -79 (dark gray). The structure partly shows how inaccessible the central iron atom of the heme is to solvent (~9% in yeast cytochrome *c*), and therefore to the binding of intrinsic amino acid ligands such as lysine groups unless there is some major unfolding and repacking of the protein.

strongly suggested to act as ligands (at elevated pH values) to the central iron atom of the heme,[11,15] and Tyr67 that may interact with residues substituted at position 80. Ness et al. have shown, using computer modeling, that refolding the phylogenetically conserved protein sequence 70−80 allows the Met80 axial ligand to be replaced by Lys72, Lys73, or Lys79 while conserving the main structural features of the native molecule. The smallest structural change is required for replacement of Met80 by Lys79 (cf. Lys72 and Lys73 that are of similar magnitude).[19]

Some Met80 mutants of cytochrome *c* have been studied previously and have proved useful for the investigation of ligand binding to the heme. For example, the Met80Ala variant of cytochrome *c* has been produced by both semisynthetic (horse) and site-directed mutagenesis (yeast) methods.[6,20,21] This molecule was shown to possess some unusual ligand binding properties due to the vacancy of the heme iron's sixth coordination site, and to the close packing of the protein moiety in the distal pocket region.

We report here the properties of the ferric forms of a set of Met80X mutants of yeast iso-1-cytochrome *c* and compare them to those of Cm cyt *c*. These include their optical and EPR (X-band) spectra and the apparent p*K* (pK_{app}) values for their pH-dependent spectral transitions. A major finding is that, at elevated pH values, either an intrinsic or an extrinsic (H_2O or OH^-) ligand can bind to the central iron atom depending on both the pH and amino acid substituted at position 80. We discuss these differences in the nature of the heme ligand set and the effect of pH in controlling this set in terms of the relative flexibility of the protein environment surrounding the distal heme pocket, i.e., the "openness" of the heme crevice.

Materials and Methods

Site-directed mutagenesis, protein expression, and purification of methionine-80 mutants of yeast iso-1-cytochrome *c* were performed as described previously.[6]

The ferric proteins were prepared by addition of excess ferricyanide followed by passage down a small (5 cm) Sephadex G25 column preequilibrated with the required buffer. Cm cyt *c* was prepared by the methods of Schejter and George.[7]

pH titrations were performed by additions of small amounts of either base (NaOH, 1.0 M) or acid (HCl, 1.0 M) to the Met80X mutant proteins contained in a quartz cuvette in a spectrophotometer. Protein concentrations were typically between 2 and 15 μM, and the buffer for all samples was 20 mM each in ethanoic acid, sodium phosphate, and sodium borate. A mini magnetic stirrer was used to mix the protein solutions during titration with H^+/OH^-, and the pH electrode remained partly immersed within the cuvette throughout. Absorbance changes in the Soret and visible regions (namely, the ~620 nm band) of the optical spectra as a function of pH were monitored, and fitted to the Henderson−Hasselbalch equation. The absolute absorbance values in the aforementioned regions were calculated by subtracting them from the absorbance value found at ~650 nm in the same spectra (i.e., where there was very little contribution from the heme). Dilution of protein in the cuvette was calculated to be not more than ~3% following additions of either acid or base, and was therefore discarded in all calculations. Wilmad SQ EPR tubes were used for EPR samples. Tubes containing protein solutions, or water (blanks), were quickly frozen in methanol kept on dry ice. Once frozen, samples were transferred to liquid nitrogen where they were stored prior to measurements. The spectra were measured on a Bruker EMX EPR spectrophotometer equipped with an Oxford Instruments liquid helium system. A spherical high-quality Bruker resonator SP9703 was used. Examination of the distal heme pocket region of the Met80Asp and -Glu mutants was performed, utilizing the ^{1}H NMR structure of the ferric cyanide derivative of the Met80Ala mutant of yeast iso-1-cytochrome *c* determined by Bren et al.[18] These structures were produced after prior mutation of the alanine at position 80 to aspartate and glutamate residues. A number of heme pocket structures of Met80Asp and -Glu were generated from the ^{1}H NMR structure of CN^--Met80Ala, by rotation of the β and γ carbon atoms (Asp80, $-CH_2COO^-$), and the β,

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γ, and δ carbon atoms (Glu80, $-CH_2CH_2COO^-$) of the replacement side chain carboxylate groups at position-80. Favorable hydrogen-bonding interactions were formed between the negatively charged carboxylates of Met80Asp and -Glu and the side chain hydroxyl of Tyr67. For each acidic 80 mutant, one oxygen atom of the side carboxylate was directed toward the Tyr67 hydroxyl group, the other oxygen atom being directed toward where a water molecule would occupy the sixth coordination site on the distal side. Swiss pdb viewer was used to create the structures.

Results

EPR Properties. (a) pH Dependence of the Ferric Forms. The ferric forms of all the Met80X mutants, wild type, and Cm cyt *c* exhibited well-resolved EPR spectra at liquid He temperatures. The nature of these spectra is dependent on the amino acid substituted at position 80 and on the pH of the sample. We illustrate this in Figure 2 where the EPR spectra of the Met80Asp mutant are given at a range of pH values. At pH 4 the heme iron is high-spin ($g_x \approx 6$, $g_z \approx 2$) and axially symmetric (Figure 2a). On increasing the pH the high-spin signal decreased and low-spin forms ($g_x \approx 3$, $g_y \approx 2$) appeared (Figure 2b). This figure also shows that at pH 7.4 the heme iron that remains high-spin is rhombically distorted. All the mutants exhibited this property to differing extents. For the Met80Asp and -Glu mutants, wild-type, and Cm cyt *c*, increasing pH generated three low-spin forms that were themselves in pH-dependent equilibria. This is also illustrated in Figure 2 where comparison of the spectra obtained at pH 10.65 (Figure 2c) and pH 13 (Figure 2d) shows that, although the heme iron remains low-spin, the nature of the ligand set has changed (see below and Table 1). The Ala mutant behaved somewhat differently in that only one low-spin form was observed at high pH values (Figure 2e). The spectra of two low-spin forms of Met80Ser at pH 11 are given in Figure 3. In addition, a further non-heme iron signal at $g = 2.065$ was observed in the Met80Ser mutant sample, but not in any of the other Met80X derivatives. The signal at $g \approx 4.3$ reports the presence of a small fraction of non-heme iron in rhombic coordination. This signal increased at very high pH values (Figure 2d), probably indicating some heme degradation under these conditions. Table 1 reports the *g* values of the high- and low-spin species for all the mutants and compares them with those seen for the wild-type protein and for Cm cyt *c*.

(b) Optical Properties. In keeping with the EPR studies described above, the optical absorbance spectra of the ferric mutants and Cm cyt *c* were pH dependent, and the spectral changes observed were consistent with high- to low-spin transitions on increasing the pH. Figure 4a,b shows the titration of the Met80Asp mutant. At low pH values (pH < 7) the spectrum displays a distinct band at ~620 nm, characteristic of high-spin ferric heme iron with water bound in the sixth coordination position (see Figure 4a).[21] On increasing the pH this band was bleached and the Soret band moved to longer wavelengths and diminished in intensity. This transition conformed to a simple ($n = 1$) deprotonation with p$K \approx 9.25$ (see the inset to Figure 4a and Table 2). Further elevation of pH led to a second optical transition that also conformed to a simple titration with p$K \approx 12.75$ (see the inset to Figure 4b and Table 2). The Met80Glu mutant and Cm cyt *c* behaved in a similar fashion, and the p*K* values are also given in Table 2.

The Ser and Ala mutants exhibited only one optical transition on increasing the pH, as shown in Figure 4c for the Met80Ser mutant. The p*K* values for this transition are reported in the inset to Figure 4c and Table 2.

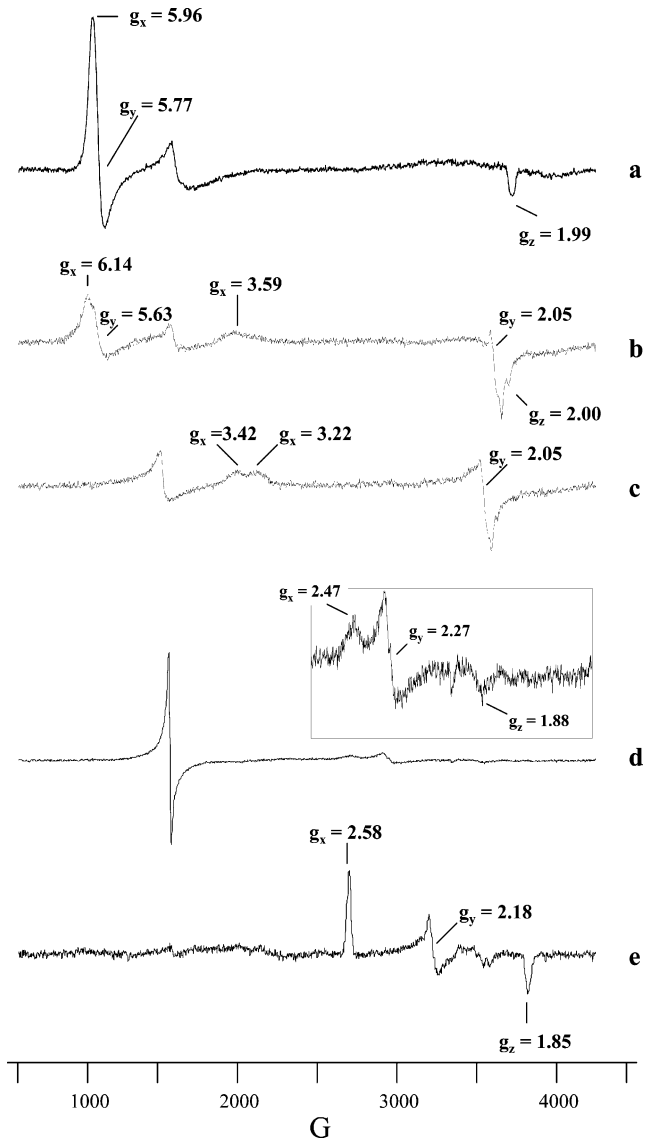

Figure 2. EPR spectra of methionine-80 mutants of yeast iso-1-cytochrome *c*: (a) Met80Asp (pH 4.0), gain 3.99×10^4; (b) Met80Asp (pH 7.4), gain 3.99×10^4; (c) Met80Asp (pH 10.65), gain 3.99×10^4; (d) Met80Asp (pH 13.2), gain 1×10^4; the inset is the high-field region of the spectra; (e) Met80Ala (pH 8.0), gain 1×10^4. The *g* values for the spectra are given in Table 1. Conditions: [protein] = 10 μM (a−c), 50 μM (d, e); the temperature for all samples was 10 K; the buffer for all samples was 20 mM each in ethanoic acid, sodium phosphate, and sodium borate; the microwave frequency and power for were 9.47 GHz and 3.18 mW, respectively.

Discussion

Assignment of Ligand Sets (Ligands in the Sixth Coordination Position). The EPR spectra for all Met80 mutants and for Cm cyt *c* show that the ferric heme iron is high-spin at pH values below the p*K* of the first spin-state transition, calculated from the optical spectra. The optical spectra support this conclusion, the 620 nm bands being diagnostic of a ferric high-spin heme iron with a water molecule coordinated in the sixth position.[21] We therefore assign the ligand set His/H_2O to all the mutant proteins at low pH (see Table 1).

On increasing the pH low-spin forms grow-in at the expense of the high-spin forms. The details of these processes depend,

Table 1 [a]

Met80X mutant	pH	spin state: high-spin	spin state: low-spin	proposed ligand set
Glu80	7.4	6.53, 5.34, 1.98		His/water
	13.0		3.45/3.27, 2.05, nd	His/Lys
			2.53, 2.14, 1.85	His/OH$^-$
Asp80	4.0	5.96, 5.77, 1.99		His/water
	7.40	6.14, 5.63, 2.00		His/water
			3.59, 2.05, nd	His/Lys
	10.65	3.42/3.22, 2.05, nd		His/Lys
	13.2	2.47, 2.27, 1.88		His/OH$^-$
Ala80	4.0	6.05, 5.66, 1.99		His/water
	8.0		2.58, 2.18, 1.85	His/OH$^-$
Ser80	4.2	5.99, 5.75, 2.04		His/water
	11.0		2.48, 2.25, 1.87	His/OH$^-$
			2.74, 2.22, 1.75	*His$^-$/OH$^-$
Cm cyt *c*	4.2	5.99, 5.79, 2.00		His/water[1]
(horse)	7.4	6.10, 5.78, 1.98		His/water[2]
			3.48/3.32, 2.06, nd	His/Lys
	13.0	6.15, 5.87, 1.97		His/water[3]
			3.44/3.27, 2.05, nd	His/Lys
			2.94, 2.24, 1.53	*His$^-$/OH
cyt *c* (yeast)	5.2		3.08, 2.14, 1.35	His/Met
	10.0		3.31, 2.04, nd	His/Lys
	12.7		2.59, 2.16, 1.86	His/OH$^-$

[a] nd = signal not detected (note, the different *g* values in the His/water[1], water[2], and water[3] forms of Cm cyt *c* are believed to be due to small protein structural changes in the immediate environment of the ferric iron as a result of changing the pH); * = deprotonated N1 of proximal histidine.

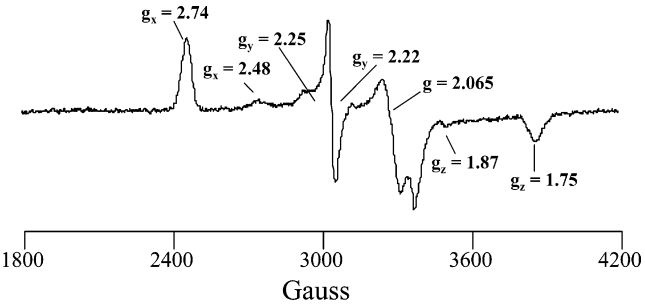

Figure 3. High-field EPR spectra of the Met80Ser yeast cytochrome *c* mutant. Clearly shown are the g_x, g_y, and g_z values for the two species that are His/OH$^-$ (2.48, 2.25, 1.87) and His$^-$/OH$^-$ (2.74, 2.22, 1.75) coordinated (see Tables 1 and 2). Indicated also in the spectrum is the *g* signal at g = 2.065 (see the text for the assignment). Conditions: [protein] = 20 μM; temperature 10 K; the buffer was K$^+$CAPS (20 mM, pH 11.0); microwave frequency 9.47 GHz; power 3.18 mW.

however, on the nature of the mutant under study. With Met80Asp and Met80Glu the pH-dependent spin-state transition leads to species that have EPR signals with g_x values at ~3.5 and ~3.25 and g_y values at ~2.05. These values are very close to those reported by others and are indicative of His/Lys ligation.[10,11,14,22,23] The two sets of g_x values show that there is a mixture of species (see Figure 2c and Table 1).[10,11] This mixture may comprise forms in which different lysine residues are bound to the iron or a single lysine is bound in two distinct conformations. Increasing the pH further led to changes in the low-spin EPR spectra, indicating the loss of lysine ligation, a conclusion that is consistent with the second optical transition seen at high pH values (Figure 4b). Both the Asp and Glu mutants yielded at high pH values (pH > 12.5) essentially the

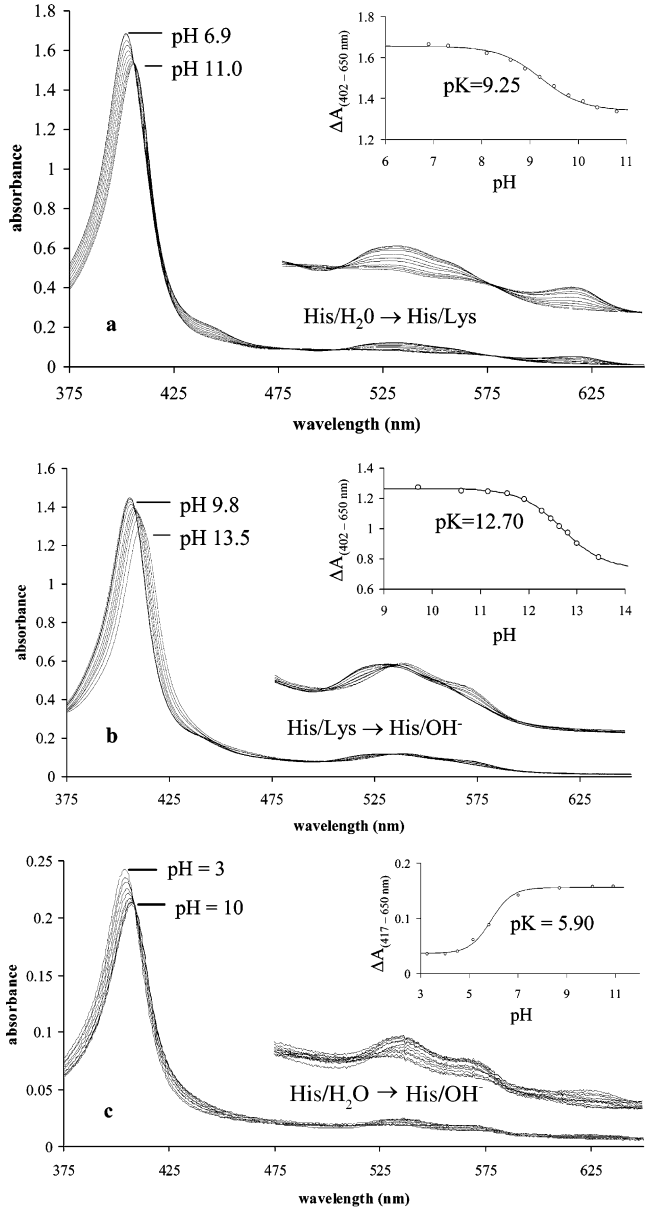

Figure 4. pH titrations of the ferric forms of Met80Asp and Ser: (a) Met80Asp (pH range 6.9−11.0); the inset shows the pK_{app} curve for the high- to low-spin ligand switch; (b) Met80Asp (pH range 9.8−13.5); the inset shows the pK_{app} curve for the low-spin ligand switch; (c) Met80Ser (pH range 3.0−10.0); the inset shows the pK_{app} curve for the high- to low-spin ligand switch. Conditions: [protein] = 2−15 μM; temperature 20 °C; the buffer was 20 mM each in ethanoic acid, sodium phosphate, and sodium borate.

same homogeneous spectrum with g_x, g_y, and g_z values at ~2.5, ~2.2, and ~1.85, respectively (see Table 1 for exact values). These values have been reported previously and are characteristic of His/OH$^-$ ligation of the ferric heme iron.[21,24−26] The optical spectrum at high pH showing absorption maxima at ~406, ~538, and ~570 nm also indicates OH$^-$ binding to the iron.[21] However, at these very alkaline pH values it was

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Table 2 [a]

cyt *c*	pK_{app}(Soret) ± sd ($n = 3$)	pK_{app}(~620 nm) ± sd ($n = 3$)	assigned ligand exchange
Met80Glu	11.55 ± 0.11	11.70 ± 0.08	$His/H_2O \rightarrow His/Lys$
	12.88 ± 0.05	−	$His/Lys \rightarrow His/OH^-$
Met80Asp	9.25 ± 0.08	9.33 ± 0.10	$His/H_2O \rightarrow His/Lys$
	12.75 ± 0.07	−	$His/Lys \rightarrow His/OH^-$
Met80Ala	5.55 ± 0.13	5.62 ± 0.17	$His/H_2O \rightarrow His/OH^-$
Met80Ser	5.90 ± 0.15	5.93 ± 0.19	$His/H_2O \rightarrow His/OH^-$
Cm cyt *c*	6.08 ± 0.05	6.05 ± 0.12	$His/H_2O \rightarrow His/Lys$
	12.10 ± 0.07	−	$His/Lys \rightarrow His/OH^-$
native yeast	8.45 ± 0.06	*8.51 ± 0.08	$His/Met \rightarrow His/Lys$
	11.7 ± 0.05	−	$His/Lys \rightarrow His/OH^-$

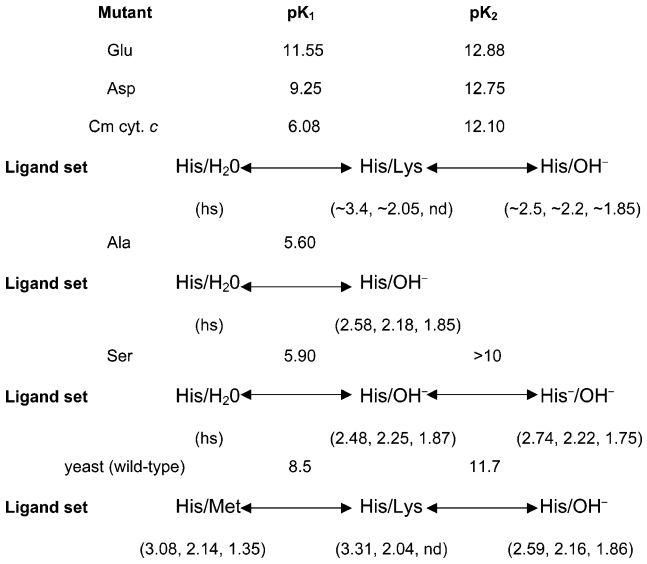
[a] sd = standard deviation; − = no ligand exchange process that gives optical data in this region; * = the given pK_{app} for native yeast cyt *c* calculated at 695 nm. The assignments of the ligand sets are made by reference to the EPR studies.

observed for the Asp and Glu mutants that the intensity of the EPR signals assigned to the His/OH^- ligand set was diminished (see Figure 2d). Integration of the EPR spectra of the Met80Asp mutant showed that ~60% of the His/OH^--coordinated heme iron was lost on increasing the pH from 10.65 to 13.2 despite the concentration of protein in both samples being the same. It is suggested this was due to unwinding of the protein and loss of the histidine ligand at high pH values (>13).

The Ser and Ala mutants behaved in a comparable fashion. On increasing the pH the high-spin signals decreased and were replaced by spectra with g_x, g_y, and g_z values at 2.58, 2.18, and 1.85, respectively, for the Met80Ala mutant or with g_x, g_y, and g_z values at 2.48, 2.25, and 1.87 for the Met80Ser mutant. Again these values are the signature for the His/OH^- ligand set.[21,24−26] Optically we observe a single transition for these mutants (see Figure 4c) and a final spectrum consistent with the assigned His/OH^- ligation.[21] For the serine mutant a second transition was observed by EPR spectroscopy (but not by optical methods) on increasing the pH above ~10. This transition led to the formation of a species with g_x, g_y, and g_z values at 2.74, 2.22, and 1.75 (see Figure 3). By reference to the work of Gadsby et al. we have assigned this to the His^-/OH^- ligand set, the His18 deprotonated on the N1 atom.[14]

Taking the assignments discussed above, and reported in Table 1, together with the p*K* values determined optically and reported in Table 2, we summarize the pH-dependent spin-state and ligation-state transitions in Scheme 1.

Scheme 1. Ligand Sets for Met80X Mutants and the p*K* Values for Ligand Set Changes[a]



[a] Values in parentheses refer to g_x, g_y, and g_z values of the low-spin forms; hs = high-spin.

How May We Account for the p*K* Values and Ligand Sets of the Met80 Mutants and for Cm cyt *c*? To interpret the data given in Tables 1 and 2 in terms of protein structure, it is useful first to consider the native protein in which the ferric iron is coordinated to the sulfur of methionine-80 and the N1 of histidine-18. These ligands coordinate at pH ≈ 7, but on increasing the pH the protein undergoes what has been termed the "alkaline transition" in which the methionine dissociates and is replaced by a lysine.[9−16] The alkaline form is heterogeneous in that one of several lysine residues may fill the sixth coordination site.[10,11] The p*K* of the alkaline transition for yeast iso-1-cytochrome *c* is (depending on the solvent conditions) ~8.5 (see Table 2).[27] This p*K* results from the combination of (at least) two separate equilibria. One equilibrium is that governing the deprotonation of what has been termed the trigger for the alkaline transition. This term refers to the group, or groups, that on loss of a proton initiates the ligand set change. The proximal histidine, a propionate carboxylate, or a buried water molecule (among others) have all been suggested to act as the trigger.[9] Coupled to this deprotonation is a conformational equilibrium in which the protein on the distal side of the heme refolds to bring a lysine residue close to the heme iron. During these processes the methionine dissociates and is subsequently replaced by the lysine.

For all the mutants studied here, and for Cm cyt *c*, the native methionine-80 residue is either substituted or chemically altered such that the replacement or modified residue does not coordinate the heme iron (Ala, Ser, and Cm cyt *c*) or may coordinate only weakly (Glu and Asp). If the mechanism that explains the alkaline transition holds for the mutants, and for Cm cyt *c*, it would be expected that on increasing the pH a lysine would more easily bind to the central iron. This should therefore lead to a lower p*K* for the formation of the His/Lys ligand set. Indeed, for Cm cyt *c* this expectation is met as the pK_{app} for the alkaline transition is ~6, ~3.5 pH units lower than the same ligand switch for the wild-type protein (horse pK_{app} ≈ 9.5).[28,29] The large difference in the value of this pK_{app} could be explained in terms of the relative "openness" of the distal heme cavity. In the synthesis of Cm cyt *c*, the methionine-80 residue is displaced from the ferric heme iron atom, and this results in a conformational change as residues 77−85 "swing out", widening the distal heme cavity.[30] The binding of the incoming lysine residue (probably Lys73 or -79) to the ferric iron in Cm cyt *c* is now much more favorable. For Cm cyt *c*, therefore, not only is the sixth coordination site of the high-spin ferric iron essentially vacant (except for H_2O), but the distal heme cavity is widened, allowing lysine binding to be favorable. However, for the mutant proteins examination of Tables 1 and

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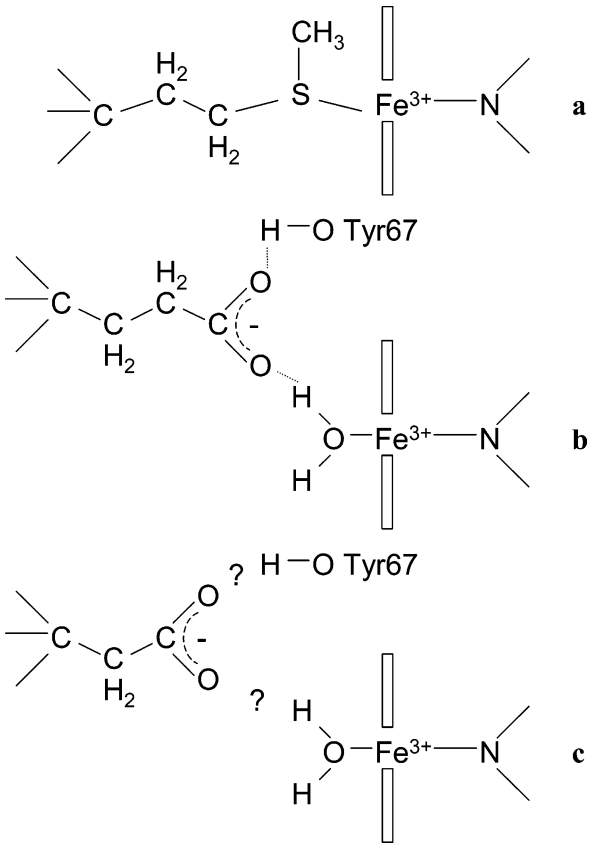

Figure 5. Schematic representations of the possible hydrogen-bonding interactions among the negatively charged side chain carboxylates of Met80Glu (b) and Asp (c), the water molecule bound to the central ferric iron atom, and the hydroxyl group of Tyr67. Also shown is wild-type cytochrome *c* (a).

2 and Scheme 1 shows that this explanation requires modification. For example, although the Met80Asp and -Glu mutants both bind a lysine, the p*K* for this (alkaline) transition is unexpectedly elevated, whereas for Met80Ala and Met80Ser the p*K* of the first pH transition is lowered but a lysine does not bind. To explain these behaviors, we consider a simplified model of the heme pocket.

We will discuss the mutants in turn starting with the Met80Asp and -Glu derivatives. For these mutants the ligand set at pH ≈ 7 is assigned as His/H_2O, an assignment consistent with both EPR spectroscopy and the well-pronounced 620 nm band in the optical spectra. This latter band is bleached in a process conforming to a simple $n = 1$ deprotonation as the pH is raised, yielding eventually a species with a His/Lys ligand set. The p*K* values for this transition are 9.25 and 11.55 for the Asp and Glu mutants, respectively. These values are higher than that of the wild-type molecule, p*K* $\approx$ 8.5. We interpret this behavior by proposing that the negatively charged side chain carboxylate groups of Met80Asp and -Glu interact to stabilize the net positive (+1) charge that is delocalized over the heme group and its iron-coordinated H_2O molecule. In addition, it is possible the carboxylate groups hydrogen bond to the coordinated water molecule and the side chain hydroxyl (−OH) of Tyr67 located nearby in the distal heme pocket (see Figure 5). These proposals are based on examination of the ^{1}H NMR structure of CN^--Met80Ala yeast iso-1-cytochrome *c* following mutation of the alanine-80 to glutamate and aspartate residues. Increasing the pH leads to deprotonation of the iron-bound H_2O molecule, leaving a coordinated OH^- ion. With the negatively charged carboxylate groups of Met80Asp and -Glu stabilizing the positive (+1) charge delocalized on the heme, the system would resist the dissociation of a proton from the water molecule to leave a hydroxyl anion coordinated to the central iron atom. This resistance to deprotonation explains the elevated p*K* values for the first transition for Met80Asp and -Glu. However, with further increases in pH it seems a lysine does eventually bind to the central iron atom, driving it to low-spin with the consequent displacement of the H_2O molecule (see later discussion). This results in the formation of the His/Lys species, the heme gaining stability from the lone pair of electrons that reside on the ϵ-nitrogen ($-NH_2$) of the lysine residue. The pK_{app} for the His/H_2O to His/Lys ligand transition (the alkaline transition) for Met80Glu is 11.55 compared to 9.25 for Met80Asp. This reflects the closer proximity ($\sim$1 Å) the side chain carboxylate of Glu80 can attain to the iron-coordinated H_2O and Tyr67 residue in the heme pocket, because it contains an extra methylene ($-CH_2-$) group in its side chain (see Figure 5). Examination of the Met80Glu and -Asp structures generated reveals that one of the carboxylate oxygen atoms of Glu80 can form a hydrogen bond to the hydroxyl of Tyr67 with a bond length of $\sim$2 Å, whereas in the Asp80 mutant the same interaction is $\sim$3 Å. It is possible that the carboxylate group of Met80Glu can share its hydrogen-bonding between the iron-bound water molecule and the hydroxyl of Tyr67, whereas for Met80Asp its carboxylate may only be hydrogen-bonded to one or the other. For Cm cyt *c*, it would seem that the carboxylate added to the sulfur atom of methionine-80 on chemical modification does not stabilize the +1 charge delocalized on the heme via hydrogen-bonding interactions to the iron-coordinated H_2O molecule. This is likely to be due to steric constraints placed upon carboxylated Met80, rendering it unable to enter the distal heme pocket.[30]

For the Glu and Asp mutants, Cm cyt *c*, and the wild-type molecule, the high pH transitions that lead to the replacement of lysine by OH^- have p*K* values all in the region of $\sim$12.5, reflecting the gross disruption of the protein unwinding on the distal side (see Table 2).

For the Met80Ala mutant the situation is very different, and a number of factors account for this. First, the absence of a negatively charged carboxylate group at position 80 means that this residue cannot stabilize the positive (+1) charge on the heme group. Titration of the water molecule bound to the ferric iron leaves a coordinated hydroxyl anion, and this is favored by the interaction with the positively charged heme group. Thus, the titration of H_2O to OH^- occurs with a relatively low p*K* (see Table 2).[20,21] In addition, protein packing in the alanine mutant in the region of the heme is very tight, making rotation of the peptide backbone and approach of the incoming lysine so difficult that this ligand never coordinates to the central iron atom. Tight packing in the ferrous derivative of Met80Ala has been previously demonstrated.[6,21] A similar argument can be advanced to explain the behavior of the Met80Ser mutant, which has a p*K* $\approx$ 5.9 for the water to hydroxyl ligand switch. In addition, a second transition was observed by EPR for the Met80Ser mutant that was not present for the other mutant proteins. This transition, with a p*K* > 10, we assign to the deprotonation of the N1 atom of the proximal His18 to give the His^-/OH^- ligand set.[14]

Alkaline Transition. Although having been studied for over 40 years, the alkaline transition in cytochrome *c* remains a subject of considerable interest. This stems from the fact that it represents what has been termed a "binary molecular switch", in which the redox potential of the protein is dramatically and reversibly altered by undergoing a transition between two conformational states,[31−33] triggered by the protonation/deprotonation of a group, or groups (see below). Interest is enhanced because this transition takes place in a well-studied protein for which there exists a wealth of structural and spectroscopic information, and because of the possible functional role it plays in vivo.

The alkaline transition involves the replacement of the intrinsic ligand to the ferric iron, methionine-80, with a lysine residue, both Lys73 and Lys79 being likely candidates.[11,15] From an examination of the structure of cytochrome *c* it is apparent that this requires repacking of the protein loop on one side of the heme crevice. It also seems clear from the location of the residues that there must exist an intermediate in which the methionine has dissociated and the lysine has yet to bind.[34] The Met80X mutants that we have studied may provide useful models of this intermediate. In the first place it is worth noting that in the mutants discussed above (and others we have made) there is no instance of a pentacoordinate ferric heme cytochrome *c*. If a protein ligand is not available, then the sixth site is filled with either H_2O or OH^-, and we suggest that these are the likely candidates for ligands to the intermediate in the alkaline transition. This being so, the intermediate may have features in common with the Met80Ala and Met80Ser mutants and coordinate a water molecule/hydroxyl ion (see Scheme 1). At pH values around neutrality and above there would therefore be a hydroxyl anion bound to the iron. The structure of wild-type ferricytochrome *c* with a cyanide anion bound (also a low-spin complex) shows that in this derivative the heme pocket is significantly disrupted and the methionine residue is displaced from the pocket into the bulk phase.[30] We speculate that a similar situation pertains in the intermediate where an OH^- is bound and the methionine displaced from the pocket. This displacement leads to a rearrangement of the protein backbone that brings the amino group of a lysine close to the heme where it displaces the OH^- and binds. The process is favored by the stability conferred on the structure by the reorganization of the protein and the binding of lysine, a process that contributes to what has been termed the conformational equilibrium constant. A similar process is encountered with the Met80Asp and -Glu mutants where the titration of the bound water molecule, leaving an OH^-, dispels the acidic side chains from the distal pocket, initiating refolding of the protein and rebinding of a lysine to the iron. Where there is no bulky or charged group at position 80 that must be displaced from the pocket, as in Met80Ala or Met80Ser, then the rearrangement is not initiated and OH^- remains bound to the iron.

There remains the question regarding the nature of the trigger group, or groups, that on deprotonation initiates the conformational change in the native molecule. A number of candidates have been suggested, among which are the heme propionates, the proximal histidine, or an internal water molecule (Wat166 in eukaryotic cytochromes *c*). The latter is a highly conserved buried water positioned within the distal heme pocket, associated through a network of hydrogen bonds to Tyr67, Asn52, and Thr78, and can also interact electrostatically with the sulfur atom of Met80 and the iron atom of the heme (see Figure 6). In agreement with others we believe that Wat166 plays a central role in mediating the mobility of certain regions of protein structure, certainly those segments of polypeptide involved in the alkaline transition, due to its association in the hydrogen-bonding and electrostatic interactions network.[35−37] Indeed, it has been suggested that Wat166 appears to mediate increases in the mobility of three nearby segments of polypeptide chain when cytochrome *c* is in its oxidized state, and that this may be related to oxidation-state-dependent interactions between cytochrome *c* and its redox partners.[32,33] Furthermore, mutagenesis studies have shown that, by eliminating Wat166 in the Asn52Ile and Asn52Ile/Tyr67Phe variants, such mobility in these polypeptide regions is diminished.[35,36] If this water is indeed the trigger, an attractive possibility is that once deprotonated (p$K \approx 11$) the resulting OH^- formed in the vicinity of the heme migrates to the iron, displacing the methionine out of the pocket (cf. cyano ferricytochrome *c*) and thus initiating the conformational transition. Some support for the idea that the titration of an internal water followed by transient binding of hydroxyl is afforded by the fact that where no internal water is found in the location in the heme pocket, e.g., *Pseudomonas* cytochrome c_{551}, then the alkaline transition does not occur until the protein is beginning to unwind through denaturation, i.e., pH $\approx$ 11.[9]

Support for the view that a water molecule plays an important role may also be provided by the recent work of Martinez and Bowler, who have reported the results of stopped-flow experi-

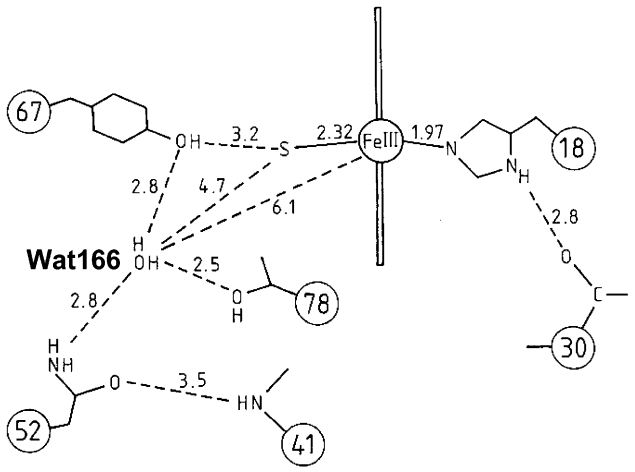

Figure 6. Structure of the heme pocket in tuna ferricytochrome *c*. Clearly shown are the Wat166 molecule and the distances between it and some of its neighboring atoms. Hydrogen bonds are formed to Asn52, Tyr67, and Thr78 as well as electrostatic interactions to the sulfur atom of methionine-80 and the iron atom of the heme group. Adapted from Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 95−115.

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ments in which the pH of solutions of the ferric form of Lys73His yeast iso-1-cytochrome *c* was rapidly changed.[38] On increasing the pH this protein undergoes an alkaline transition in which the intrinsic Met80 ligand is replaced. Up to pH 8 the replacement ligand is His73 and at higher pH values Lys79. Analysis of the pH dependencies of the observed rate constants and amplitudes for the initial event has revealed that this is influenced by three ionizable groups, one with a pK = 6.4, identified as His73, and two others with pK = 5.6 and 8.7. Thus, the authors conclude that the alkaline conformational transition appears to involve more than a single trigger group. Furthermore, and of particular interest in the context of the work presented in this paper, deprotonation of the group with pK = 8.7 is shown to influence the kinetics of the alkaline transition by stabilizing the transition state, i.e., that form of the ferric protein that does not have an amino acid residue occupying the sixth coordination site. The nature of this group was not identified, but the role it plays is the same as that we propose for the buried water molecule (Wat166), namely, that the transition state is stabilized by binding of an OH^- ion to the ferric iron. We suggest, therefore, that the group identified by Martinez and Bowler as important in controlling the alkaline transition is in fact this water molecule.

The design of proteins with new functions is of increasing academic and, potentially, commercial interest. This task is difficult, however, as is well exemplified by our studies of the mutants of cytochrome *c*. The native protein from which the mutants are constructed is small and relatively rigid. This latter property is illustrated by the low apparent quantum yields for CO photodissociation from the ferrous form, indicating that even small neutral gaseous molecules find difficulty in escaping the interior of the protein. This rigidity is advantageous for electron transfer, the native function, as it limits conformational change during redox reactions and hence the reorganization energy. Despite this, the mutant proteins, albeit very similar in overall sequence, display a wide range of behaviors and respond quite differently to the same environmental stimulus, i.e., pH change. The ligand sets to the iron differ, and the pH-induced changes occur with very different pK values. Although it has been possible to rationalize these differences, given knowledge of the structure, they could not easily have been predicted in advance of the experiments being performed. This is another example of small changes in critical positions in proteins having unexpected and far reaching consequences.

Acknowledgment. This work was supported by grants from the BBSRC (U.K.) and the Wellcome Trust. M.T.W. dedicates this paper to the memory of Eraldo Antonini.

JA045719B

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