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Role of Met80 and Tyr67 in the Low-pH Conformational Equilibria of Cytochrome *c*

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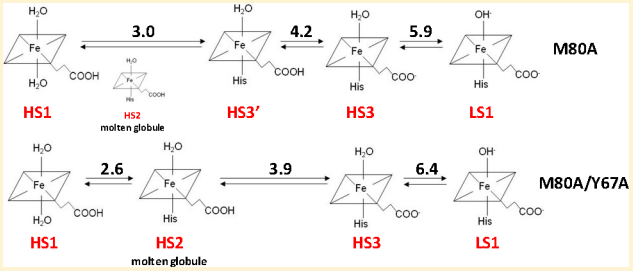
Supporting Information

ABSTRACT: The low-pH conformational equilibria of ferric yeast iso-1 cytochrome *c* (ycc) and its M80A, M80A/Y67H, and M80A/Y67A variants were studied from pH 7 to 2 at low ionic strength through electronic absorption, magnetic circular dichroism, and resonance Raman spectroscopies. For wild-type ycc, the protein structure, axial heme ligands, and spin state of the iron atom convert from the native folded His/Met low-spin (LS) form to a molten globule His/H_2O high-spin (HS) form and a totally unfolded bis-aquo HS state, in a single cooperative transition with an apparent pK_a of ∼3.0. An analogous cooperative transition occurs for the M80A and M80A/Y67H variants. This is preceded by protonation of heme propionate-7, with a pK_a of ∼4.2, and by an equilibrium between a His/OH^--ligated LS and a His/H_2O-ligated HS conformer, with a pK_a of ∼5.9. In the M80A/Y67A variant, the cooperative low-pH transition is split into two distinct processes because of an increased stability of the molten globule state that is formed at higher pH values than the other species. These data show that removal of the axial methionine ligand does not significantly alter the mechanism of acidic unfolding and the ranges of stability of low-pH conformers. Instead, removal of a hydrogen bonding partner at position 67 increases the stability of the molten globule and renders cytochrome *c* more susceptible to acid unfolding. This underlines the key role played by Tyr67 in stabilizing the three-dimensional structure of cytochrome *c* by means of the hydrogen bonding network connecting the Ω loops formed by residues 71−85 and 40−57.



Conformational changes in proteins caused by binding events and/or modification of the properties of the medium are essential for biological functions.[1−5] Moreover, biotechnological applications of proteins may rely on reversible conformational transitions associated with changes in property and function (biomolecular switches).[6−9] Therefore, elucidating the trigger event(s) and the mechanism of conformational equilibria in proteins has a fundamental and applicative relevance.[4] The conformational equilibria leading to non-native states of cytochrome *c* (cytc) have been studied in depth,[10,11] as they are likely involved in some as yet not fully understood functions of cytc beyond electron transfer, such as apoptosis and cellular oxidative stress.[13−20] Moreover, cytc has been widely used as a model to gain information about the mechanism of protein folding and unfolding processes.[21−34] The effects of pH on cytc structure and function have been extensively investigated.[10,35] The low-pH conformational changes start with disruption of the axial methionine−Fe(III) bond and formation of a molten globule state, followed by extensive protein unfolding.[10,29,35−52] This complex process is affected by the ionic strength and the nature of the anions in solution,[4,29,36−44,46,47,49−52] which influence heme coordination and the structure and stability of the protein conformers. Indeed, the molten globule state is stabilized at high ionic strengths, while at low salt concentrations, the acid transition appears as a single cooperative transition between the native and low-pH unfolded forms.[4,29,36−39,42−44,46,49,50] In the latter case, the nature of the axial heme iron ligands is still debated.[4,51] It is known that cytc at low pH displays a peroxidase-like activity,[53] which at neutral pH can be obtained by replacing the axial Met80 with a noncoordinating Ala.[54−56] Moreover, upon binding to cardiolipin in vivo, cytc acts as a cardiolipin oxygenase and peroxidase, thus playing a role in the apoptotic events in mitochondria.[12,18−20] The conserved Tyr67 was proposed as a possible apoptotic trigger.[5] Indeed, Tyr67 plays a key role in stabilizing the three-dimensional (3D) structure of cytc at pH 7, as its OH group participates in the hydrogen bond (H-bond) network in the distal side of the heme connecting the Ω loop containing Met80 (from residue 71 to 85) with that formed by residues 40−57,[5,57−63] which is the least stable part of cytc and triggers both acid and alkaline unfolding.[27,31,48,64,65] *Saccharomyces cerevisiae* cytochrome *c* is not involved in apoptosis. However, investigating the low-pH conformational transition in yeast cytc mutated at Met80 and

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Tyr67 can contribute to the understanding of the role that these residues play in stabilizing cytc conformation.

Electronic absorption spectra provide information about the spin state, oxidation state, and coordination number of the iron atom in heme proteins.[66−68] However, elucidation of the molecular processes associated with pH-induced conformational equilibria requires methods that are more sensitive to structural changes of the heme center and its protein environment, such as magnetic circular dichroism (MCD)[68−89] and resonance Raman (RR) spectroscopies.[4,46,80,81,90,91]

In this work, the spectral properties of oxidized wild-type (wt) yeast iso-1 cytochrome *c* (ycc) and its M80A, M80A/Y67A, and M80A/Y67H mutants (Figure 1) were investigated

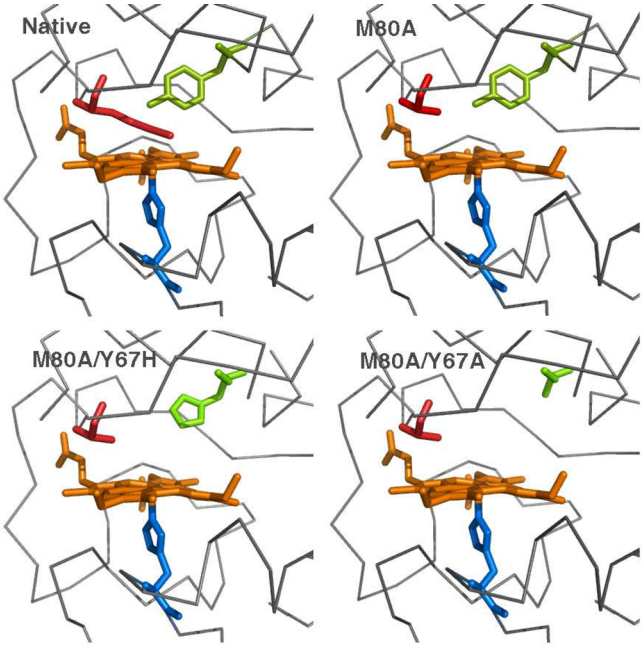

Figure 1. Heme cavity in wild-type, M80A, M80A/Y67H, and M80A/Y67A *S. cerevisiae* cytochrome *c*. Structural data are from Protein Data Bank entries 1YCC (native cytc) and 1FHB (M80A variant).

from pH 7 to 2 at low ionic strength. The analysis of the spectral data offers new insight into the pH-dependent axial heme ligand swapping and conformational equilibria of mitochondrial cytochrome *c* and into the crucial role of Tyr67 in stabilizing the folding of cytc. Furthermore, it contributes to a comprehensive understanding of the electronic and structural properties of the heme group in non-native states of the protein imparted with biologically relevant peroxidase-like activity.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of reagent grade. Doubly distilled water was used throughout this work.

Protein Production and Isolation. Wild-type recombinant *S. cerevisiae* iso-1 cytochrome *c* and its M80A, M80A/Y67H, and M80A/Y67A mutants were expressed and isolated as described previously.[53−56] All protein variants are non-trimethylated and carry the C102T mutation, to prevent protein dimerization.[56]

Spectroscopic Measurements. Electronic absorption and MCD spectra were recorded in the Soret (360−450 nm) and visible (450−710 nm) regions with a Jasco J-810 spectropolarimeter. The magnetic field was provided by a GMW Magnet system model 3470 split coil superconductivity magnet with a maximum field of 1 T (T). The MCD spectra were measured in $[\theta]$ (millidegrees) and converted to $\Delta\varepsilon$ (M^{-1} cm^{-1} T^{-1}) using the conversion factor $\Delta\varepsilon = \theta/(32980 c\ d\ B)$, where c is the protein concentration, B is the magnetic field (1 T), and d is the thickness of the sample (path length, 0.2 cm).[69] All experiments were conducted at 25 °C with protein solutions freshly prepared before use in 0.5 mM phosphate buffer (pH 7), and the protein concentration was checked spectrophotometrically, using an ε_{410} of 106100 M^{-1} cm^{-1} for the wild-type protein and an ε_{405} of 121700 M^{-1} cm^{-1} for the M80A, M80A/Y67A, and M80A/Y67H mutants.[92,93] RR spectra were obtained with 413 nm excitation (Krypton ion laser, Coherent Innova 300c) using a confocal Raman spectrometer (Jobin Yvon, LabRam 800 HR) equipped with a liquid nitrogen-cooled back-illuminated CCD detector. The total acquisition time for each spectrum was between 300 and 1600 s with a wavenumber increment per pixel of ~0.4 cm^{-1}. The laser beam (3−10 mW) was focused onto the sample contained in a rotating cuvette using a Nikon 20× objective (N.A. 0.35) with a working distance of 20 mm.[91] For RR experiments, protein concentrations were ~150 μM. The pH of the samples was changed by adding small amounts of concentrated HCl under fast stirring.

RESULTS AND DISCUSSION

Wild-Type Cytochrome *c*. The UV−vis and MCD spectra of ferric wt ycc are essentially pH-insensitive between pH 7.0 and 4.0 and coincide with those for horse heart cytc at pH 7.0 (Figure S1 of the Supporting Information and Table 1).[76,80,86,93,94] The MCD derivative-shaped signals in the Soret and in the visible regions are typical of a low-spin (LS) six-coordinate (6c) ferric heme,[76,77,80,93] as expected from the His/Met axial heme iron ligation.[10,11] When the pH is reduced to <3.5 (Figure S1 of the Supporting Information and Table 1), the Soret band increases in intensity and shifts to shorter wavelengths (an isosbestic point is observed at 404 nm), while the peak and the trough of the corresponding MCD signal are weakened and shift to shorter wavelengths. At the same time, in the visible part of the spectra, the intensities of the absorption bands at 528 and 555 nm decrease, the peak-to-trough distance of the S-shaped MCD signal decreases, and a charge-transfer band and a new MCD trough are observed at 618 and 632 nm, respectively (Figure S1 of the Supporting Information and Table 1). The features at pH 2.7 indicate that the heme of ycc progressively shifts to a predominantly 6c high-spin (HS) state, in agreement with NMR data.[95] With the decrease in pH to 2.0, the Soret band and the corresponding MCD signal undergo a further blue shift with an isosbestic point at 400 nm, concomitant with a red shift of the charge-transfer band to 617 nm, and a new absorption band at 492 nm replaces those at 528 and 555 nm (Figure S1 of the Supporting Information and Table 1). These spectral changes closely parallel those of horse heart cytc[4,36−39,41−44,46,47,50,96] and are consistent with the presence of three different conformers below pH 4,[4,36−39,41−44,46,47,50,96] involved in two conformational equilibria.[4,36−39,41−44,46,47,50,51] The LS His/Met-ligated form (**LS1**) converts upon Met swapping by a water molecule to a HS-heme containing molten globule conformer (**HS2**),[4,36−38,50,51,97] prevailing from pH 3.4 to 2.6, which represents a globular and compact state with a high content of

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Table 1. Wavelengths of the Relevant Spectral Features of the UV−Vis and MCD Spectra of *S. cerevisiae* Cytochrome *c* and Its M80A, M80A/Y67H, and M80/Y67A Variants as a Function of pH[a]

			MCD							
			Soret			vis			absorption	
variant	species	pH	peak	trough	zerocross	peak	trough	zerocross	Soret	vis
wt	LS1	7.0	401	415	409	548	567	556	408	528, 555
	HS2	2.7	393	414	404	545	545, 567, 632		396	528, 555, 618_{CT}
	HS1	2.0	389	402	396		550, 629		394	492, 530_s, 617_{CT}
M80A	LS1	7.3	399	413	407	554	574, 539	561	405	532, 554
	HS3	5.0	398	413	406	553	536, 570, 630	559	402	526, 557, 621_{CT}
	HS3′	4.0	397	412	406	553	535, 567, 629	558	401	524, 557, 621_{CT}
	HS2	3.3	395	411	404	553	535, 565, 629	558	398	522, 556, 620_{CT}
	HS1	2.0	390	402	397	554	539, 565, 628	547, 558	394	491, 525_s, 556, 617_{CT}
M80A/Y67H	LS1	7.0	399	413	407	548	574	559	405	532, 563
	HS3	5.0	398	414	406	549	565, 572, 628	555	402	524, 563, 617_{CT}
	HS3′	3.9	397	412	406	548	565, 572, 628	554	401	524, 563, 617_{CT}
	HS2	3.0	395	411	405	548	566, 570, 630	555	398	524, 563, 614_{CT}
	HS1	2.0	390	402	398	–	537, 564, 630		394	491, 524_s, 563, 616
M80A/Y67A	LS1	7.0	398	413	406	560, 552	573	566	405	533, 562
	HS3	4.9	398	413	405	550	572, 630	562	402	527, 562, 622_{CT}
	HS2	3.4	395	411	404	550	572, 629	560	398	527, 562, 622_{CT}
	HS1	1.9	391	402	397		538, 564, 627		394	491, 525_s, 560, 617_{CT}

[a]Spectra were recorded in 0.5 mM phosphate buffer at different pH values. Protein concentrations ranged from 17 μM to 0.15 mM. Subscripts s and CT stand for shoulder and charge transfer, respectively.

ordered secondary structure and a fluctuating tertiary conformation.[32,33,49−51] These spectra at low ionic strengths are closely similar to those for *N*-acetyl microperoxidase-8 and -11 at pH 7, which include a His/H_2O axially ligated HS heme.[66,67,98] The presence of this coordination state in the **HS2** conformer is in line with literature data for horse heart cytc that show that the HS form of the molten globule species prevails at low ionic concentrations.[37,38,50,97] Below pH 2.6, a new conformer, **HS1** prevails that still contains a predominantly 6c HS heme, as the Soret band is sensibly narrower and more intense than that for 5c heme[67,68] and the corresponding MCD signal has a typical S shape (Figure S1 of the Supporting Information).[68,76−78,80−86,88,89,99] This conformer would correspond to the totally unfolded form. The absorption spectrum at pH 2 almost coincides with those of *N*-acetyl microperoxidase-8 and -11 under the same conditions, which contain a 6c HS heme with a H_2O/H_2O axial ligation,[66,67,98,100] while some differences exist compared to the state of horse cytc at pH 2 in the presence of 9 M urea, which includes a H_2O and His as axial ligands.[80] Therefore, at this pH, two water molecules most likely bind axially to the heme iron in **HS1**, although minor contributions of a His/H_2O-ligated HS heme cannot be excluded, as indicated by resonance Raman studies.[4,47] Horse heart cytc at pH 1.8 displays different MCD spectra,[76] likely because of the effects of a high concentration of Cl^- (0.6 M) that induce a mixed spin-state population.[76]

The pH-dependent changes in the peak-to-trough distance of the MCD Soret signal (Figure 2a) and in the absorbance of the Soret band for the **LS1** (408 nm) and **HS1** (394 nm) conformers of wt ycc (Figure 2b,c) indicate that the electronic properties of the heme are influenced by a single acid−base equilibrium (Scheme 1). The absorbance and peak-to-trough changes versus pH profiles can be described well on the basis of a one-proton equilibrium yielding an average pK_a value of 3.0 ± 0.2 for the overall **HS1** to **LS1** transition, which compares favorably to previously reported values for native and wt recombinant yeast cytochrome *c*.[65,101] The value is slightly higher than the respective pK_a of horse heart cytc (2.5), confirming the lower conformational stability of ycc compared to its mammalian homologues.[36,38,42,43,45−47,60,102−106] The observation of a single cooperative transition from the native to the unfolded form is due to the low ionic strength at which the measurements were made. Indeed, it is known that the stability of molten globule conformer **HS2** for horse heart cytochrome *c* increases at high ionic strengths. Under these conditions, two conformational equilibria among the three protein conformers are observed.[36,38,42,43,46,47]

M80A Variant. The MCD, absorption, and RR spectra of oxidized M80A ycc (M80A hereafter) from pH 7 to 2 are shown in panels a and b of Figures 3 and 4, and in Figure 5, respectively. The UV−vis and MCD spectral parameters are listed in Table 1. At pH 7, the MCD spectrum displays the intense derivative-shaped Soret-related signal typical of a 6c LS ferric heme,[68,71,76−81,83−89] which is slightly blue-shifted compared to that of wt ycc. The signals in the visible region are generally red-shifted compared to those of wt ycc, and new peaks are observed between 450 and 500 nm. These features are consistent with the replacement of the axial methionine ligand with an OH^- ion (**LS1** conformer), in agreement with literature data.[54,56,93,96,107] In the high-frequency region of the RR spectra, peaks at 1374 (ν_4), 1502 (ν_3) 1586 (ν_2), and 1636 (ν_{10}) cm^{-1} are characteristic of a 6c LS heme center.[4] Whereas this part of the spectrum is not significantly different from that of the His/Met wt ycc protein at pH 7, the low-frequency region between 300 and 450 cm^{-1} confirms the identification of a hydroxide as the sixth ligand in the M80A variant,[93−96,107] because the vibrational band pattern is closely related to that of the His/OH^--ligated state of wt ycc formed at pH >10.5

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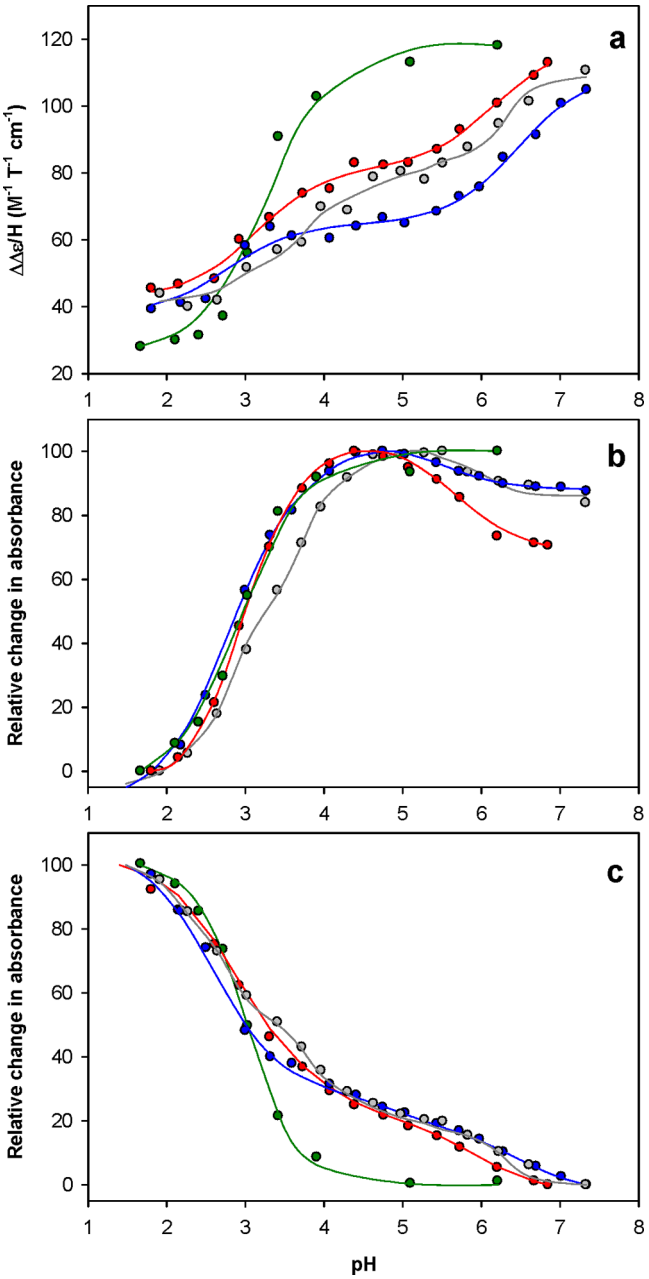

Figure 2. pH-induced changes in (a) the peak-to-trough difference for the MCD signal associated with the Soret band, (b) the relative intensity of the absorbance at 405 nm (408 nm for wt protein), which corresponds to the maximum of the Soret band at neutral pH, and (c) the relative intensity of the absorbance at 394 nm, which corresponds to the maximum of the Soret band at acidic pH for the oxidized form of wild-type (green), M80A (red), M80A/Y67H (blue), and M80/Y67A (gray) *S. cerevisiae* cytochrome *c*. Solid lines are least-squares fits to a one-equilibrium (wt) or a three-equilibria (M80A, M80A/Y67H, and M80A/Y67A) equation.

(Figure 5).[108] Specific spectral markers for the replacement of the Met ligand with a hydroxide are the significant frequency downshift of mode ν_8 by ~5 cm^{-1} to 344 cm^{-1} and the loss of intensity of the prominent band at 398 cm^{-1}.[108]

These findings indicate that in M80A a water molecule has taken over the role of Met80 as an axial ligand of the heme and, furthermore, participates in the H-bond network in the heme pocket. At pH 7, the water molecule acts as a proton donor and thus becomes a sufficiently strong ligand (OH^-) to induce a LS state of the heme iron. Conversely, the hydroxide ligand serves as a proton acceptor below pH 7, contributing to the more complex pH-dependent conformational equilibria of M80A compared to those of wt ycc. When the pH is lowered, at least three additional different conformers can be distinguished.

Lowering the pH to 5 results in a decrease of the peak-to-trough distance and a blue shift of both S-shaped MCD signals (Figures 3a and 4a and Table 1). In addition, an intense trough appears at 630 nm (Figure 4a and Table 1). The Soret absorption band increases in intensity and shifts to shorter wavelengths with an isosbestic point at 408 nm (Figure 3b and Table 1). The α and β bands are replaced by new absorptions, and a new charge-transfer band appears at 621 nm (Figure 4b and Table 1). These variations, due to protonation of the axial OH^- ligand,[54,56,93,96,107] yield spectral features that are indicative of an equilibrium between low-spin and high-spin 6c ferrihemes,[68,78,79,83−87,99] corresponding to the transition from **LS1** to a high-spin 6c conformer (**HS3**). This interpretation is confirmed by the RR spectrum at pH 6.2 that demonstrates the rise of two peaks at 1476 and 1568 cm^{-1}, which can be attributed to the ν_3 and ν_2 modes of a His/H_2O-coordinated HS ferric heme (Figure 5).[4] The equilibrium is further shifted toward the HS configuration when the pH is lowered. Between pH 5 and 4, the Soret band and the S-shaped MCD signals are slightly blue-shifted (Figures 3a,b and 4a and Table 1), while their intensities and peak-to-trough distances remain nearly unchanged. At pH 3.3, the spectroscopic properties are very similar to those of the HS heme-containing molten globule conformer of native ycc (conformer **HS2**).

Consistent with the UV−vis and MCD data, the RR spectra show a relative increase in the intensity of the 6c HS marker bands (ν_3 at ca. 1478 cm^{-1}; ν_2 at 1568 cm^{-1}) at the expense of their 6c LS counterparts at 1502 and 1586 cm^{-1} when the pH is lowered from 6.2 to 3.8 (Figure 5). Note that the intensity ratios of the conjugate bands (i.e., 1478/1502 and 1568/1586 cm^{-1}) are not identical to the 6c HS/6c LS concentration ratio because at 413 nm excitation, which was used in our experiments, the resonance enhancement is stronger for modes of the 6c LS than for those of the 6c HS configuration.[90]

When the pH is further lowered, the spectroscopic properties of M80A dramatically change. At pH 2, the Soret band moves to 394 nm and increases in intensity with an isosbestic at 399 nm, and several changes occur in the visible region (Figures 3b and 4b and Table 1). Moreover, the derivative-shaped MCD Soret signal shifts at shorter wavelengths and becomes less symmetric, and its peak-to-trough distance decreases (Figure 3a and Table 1). These data suggest that at pH 2 the M80A mutant exists as a mixture of two different species, formed by approximately 25% of the **HS2** conformer and 75% of a new **HS1** form stabilized under strongly acidic conditions. The spectroscopic properties of the latter (obtained by subtracting the contribution of the species stable at higher pH from the experimental spectrum) are very similar to those of the unfolded conformer **HS1** of wt ycc under the same conditions, which includes a 6c HS H_2O/H_2O ligand set. Accordingly, the RR spectrum at this pH (Figure 5) shows broad and poorly structured features in the ν_3 and ν_2 mode region with maxima at 1486 and 1573 cm^{-1}, respectively, suggesting the involvement of two different 6c HS configurations. In addition, there may be a small contribution of a 5c HS configuration. The pronounced broadening of modes ν_3 and ν_2 is consistent with a considerable conformational flexibility of the protein matrix surrounding the heme that facilitates ligand exchange.

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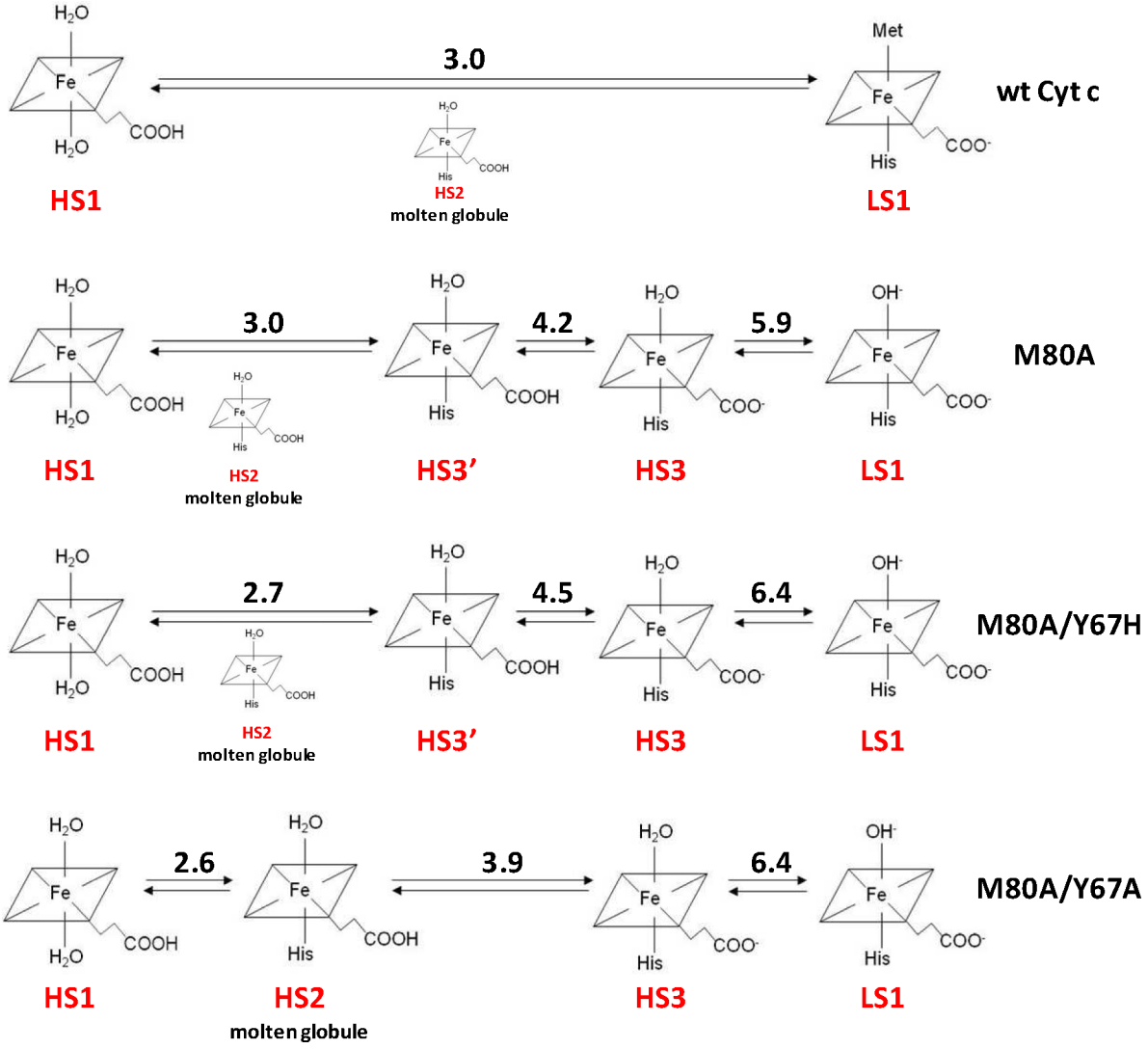
Scheme 1. pH-Dependent Axial Ligation and Spin State of the Ferric Heme Center for *S. cerevisiae* Iso-1-cytochrome *c* and Its M80A, M80A/Y67H, and M80A/Y67A Variants[a]



[a]The average pK_a values, determined from MCD and electronic absorption spectra, are indicated and correspond to each acid−base equilibrium (see the text). Errors in pK_a values are ±0.2.

The pH-induced changes in the peak-to-trough distance of the MCD Soret signal (Figure 2a) and in the relative absorbance of the Soret absorption band at 405 nm (Figure 2b) and 394 nm (Figure 2c) (vide infra) were fit (Scheme 1) by a three-proton equilibrium equation. In order of decreasing pH values, the first equilibrium corresponds to the **HS3** ⇄ **LS1** transition, which occurs with a pK_a of 5.9 ± 0.2, in close agreement with previously reported values.[54,93,96,107] The second equilibrium (pK_a = 4.2 ± 0.2) is responsible for the small spectroscopic changes observed between pH 5 and 4. The third is related to the overall **HS1** ⇄ **HS3** transition, which appears as a single cooperative transition, as for the native protein, with a pK_a of 3.0 ± 0.2. The good agreement of the apparent pK_a values for the **HS1** ⇄ **HS3** transition of M80A and for the **HS1** ⇄ **LS1** transition for wt ycc demonstrates that the removal of the axial methionine ligand does not significantly alter the mechanism of acidic unfolding of cytochrome *c* and the intervals of stability of the acidic **HS2** and **HS1** conformers. Therefore, rupture of the Met80−Fe(III) bond does not trigger the acid unfolding of cytochrome *c*, although breaking of the same bond is involved in the formation of the molten globule conformer **HS2**.[4,36−39,41−44,47,50,51,97] This finding supports the proposal that the acid unfolding of the Ω loop containing the axial Met (i.e., from residue 71 to 85) is triggered by the unfolding of the Ω loop formed by residues 40−57, which in turn is most probably induced by the pH-induced breaking of the H-bond connecting the imidazole ring of His26 with the carboxylate side chain of Glu44.[27,48,49] Because the structural effect of the M80A mutation is limited to residues in the heme distal cavity,[109] it is conceivable that the interactions described above are not seriously affected by the replacement of the native Met with an Ala.

The acid−base equilibrium influencing the spectroscopic properties of the M80A mutant in the pH range of 3.5−5 is not observed for the wt protein and does not influence the spin state or axial ligation of the heme iron. It is well-known that in mitochondrial cytochromes *c*, the evolutionarily conserved His26 and the heme propionate-7 are characterized by low pK_a values.[10,110] Spectroscopic studies indicate that in oxidized mitochondrial cytc, the pK_a of His26 is <3.6 as a consequence

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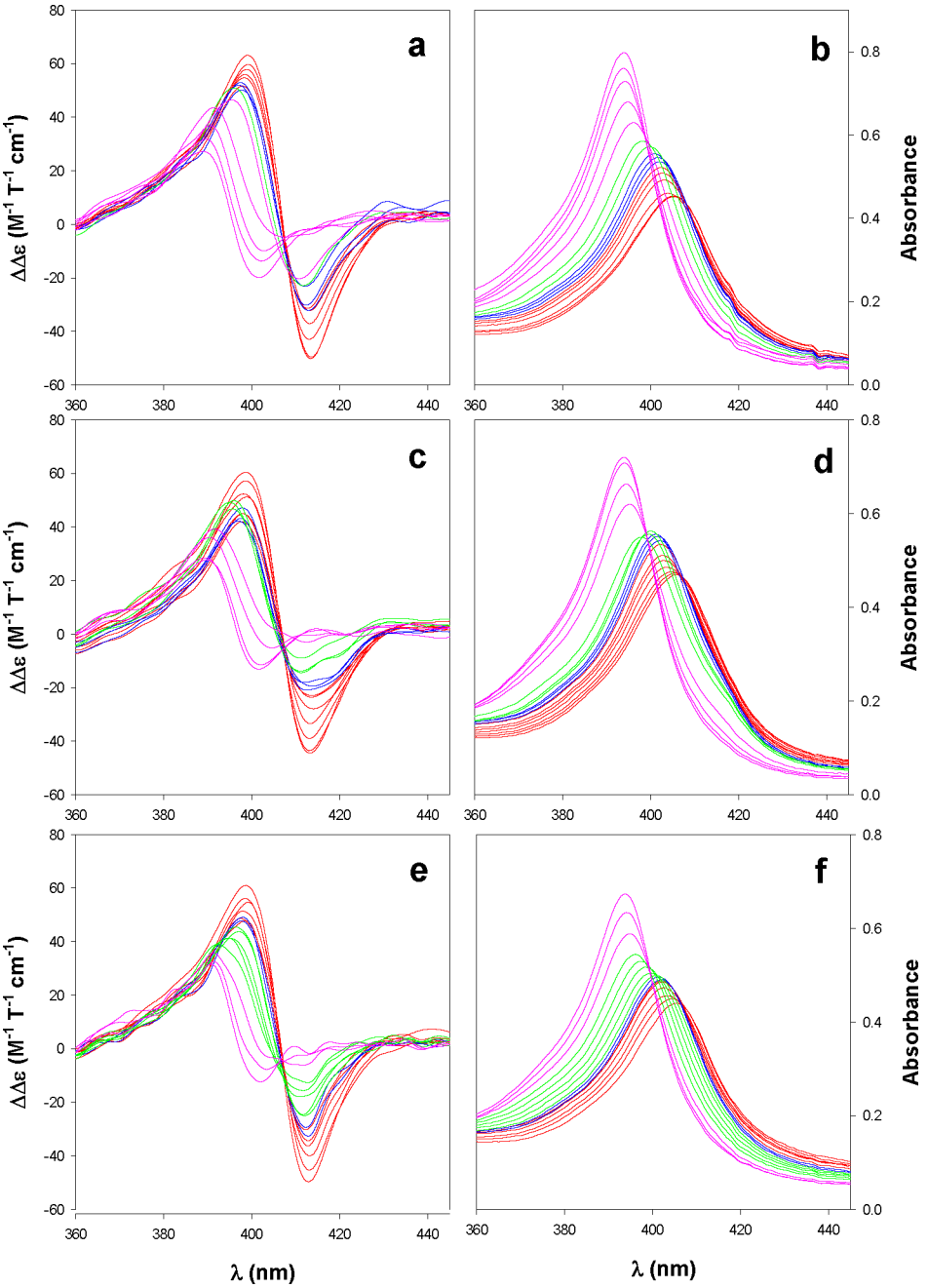

Figure 3. MCD and electronic absorption spectra in the Soret region of the oxidized form of the M80A (a and b), M80A/Y67H (c and d), and M80A/Y67A (e and f) mutants of *S. cerevisiae* cytochrome *c* in 0.5 mM phosphate buffer at different pH values: (a and b) red for 5.0 < pH < 7.0, blue for 4.0 < pH < 5.0, green for 3.00 < pH < 3.90, and pink for pH < 3.0, (c and d) red for 5.0 < pH < 7.3, blue for 4.0 < pH < 5.0, green for 3.0 < pH < 3.9, and pink for pH < 3.0, and (e and f) red for 5.2 < pH < 7.0, blue for 4.5 < pH < 5.2, green for 3.0 < pH < 4.5, and pink for pH < 3.0. Protein concentrations were 20, 20, and 17 μM, respectively.

of its nonpolar environment.[10,110] Because this environment is conserved in the M80A variant,[109] the pK_a value of His26 should be unaffected by this mutation. On the other hand, the pK_a of the heme propionate-7 in oxidized yeast (and mammalian) cytc is <4.5, in agreement with the pK_a values of 4.2 ± 0.2 obtained in this study.[10,110] We therefore propose to assign the intermediate pH-induced transition to the **HS3′** ⇄ **HS3** acid−base equilibrium of heme propionate-7 (Scheme 1). The acid−base equilibria involving the heme propionates were not detected by optical spectroscopy in cytochromes *c*, but only in *N*-acetyl microperoxidase-8 and -11.[66,67,98] The different spectroscopic properties of **HS3** and **HS2**, which share the same His/H_2O axial heme ligation, are most probably the result of the different environments surrounding the heme, which can influence the electron donating properties of weak heme axial ligands through H-bonding and electrostatic interactions.[85] In molten globule conformer **HS2**, where the heme is surrounded by a folded protein subdomain containing the C- and N-terminal and 60s helices,[51] the electron donating properties of the axial water are weakened compared to those of the **HS3** species, suggesting altered H-bonding and electrostatic interactions with the residues in the heme cavity compared to the native form.

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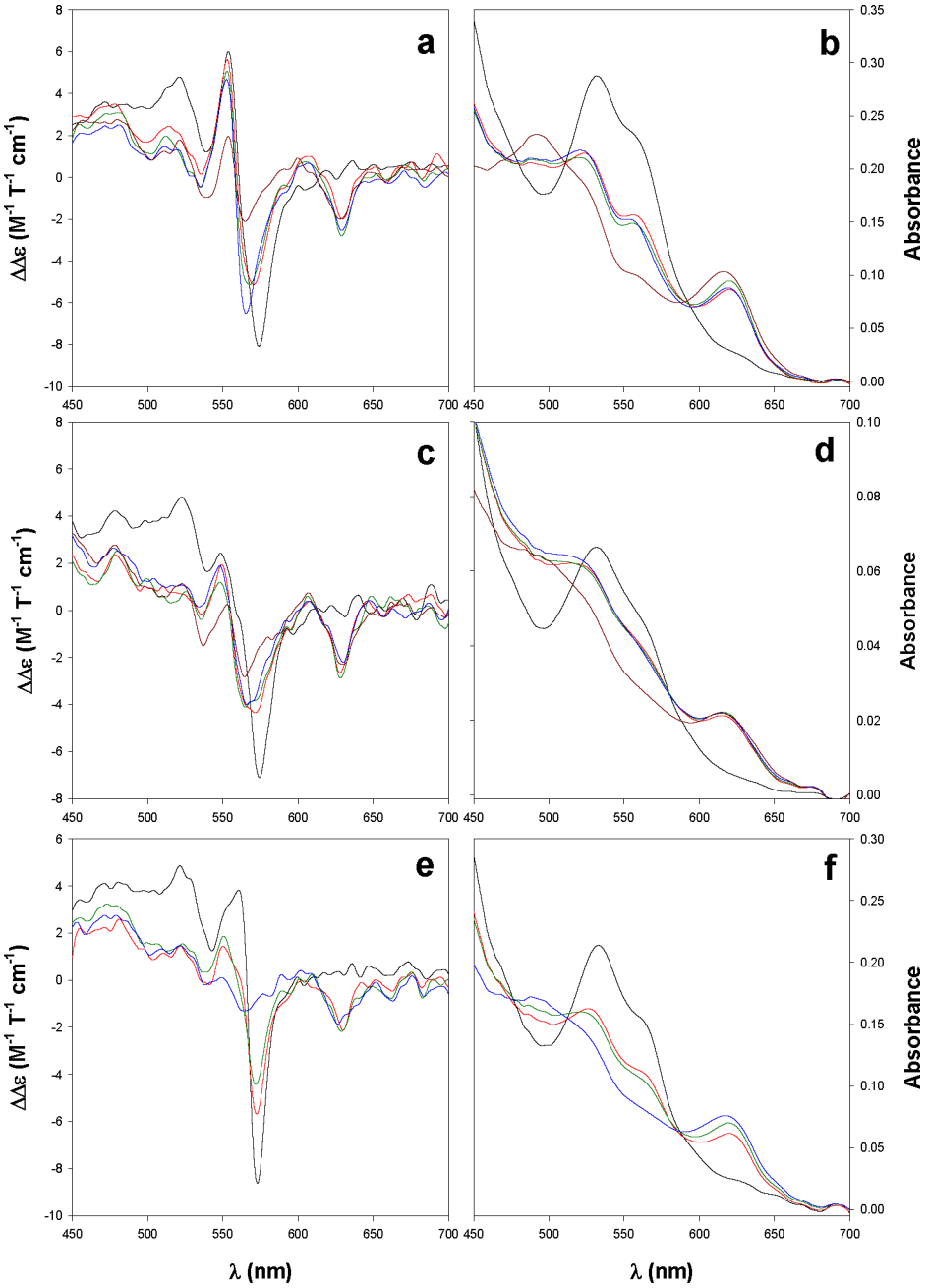

Figure 4. MCD and electronic absorption spectra in the visible region of the oxidized form of the M80A (a and b), M80A/Y67H (c and d), and M80A/Y67A (e and f) mutants of *S. cerevisiae* cytochrome *c* in 0.5 mM phosphate buffer at different pH values: (a and b) black for pH 7.0, red for pH 4.7, green for pH 3.90, blue for pH 3.0, and dark red for pH 2.0, (c and d) black for pH 7.0, red for pH 5.1, green for pH 3.80, blue for pH 2.60, and dark red for pH 2.0, and (e and f) black for pH 7.0, red for pH 5.0, green for pH 3.90, blue for pH 3.0, and dark red for pH 2.0. Protein concentrations were 0.15 mM, 37 μM, and 0.11 mM, respectively.

M80A/Y67H and M80A/Y67A Variants. The electronic absorption and RR spectra for these double variants are very similar to those for M80A over the whole pH range investigated (Figures 3c–f, 4c–f, and 6 and Table 1), indicating the same pH-dependent coordination changes of the heme center (Scheme 1). It is worth noting that the spectroscopic features of the neutral-pH conformers of both double mutants in the visible region are similar but not identical to those of the corresponding form of M80A, implying that the mutation of Tyr67 affects to some extent the electronic properties of the heme. This is not unexpected as replacement of the side chain of Tyr67 with the imidazole ring of a histidine and the methyl group of an alanine most likely results in a significant rearrangement of the H-bonding network in the distal heme cavity,[5] which includes the OH^-/H_2O heme axial ligand, resulting in an increase in the pK_a values for the **LS1** ⇄ **HS3** transition (Scheme 1).

The pH dependencies of the peak-to-trough distance of the MCD Soret signal and the relative absorbance of the Soret band for the **LS1** (408 nm) and **HS1** conformers (394 nm) for both double variants are reported in Figure 2. The behavior of the M80A/Y67H variant is closely similar to that of the M80A mutant, demonstrating that the electronic properties of the heme are influenced by the same transitions with similar pK_a

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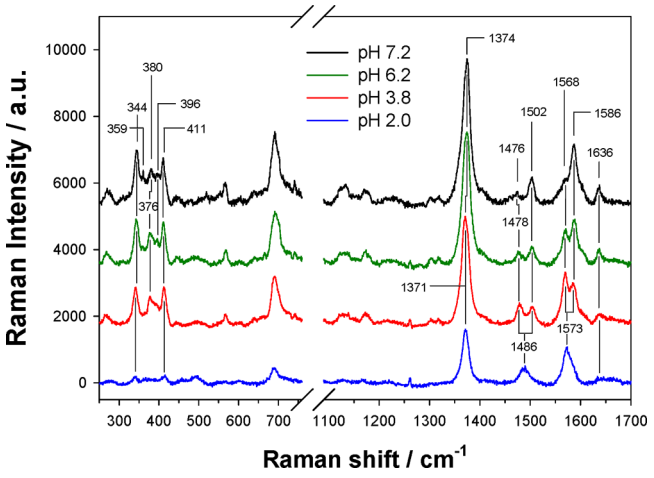

Figure 5. RR spectra of the oxidized form of M80A *S. cerevisiae* cytochrome *c* in a 30 mM phosphate buffer solution at different pH values recorded with a 413 nm excitation line. The spectra were corrected by subtracting a linear baseline. The protein concentration was 160 μM.

values (Scheme 1). On the other hand, the pH dependencies of M80A/Y67A show significant differences below pH 4.5. In particular, the pH-induced changes in the Soret part of the electronic absorption and MCD spectra observed between pH 4.5 and 3.5 (Table 1, and green spectra in Figure 3e,f) show that **HS2** (molten globule) appears at higher pH values than in the other variants, leading to two detectable acid−base equilibria between pH 4.5 and 2 instead of a single cooperative transition (Figure 2a−c and Scheme 1), as observed for the wt protein at high ionic concentrations or in the presence of chaotropic anions.[4,36,38−40,42,43,45−47,50,97] In addition, as a result of the increased pK_a for the **HS2** ⇄ **HS3** transition, the effects of protonation of heme propionate-7 are not observed.

The slightly lower pK_a value for the overall **HS1** ⇄ **HS3′** transition in M80A/Y67H compared to those of wt (**HS1** ⇄ **LS1**) and M80A indicates that replacement of the native tyrosine with a histidine disfavors only slightly the acid unfolding of cytc. This is in agreement with literature data showing that disruption of the H-bonding network connecting the heme propionate-7 with the side chain of Tyr67 enhances the free energy of unfolding and decreases the flexibility of both redox forms of yeast cytc.[5,57,59,61,62] On the other hand, the increased pK_a for the **HS2** ⇄ **HS3** transition in M80A/Y67A shows that the molten globule conformer **HS2** is stabilized upon replacement of Tyr67 with Ala. These findings underline the key role played by Tyr67 in stabilizing the 3D structure of cytc by connecting through an H-bonding network the Ω loop running from residue 71 to 85 (containing the axial methionine) with that formed by residues 40−57,[5,57,59,61,62] which triggers both acid and alkaline unfolding of cytc.[27,48,64,65] Indeed, the Y67A mutation, perturbing the H-bonding network in the heme pocket, facilitates the formation of molten globule conformer **HS2** that precedes the acidic unfolding of cytc.

CONCLUSIONS

The M80A, M80A/Y67H, and M80A/Y67A variants of yeast iso-1-cytochrome *c* undergo more complex low-pH conforma-

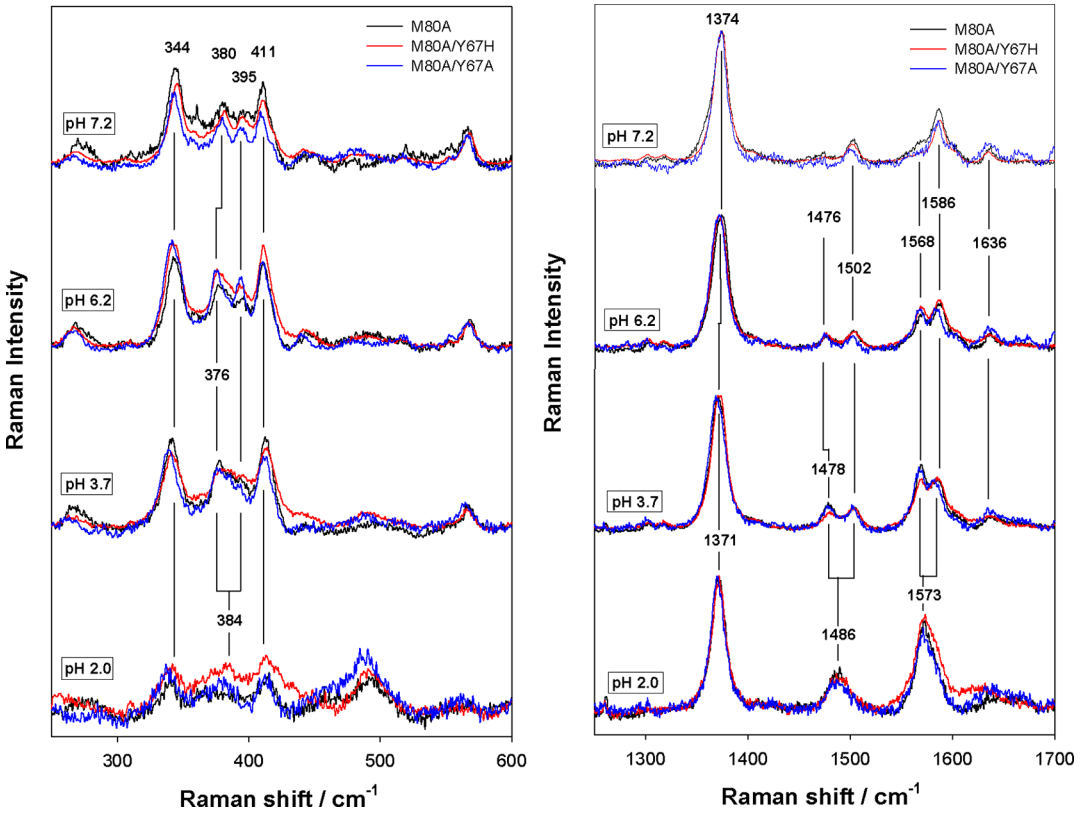

Figure 6. RR spectra of the oxidized forms of the M80A, M80A/Y67H, and M80A/Y67A mutants of *S. cerevisiae* cytochrome *c* between pH 7.2 and 2.0 in the low-frequency (left) and high-frequency (right) regions. Measurements were performed in 30 mM phosphate buffer. The spectra were corrected by subtracting a linear baseline. The protein concentration was 160 μM.

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tional transitions at low ionic strengths than the wt protein. For all mutants, at least four different conformers are observed from pH 7 to 2. In particular, the 6c LS His/OH^- form observed at neutral pH transforms into a 6c HS His/H_2O-ligated species when the pH is lowered, because of protonation of the axial hydroxide ligand. Two HS conformers whose spectroscopic (and coordination) properties are closely related to the molten globule and to the unfolded state of the native protein are formed below pH 4.5 and 2.5, respectively. The data for the M80A variant show that removal of the axial Met ligand does not significantly alter the mechanism of acidic unfolding of cytc and the pH ranges of stability of the low-pH HS conformers, thereby supporting the view that the acid unfolding of cytc is not triggered by molecular events in the Ω loop containing the axial Met80 ligand. Replacement of Tyr67 with Ala in the M80A/Y67A mutant induces a remarkable change in the low-pH behavior of cytc resulting in the formation of the molten globule HS His/H_2O-ligated conformer at pH values higher than those for the other species. Conversely, no such effect is observed upon substitution of Tyr67 with a His in the M80A/Y67H variant. Thus, Tyr67 stabilizes the 3D structure of cytc by connecting the Ω loops 71−85 and 40−57 through H-bonding. The latter loop triggers both acid and alkaline unfolding of cytc. Therefore, breaking this H-bonding network is the initial key event in the low-pH unfolding of cytc, leading to the formation of the molten globule conformer.

■ ASSOCIATED CONTENT

Supporting Information

MCD and electronic absorption spectra for oxidized recombinant wt *S. cerevisiae* cytc in 0.5 mM phosphate buffer at different pH values. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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