

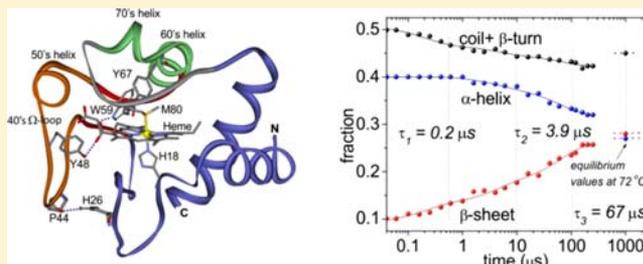
His26 Protonation in Cytochrome c Triggers Microsecond β -Sheet Formation and Heme Exposure: Implications for Apoptosis

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S Supporting Information

ABSTRACT: Cytochrome c unfolds locally and reversibly upon heating at pH 3. UV resonance Raman (UVRR) spectra reveal that instead of producing unordered structure, unfolding converts turns and some helical elements to β -sheet. It also disrupts the Met80–heme bond, and has been previously shown to induce peroxidase activity. Aromatic residues that are H-bonded to a heme propionate (Trp59 and Tyr48) alter their orientation, indicating heme displacement. T-jump/UVRR measurements give time constants of 0.2, 3.9, and 67 μ s for successive phases of β -sheet formation and concomitant reorientation of Trp59. UVRR spectra reveal protonation of histidines, and specifically of His26, whose H-bond to Pro44 anchors the 40s Ω loop; this loop is known to be the least stable ‘foldon’ in the protein. His26 protonation is proposed to disrupt its H-bond with Pro44, triggering the extension of a short β -sheet segment at the ‘neck’ of the 40s Ω loop into the loop itself and back into the 60s and 70s helices. The secondary structure change displaces the heme via H-bonds from residues in the growing β -sheet, thereby exposing it to exogenous ligands, and inducing peroxidase activity. This unfolding mechanism may play a role in cardiolipin peroxidation by cyt c during apoptosis.



1. INTRODUCTION

Cytochrome c (cyt c), a much-studied electron carrier in the mitochondrial respiratory chain, has emerged as a renewed focus of biological interest because of its role as a mediator of apoptosis. Apoptotic cell death is triggered by migration of cyt c from the mitochondria into the cytoplasm, where it induces a cascade of biochemical events.^{1–7} Cyt c release into the cytoplasm results from its association with cardiolipin, a component of the mitochondrial membrane, accompanied by membrane pore formation.⁸ Cardiolipin forms a specific association with cyt c by inserting its hydrocarbon chains into hydrophobic channels in the protein, resulting in a conformation change that induces peroxidase activity.^{9–13} Peroxidation of cardiolipin induces the release of apoptotic factors.^{14,15}

We have shown that peroxidase activity is also induced by heating horse heart cyt c at pH 3, in concert with a protein conformation change involving β -sheet formation, and with conversion of the heme to a high-spin state.¹⁶ This conformation change was proposed to be initiated by protonation of His26,^{17,18} which H-bonds to Pro44, a residue at the tip of a long omega loop (40s Ω loop) (Figure 1). This loop was characterized by Englander and co-workers^{19,20} as the least stable cooperative unfolding unit (‘foldon’) in cyt c. It is anchored at its other end by a short β -sheet ‘neck’, which we suggested could extend into the loop itself, when the His26–Pro44 H-bond is broken. Since the heme propionate substituents accept H-bonds from residues in the loop, this conformation change would be expected to perturb the heme,

inducing dissociation of the Met80 ligand and allowing access of peroxide to the vacated coordination site.

In the present work, we characterize this conformation change more fully, using several UV resonance Raman spectroscopic probes. The spectra reveal that heating cyt c at pH 3 does indeed induce protonation of His26. The accompanying shift to β -sheet structure also induces changes in signals from tryptophan and tyrosine residues, which are consistent with the 40s loop being the locus of protein conformation change, along with the adjacent 60s and 70s helices (Figure 1). These changes are reversible, and T-jump/UV resonance Raman (UVRR) experiments reveal that they occur on the microsecond time scale.

Since antibody to the Pro44 region was earlier shown to bind to apoptotic cells, as well as to cyt c when bound to lipid vesicles,²¹ the proposed unfolding mechanism may be involved in cardiolipin peroxidation and apoptosis.

2. METHODS

2.1. Materials. Horse heart ferricytochrome c (*hh* cyt c) was purchased from Sigma Aldrich and pretreated as described below. Deuterated water (D_2O), NaOD and DCl of greater than 99% purity were purchased from Cambridge Isotope Laboratories, Inc. and used without further purification. All other chemicals are of analytical grade and used as received. The protein was dissolved in 50 mM sodium phosphate buffer in H_2O or D_2O at appropriate pH or pD. Stock solutions of 1 mM protein were pretreated with 10 mM potassium

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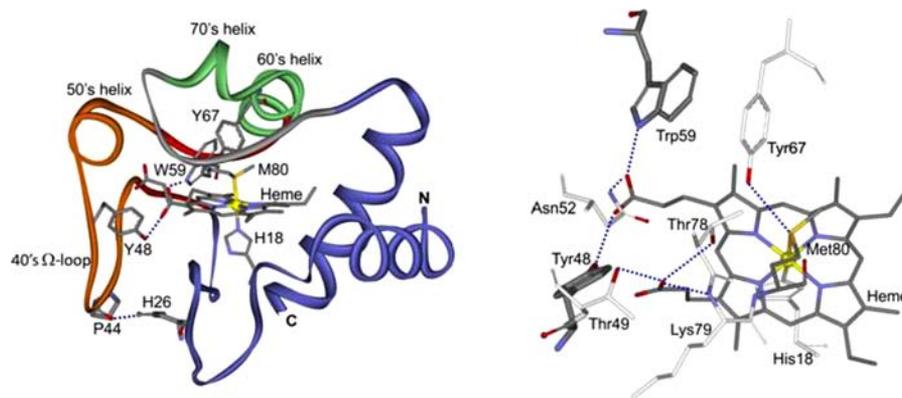


Figure 1. Left panel: Ribbon diagram of *hh* cyt *c* (pdb id: 1hrc). The 40s Ω loop is orange, its β -sheet 'neck' is red, the 60s and 70s helices are green and the Met80 loop is gray. H-bonds are shown in dashed blue lines for P44/H26, and for W59 and Y48 with a heme propionate. Right panel: The full H-bond pattern of the heme propionates with residues in the 40s and Met80 loops, and the Tyr67/Met80 H-bond.

ferricyanide to oxidize the residual reduced forms and desalted through PD10 column. Samples included 50 mM sodium perchlorate as a Raman intensity standard. The protein concentration was 50–100 μM for static RR measurements, 200 μM for T-jump experiments, 50 μM for measurements of the 695 nm absorption band, and 20 μM for CD measurements.

2.2. Visible Absorption Spectroscopy. UV–vis absorption spectra were recorded on an Agilent 8453 UV–vis Spectrophotometer with a 1 nm slit width. Temperature-dependent measurements were recorded on a thermostatted Cary 300 Bio UV–visible spectrophotometer; samples were equilibrated for 10 min. Ten millimeters path length cells were used for absorption measurements. The baseline of the absorption spectra was modeled and subtracted using a spline function.

2.3. Circular Dichroism. Variable temperature far-UV circular dichroism (UVCD) wavelength scans were collected on an AVIV 62DS spectropolarimeter, equipped with a temperature-controlled cuvette holder, using a 1 mm quartz cell. The data were recorded from 260 to 190 nm with 1 nm spacing, and every 5 $^{\circ}\text{C}$ from 5 to 95 $^{\circ}\text{C}$. The 222 nm temperature scans were carried out from 20 to 100 $^{\circ}\text{C}$ at a rate of 2 $^{\circ}\text{C}/0.5$ min. A bandwidth of 1.5 nm and average time of 5 s were used. The data were converted to mean residue ellipticity $[\theta]$ in $\text{deg} \times \text{cm}^2/\text{dmol}$ using the measured protein concentration.

2.4. Equilibrium UVRR Measurements. The experimental setup and the spectral acquisition scheme have previously been described.²² UV probe pulses (20 ns, 1 kHz) at 197 nm²³ (~ 1 $\mu\text{J}/\text{pulse}$) or 229 nm^{22,24} (~ 1 $\mu\text{J}/\text{pulse}$) were obtained by frequency quadrupling the output of a Ti:sapphire laser, which was pumped (527 nm, ~ 10 mJ/pulse, 70 ns, 1 kHz) by an intracavity frequency-doubled Nd:YLF laser (GM30, Photonics International, Inc.). Raman scattered light was collected at 135 $^{\circ}$ geometry and focused into a 1.26 m spectrograph (Spex 1269), which was equipped with a holographic grating (3600 groove/mm) and an intensified photodiode array detector (IPAD) (Roper Scientific). The sample solution was pumped through a wire-guided free-flowing cell to minimize the background. Temperature control of the sample was achieved with a water bath (RTE-100, Neslab), which regulates the temperature of the sample cell and reservoir. UVRR spectra were collected with 15 min accumulation time. The measured spectra were calibrated using the Raman spectrum of cyclohexane. The water background was subtracted from protein Raman spectra using the pure water Raman spectrum.

2.5. Time-Resolved T-jump UVRR Measurements. UV probe pulses for time-resolved UVRR measurements were generated by the UV laser described above. T-jump pulses were obtained from an intracavity diode laser pumped Nd:YLF laser optical parametric oscillator (OPO), which can generate tunable near-infrared (NIR) 1.6–2.0 μm , 0.8–1.0 mJ/pulse, 20 ns, 1 kHz pulses (Photonics International, Inc.). The final temperature of the sample after a T-jump was determined to ± 2 $^{\circ}\text{C}$ from the ~ 3400 cm^{-1} water Raman band intensity change.²⁵ The wavelength of the T-jump pump pulse is

within the near-IR water band centered at 1.9 μm . The final temperature of the sample within the laser interaction volume is achieved instantaneously after a T-jump pulse (complete thermalization is reached within 10–20 ps²⁶). The probe and pump pulse sequence was alternated to minimize artifacts from spectrometer drift and photoproduct accumulation, which is critical for this study, since no internal standard was added. The timing between T-jump and probe pulses was adjusted with a computer-controlled pulse generator (DG 535, Stanford Research Systems). The final pump–probe (with T-jump) and probe-only (without T-jump) spectra were averages over 15 min of accumulation.

All UVRR spectra were processed and analyzed using Grams/AI 7.00 (Galactic Industries Corp.) and plotted using Origin 6.0 software.

3. RESULTS

3.1. Avoidance of Aggregation. Under conditions of thermal denaturation, cyt *c* is known to be susceptible to aggregation, through intermolecular β -sheet association.^{27–31} We therefore determined the concentration range in which aggregation of horse heart cyt *c* at pH 3 could be avoided under our experimental conditions. Turbidity was monitored at 80 $^{\circ}\text{C}$ near 450–480 nm, in a trough of the cyt *c* absorption spectrum, where the optical density reflects diffuse scattering. At pH 3 and 80 $^{\circ}\text{C}$, the solution became turbid when the concentration was higher than 150 μM . The apparent time constant for turbidity growth ranged from 2 to 4 h for concentrations between 600 and 160 μM . Turbidity was not observed below 100 μM . Subsequent experiments were carried out at a concentration of 50 μM , except for the T-jump measurements. For these experiments, the reservoir temperature was held at 40 $^{\circ}\text{C}$, and only heated transiently. Consequently, a higher concentration, 200 μM , could be used without aggregation, in order to generate acceptable signal/noise in the pump–probe difference spectra.

3.2. Thermally Induced β -Sheet Formation. Thermally induced conformation change was initially monitored via UV circular dichroism (Figure 2). Cyt *c* spectra were recorded at a series of temperatures at pH 7.0 and 3.0. Progressive loss of helicity can be seen in the loss of the 222 nm trough. At pH 3.0, spectra at intermediate temperatures resemble those characteristic of β -sheet.³²

The ellipticity at 222 nm gave well-behaved melting curves (Figure 2), with midpoint temperatures of 87 and 64 $^{\circ}\text{C}$ at pH 7 and 3. Importantly, the temperature dependence was reversible, the same ellipticity being obtained on heating and cooling (arrows).

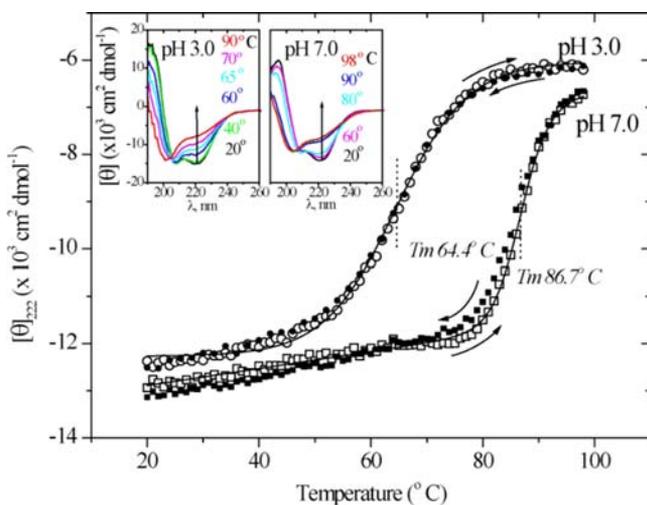


Figure 2. UVCD spectra (insets) for *hh* cyt c at pH 3.0 and 7.0, and the temperature profiles of the ellipticity at 222 nm. Reversibility can be seen from the agreement of $[\Theta]_{222}$ during heating (open symbols) and cooling (closed symbols).

While the UVCD spectra can be analyzed for secondary structure composition,^{32–38} the considerable overlap of component spectra limits the reliability. We turned instead to UVRR spectra excited at 197 nm (Figure S1). Our procedure for secondary structure analysis³⁹ is to subtract contributions from phenylalanine and tyrosine side chains (Figure S2) and then to resolve the backbone scattering regions into contributions from α -helix, β -sheet, β -turn, and coil (un-ordered) contributions, using component spectra from a training set of proteins, via least-squares fitting. The greatest discrimination is found in the 1200–1500 cm^{-1} region, where complex bands arising from the amide III and amide S vibrations^{39–43} give well-structured component spectra, as illustrated in Figure 3. The spectral fitting gave an average goodness-of-fit parameter, $R^2 = 0.962$, and relative uncertainties (1σ) in the population estimates of 2% for helix and coil, 9% for β -sheet and 11% for β -turn (the latter two being somewhat correlated).

Figure 4 shows the results of this procedure. At 20 °C, the spectra are the same at pH 7 and 3, and indicate the presence of 3% β -sheet, 16% β -turn, 41% α -helix, and 40% unordered structure. This composition is consistent with the crystal structure of native cyt c.⁴⁴ At pH 7, unordered structure increases at high temperature, at the expense of helix melting, consistent with the 87 °C T_m determined from UVCD at pH 7. (The temperature range was somewhat lower for the UVRR measurements, precluding an accurate T_m determination.)

A very different pattern emerged at pH 3 (Figure 4). Helix melting was still observed, and its T_m , 60 °C, was consistent with the UVCD determination (Figure 2). However, β -sheet, rather than unordered structure, increased with temperature, and the T_m for this increase was distinctly lower, 53 °C. The β -turn population decreased perceptibly, with an even lower T_m , 45 °C.

3.3. Associated Changes in Aromatic Residues. The 229 nm excitation selectively enhances UVRR bands of tyrosine and tryptophan (Figure S3). These signals lose intensity as the temperature increases (Figure 5). This effect is associated with exposure to solvent of buried side chains.^{45–47}

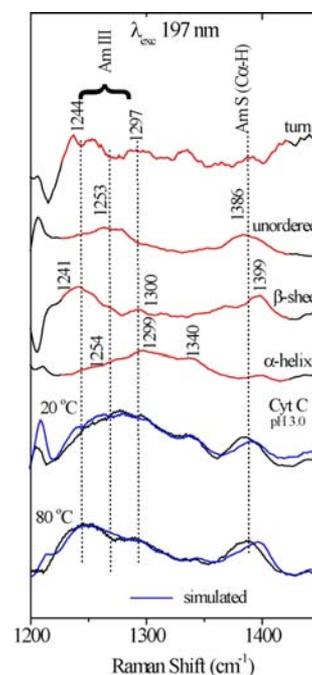


Figure 3. Illustration of secondary structure component analysis of the *hh* cyt c 197 nm-excited UVRR spectra at pH 3.0 in the amide III and S region (red). Experimental and simulated spectra are compared at 20 and 80 °C.

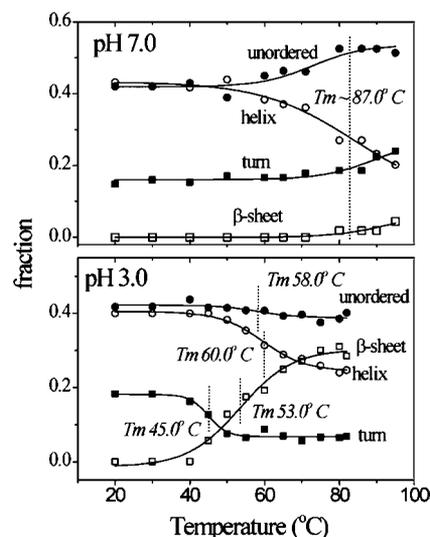


Figure 4. Temperature profiles of UVRR-determined secondary structure content in *hh* cyt c at pH 7.0 and 3.0.

In addition, the Trp W3 band shifts down in frequency and also become broader. The W3 frequency reflects the dihedral angle of the indole side chain,^{48–50} and the observed broadening indicates a wider distribution of this angle. The sole Trp residue is Trp 59, and the W3 broadening indicates an increased distribution of its side chain angles. The Trp59 indole is H-bonded to one of the heme propionate substituents (Figure 1), and its increased mobility implies weakening of the H-bond. At pH 3, the T_m for this process, 50 °C (Figure 5), matches the T_m for β -sheet formation, as does that for the W3 intensity loss.

Essentially the same T_m , 53 °C, describes an upshift of the Tyr 9a band (Figure 5) at pH 3, but in this case, the band

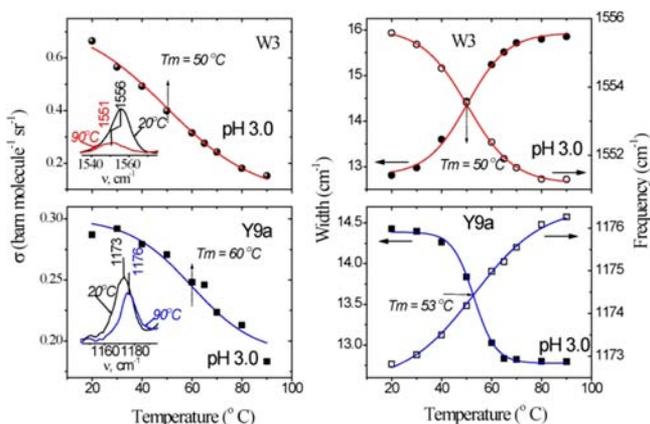


Figure 5. Temperature profiles of the Trp W3 and Tyr 9a band intensities (left), frequencies (open symbols) and bandwidths (filled symbols) in *hh cyt c* at pH 3.0. Inset to the intensity profiles shows the corresponding experimental band measured at the indicated lowest and highest temperatures. Sigmoid fits to the experimental data are shown as solid lines.

narrows. The Y9a band has been found to reflect the dihedral angle of the tyrosine OH substituent with respect to the phenyl ring of tyrosine, more in-plane orientations giving higher frequencies.⁵¹ The upshift and narrowing indicated that one or more of the Tyr OH groups shift from a largely out-of-plane to a more in-plane orientation, and thereby narrow the range of orientations among the four residues. A higher T_m , 60 °C, is found for the Y9a intensity loss, indicating that one or more of the Tyr residues retain a hydrophobic environment beyond the temperature where others change their OH orientation.

There are four Tyr residues in *cyt c*, three of which donate OH H-bonds. Jordan et al. calculated the OH dihedral angles from crystallographic coordinates by assuming standard C–O–H distances and angle, and minimizing the H-bond distances.⁵² The computed dihedral angles are 37°, 22°, and 3° for Tyr48, 67, and 74. The fourth residue, Tyr97, is at the protein surface, and likely has a large dihedral angle (steric hindrance is minimized at 90°), which, however, is unlikely to change much with temperature. Since Tyr48 has the largest angle of the H-bonded residues, it can most readily account for the observed Y9a upshift and narrowing, if its H-bond partner shifts position in a way that decreases the OH dihedral angle, bringing it to a more in-plane orientation. Also supporting the Tyr48 assignment for the Y9a shift is the coincidence of its T_m with that of the Trp59 W3 band, since both residues are H-bonded to the same heme propionate (Figure 1, see Discussion).

From the behavior of the Tyr48 and Trp59 UVR signals, it can be inferred that the heme is displaced during the temperature-induced protein conformation change, shifting the propionate and decreasing the Tyr48 OH angle, while freeing up the Trp59 indole. The Tyr67 OH orientation may also be perturbed, since it is H-bonded to the S atom of the Met80 heme ligand, which is broken upon heating at pH 3 (see below). However, Tyr67 is unlikely to contribute much to the Y9a upshift, since its starting OH orientation is more in-plane than that of Tyr48. Tyr74, which is H-bonded to an ordered water molecule near the protein surface cannot contribute since its starting OH orientation is in-plane.

Similar changes in the aromatic residue UVR signals are seen at pH 7 only near the global melting temperature (Figure 6).

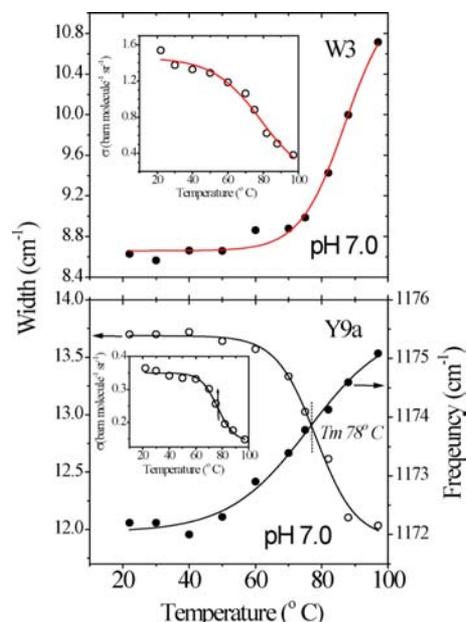


Figure 6. Same as Figure 5 but for *hh cyt c* at pH 7.0.

3.4. Met80–Fe Bond Breaking. Heme ligation was monitored via the absorption band at 695 nm associated with the Fe–methionine bond^{53,54} (Figure 7). This band diminishes as the pH is decreased, reflecting the well-known acid-induced dissociation of the Met80 ligand.⁵⁵ The 695 nm band is replaced by one at 627 nm, assigned to a charge transfer transition of the high-spin heme that results from displacement of Met80 by a water molecule.⁵⁶ At 20 °C, both absorbances follow pH titration curves with a pK_a of 3.1. The 695 nm band is also replaced by the 627 nm band when the temperature is increased, describing a series of thermal transitions that depend on pH. At pH 7, T_m for the 695 nm band is 73 °C, slightly below the global melting temperature, while at pH 3, $T_m = 48$ °C, near the T_m for the first phase of β -sheet formation. Consistent with the pH titration curve, the fraction of methionine-bound heme is 40% at 20 °C and pH 3.

3.5. Histidine Protonation. To explore the coupling of pH with protein structure change, we monitored the protonation status of histidine residues, using the intensity of the 220 nm-excited UVR band^{57,58} of HisD_2^+ at 1408 cm^{-1} . The histidine UVR spectrum is complex and weak, but it is simplified upon protonation, and further simplified upon H/D exchange, which eliminates mixing of the N–H bending coordinate into the imidazole ring modes.^{59,60} A single prominent band remains, which, although weaker than other protein bands, is sufficiently isolated to permit measuring the number of protonated histidine residues^{57,58} (Figure S4).

The pD values obtained after correction ($pD = 0.929 \times \text{pH}^* + 0.42$, where pH^* is the pH meter reading in D_2O)⁶¹ shown in Figure 8 reveals a marked dependence of histidine protonation on protein unfolding. *Cyt c* contains three His residues, 18, 26, and 33. His18 is one of the heme ligands, His26 forms a H-bond with Pro44, and His33 is exposed to solvent. At 20 °C, the HisD_2^+ titration curve reveals protonation of one His residue with an apparent pK_a of 6.3, and two His residues with an apparent pK_a of 3.4. The first pK_a is close to that of aqueous HisD_2^+ (Figure 8 inset), and is assigned⁶² to the exposed His33. The second pK_a is assigned to both His18 and His26. This pK_a is lowered to about the same extent from the free HisD_2^+ value

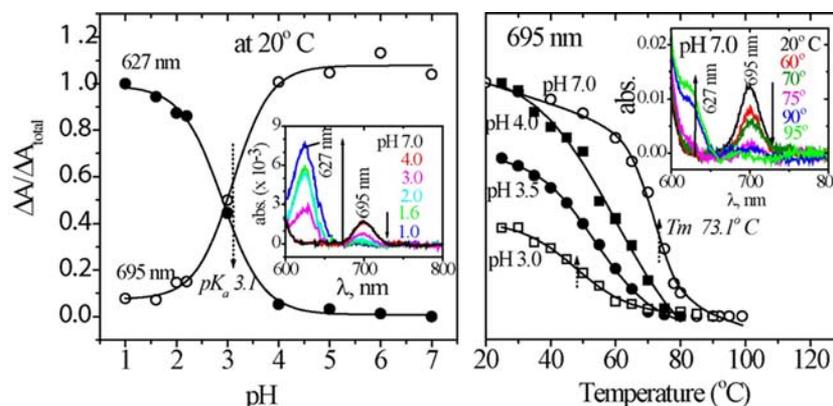


Figure 7. Left panel: pH titration curve for the 695 (heme–Met80, open circles) and 627 nm (heme–H₂O, filled circles) absorption bands of *hh* cyt c. Right panel: Temperature profiles of the 695 nm absorbance at pH = 7.0 (open circles), 4.0 (filled squares), 3.5 (filled circles), and 3.0 (open squares). Inset show the baseline-corrected absorption spectra, as a function of pH and temperature (various colors).

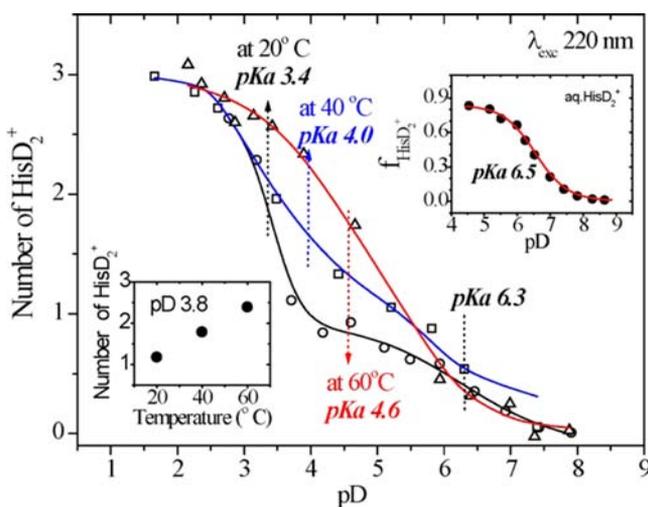


Figure 8. pD titration curves for the 1408 cm⁻¹ HisD₂⁺ of *hh* cyt c at 20 °C (circles), 40 °C (squares) and 60 °C (triangles). The insets show the aqueous histidine titration curve (upper) and the intensity-derived average protonation number as function of temperature (bottom). [The pH meter readings (pH*, in D₂O) were converted to pD = 0.929 × pH* + 0.42 as described in ref 61.]

by coordination to the heme Fe and by the H-bond from Pro44. Previous studies had indicated a pK_a of <3.5 for His26⁶² and 2.5 for His18.⁶³ The differences between the latter estimate and ours is likely not significant.

As the temperature is raised, the titration curve shifts markedly to higher pD values. The first His residue still titrates with an apparent pK_a ~ 6.3, as expected for the exposed His33, but the pK_a shifts steadily higher for the second and third residues at 40 and 60 °C. At pH 3.8, the proton number rises from one at 20 °C toward three at 60 °C. This behavior is consistent with an increase in the His26 pK_a as the H-bond from Pro44 is broken due to temperature-induced protein conformation change; the His18–Fe bond is also weakened in this process, resulting in His18 protonation at high temperatures.

3.6. T-jump/UVR Spectroscopy. Figure 9 displays UVR difference spectra induced by a 40–72 °C temperature jump in cyt c, at 197 and 229 nm probe wavelengths. Figure 10 displays the time course of secondary structure change, obtained via the same analysis of the 197 nm UVR spectra

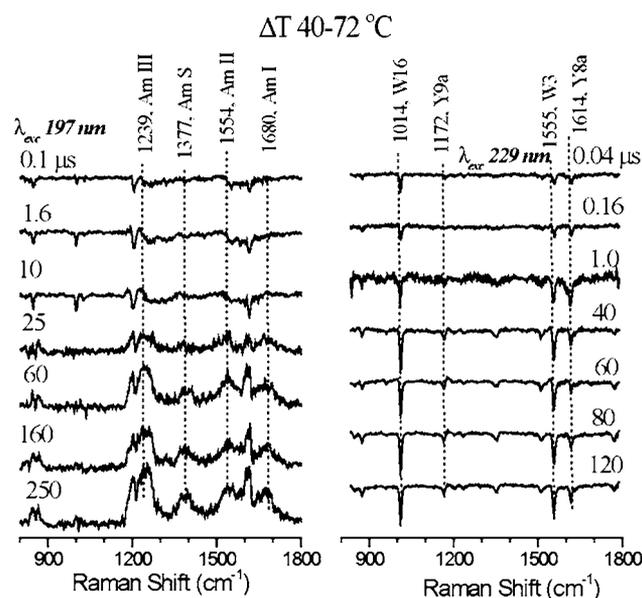


Figure 9. Pump–probe UVR difference spectra with 197 (left) and 229 (right) nm excitation, induced by the indicated T-jump, at the indicated delay intervals, for *hh* cyt c at pH 3.0.

as was used to determine temperature profiles from the static spectra. However, because of a higher noise level induced by the T-jump, the procedure failed to reliably separate coil and β -turn contributions, which are therefore summed in Figure 10. Also indicated in Figure 10 are the secondary structure fractions obtained from the equilibrium spectrum at the final temperature 72 °C. These values show that β -sheet formation is essentially completed in the 200 μ s time window of the T-jump measurements, but some conversion of α -helix to unordered structure (~5%) continues after 200 μ s.

The growth of β -sheet structure, at the expense of the other secondary structures, occurs in a multistage fashion, over four decades in time. The dotted lines indicate best-fit time constants, 0.2, 3.9, and 67 μ s, for three sequential first-order processes. About half of the β sheet amplitude is encompassed by the first two processes.

Similar multiexponential evolution is seen for the TrpW3 band intensity and broadening, (Figure 11); at 200 μ s the data approach the 72 °C equilibrium values. The curves drawn through the points are computed with the time constants

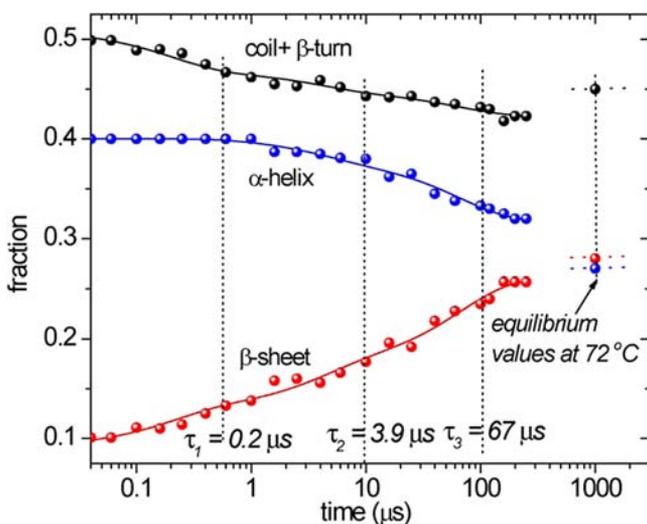


Figure 10. Time evolution of UVRR-determined secondary structure content in *hh* cyt *c* at pH 3.0 following a 40–72 °C T-jump. Solid curves are fits to successive exponentials, with the indicated time constants.

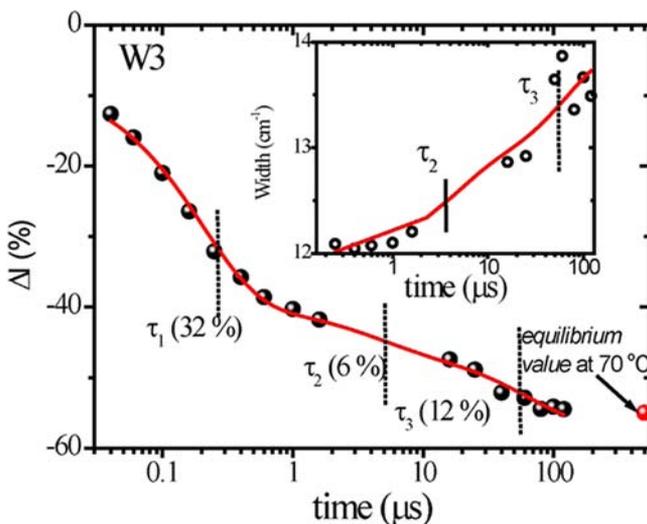


Figure 11. Time evolution of Trp W3 intensity in *hh* cyt *c* at pH 3.0 following a 40–72 °C T-jump. Solid curve is obtained using the time constants obtained from 197 nm excited UVRR spectra with the indicated time constants. Inset shows the time-dependent bandwidth change for W3 band.

obtained from the secondary structure fits. The fastest process (0.2 μs) accounts for most of the W3 intensity loss, but produces little W3 broadening and only a small change in the secondary structure (Figure 10). We attribute this process to solvent penetration into the interior of the protein, exposing Trp59 to a hydrophilic environment. Similar submicrosecond decays of aromatic residue intensity have been seen in other protein T-jump studies.^{64,65} The second (3.9 μs) and third (67 μs) processes account for most of the β -sheet formation, as well as of the W3 broadening, consistent with coupling of the protein conformation change and the Trp59 mobility.

4. DISCUSSION

4.1. His26 Protonation Triggers 40s Ω Loop Conversion to β -Sheet. The β -sheet is an important structural element in many proteins, and has become a focus of interest

because of its role in protein aggregation, including fibril formation in amyloid diseases.^{66,67} The interstrand H-bond motif of the β -sheet lends itself to interprotein association. β -sheet peptides and proteins are therefore prone to aggregation.^{68,69}

The present work reveals a novel instance of *intra*-protein β -sheet formation as a stage in protein unfolding. The UVRR analysis of secondary structure shows melting of the cyt *c* helices and turns to unordered structure at neutral pH, but at pH 3, heating produces β -sheet at the expense of turns and helices. This conformation change is reversible, provided the solution is dilute. As the cyt *c* concentration is raised above 150 μM , heating leads to protein aggregation, probably due to intermolecular association of the thermally induced β -sheet strands. The reversibility of the transition in dilute solution, where the protein molecules are isolated, implies that β -sheet interstrand H-bond requirements are satisfied intramolecularly. T-jump/UVRR spectra show this process to occur in microseconds, consistent with unfolding rates in other small proteins.^{70–77}

The UVRR analysis of histidine protonation reveals that one of the three His residues in cyt *c* titrates with a normal $\text{p}K_{\text{a}}$, 6.3. This $\text{p}K_{\text{a}}$ is assigned to His33,⁶² whose side chain is exposed to solvent in the native cyt *c* structure. The remaining two His residues have greatly depressed apparent $\text{p}K_{\text{a}}$'s, both around 3.4 at 20 °C. One of these, His18, is ligated to the heme Fe, and the Fe–His bond must be broken to permit its protonation at $\text{p}K_{\text{a}}$ of 2.5.⁶³ The remaining residue, His26, is H-bonded to the backbone carbonyl of Pro44. Evidently this interaction is strong enough to depress the His26 $\text{p}K_{\text{a}}$ by ~ 3 units.⁶² As the temperature is raised, both of the depressed $\text{p}K_{\text{a}}$'s are also elevated, reaching an average value of 4.6 at 60 °C.

We infer that protonation of His26 disrupts its interaction with Pro44 and triggers the conformation change that induces β -sheet formation. Pro44 sits at the tip of a large Ω loop (40s Ω loop) containing residues 40–57 (Figure 1). Using H-exchange NMR measurements, Englander and co-workers¹⁹ have identified this loop, along with another loop containing the Met80 ligand, as being the least stable 'foldons' of cyt *c*. The 40s Ω loop was found to be strongly destabilized at low pH, an effect attributed to protonation of His26, and possibly one of the heme propionates.²⁰ Our observed shift of ~ 3 units in the $\text{p}K_{\text{a}}$ of His26 supports this view, as does the finding by Sinibaldi et al.¹⁷ that mutation of the His26 significantly destabilizes cyt *c* structure.

Disruption of the His26/Pro44 H-bond thus leaves the 40s Ω loop only marginally stable. Heating the solution then overcomes the remaining stabilization. Instead of melting to unordered structure, however, the 40s Ω loop converts to a β -sheet structure. In the native cyt *c* structure (Figure 1), the 'neck' of the 40s Ω loop consists of a short antiparallel β -sheet, consisting of residues 37–39 and 58–61. We envision that this β -sheet segment extends itself into the remainder of the now-labile 40s Ω loop, at the expense of the three β -turns and the short 50s helix contained in the native loop.

The 40s Ω loop, including its β -neck (residues 37–61) contains 20% of the cyt *c* residues, whereas the extent of thermally induced β -sheet structure is $\sim 30\%$ (Figure 4). Moreover the 50s helix (residues 49–55) contains only 5% of the cyt *c* residues, while heating induces a loss of helix from 40% to 25%. The remaining 10% loss could result from melting of the short 60s and 70s helices. These are immediately upstream of the β -neck, and we envision the β -sheet formation

extending from the 40s Ω loop on through the 60s and 70s helices. It is notable that the T_m for helix melting, 60 °C, is higher than the T_m for β -sheet formation, 53 °C, whereas the β -turn T_m , 45 °C is lower (Figure 4). These data indicate that the 40s Ω loop conformation change is initiated at 45 °C, and that additional thermal energy is needed to extend the sheet from the 40s Ω loop into the 60s and 70s region. The 53 °C T_m for β -sheet formation represents an average for these two processes.

4.2. Aromatic Signals Reveal Heme Displacement.

T_m 's of 50 and 53 °C are observed for frequency shifts in the Trp W3 and Tyr 9a UVRR bands (Figure 5), coincident with the β -sheet T_m . The W3 signal arises from the sole Trp residue, Trp59. Its shift and broadening indicates increased mobility of the indole side chain.^{48,49} The Y9a band arises from the four Tyr residues; its upshift and narrowing implicate a more in-plane average orientation of the OH substituents, and a narrowing of its distribution.⁵² This effect is likely associated mainly with Tyr48, which has the most out-of-plane OH orientation in the native structure.⁵²

Both Trp59 and Tyr48 are H-bonded to the same heme propionate substituent. Trp59 sits in the middle of the β -neck of the 40s Ω loop, while Tyr48 is in the loop, just downstream of the 50s helix (Figure 1). The same propionate also forms H-bonds with Asn52, in the 50s helix, and with a backbone amide adjacent to Gly41, further into the loop. As well, the residue adjacent to Tyr48, Thr49, is H-bonded to the second propionate substituent (Figure 1). This extensive set of H-bonds with residues in the 40s Ω loop, provide a mechanism for heme displacement when the loop converts to β -sheet, thereby altering the side chain positions. The Y9a upshift indicates that the displacement forces the Tyr48 OH into a more in-plane orientation, while the W3 broadening indicates an increase in the Trp59 mobility, due to weakening of its H-bond.

The second propionate also form H-bonds with Lys79 and Thr78 (Figure 1), which are in the loop containing the Met80 ligand. Thus, the heme displacement resulting from the 40s Ω loop/ β -sheet conversion provides a mechanism for breaking the Met80-Fe bond. The T_m for Met80-Fe bond breaking, 48 °C, corresponds to the first stage of β -sheet formation.

We note as well the likely involvement of Tyr67, which H-bonds to Met80. Breaking the Met80-Fe bond would destabilize the 60s helix, contributing to its melting, proposed to occur at a slightly higher temperature ($T_m = 60$ °C).

4.3. Microsecond Conformational Rates. The T-jump/UVRR analysis reveals a rapid process, 0.2 μ s, during which the W3 signal from Trp59 loses significant intensity (Figure 11); other Trp and Tyr bands lose intensity as well (Figure 9). This phase is attributed to thermally driven solvent penetration, which exposes the aromatic side chains to an aqueous environment.^{64,65}

During this early phase, there is little change in secondary structure (Figure 11), whose subsequent alteration occurs with two time constants, 3.9 and 67 μ s. The microsecond time scale is consistent with intramolecular unfolding processes that have been reported for small proteins.^{70–77} The early phases account for about half of the β -sheet formation, while the 67 μ s phase accounts for the remainder. These amplitudes are roughly congruent with the two successive thermal equilibrium phases (Figure 4), which we assign to the 40s Ω loop and the 60s and 70s helices. The Trp59 W3 band broadens during these two phases (Figure 11), consistent with increased Trp59 mobility as a result of the protein conformation change.

4.4. Possible Mechanism of Cardiolipin-Induced

Apoptosis. We suggest that the 40s Ω loop β -sheet conversion triggered by loss of the Pro44–His26 H-bond may also initiate apoptotic events associate with cardiolipin binding to cyt c. Jemmerson et al.²¹ found that cyt c in apoptotic cells, or in association with lipid vesicles, binds to an antibody that recognizes a region around the Pro44 residue in non-native forms of cyt c, indicating exposure of Pro 44. Moreover, cyt c bound to cardiolipin vesicles has been reported to contain substantial fractions of β -sheet, on the basis of UVCD measurements.⁷⁸

Cardiolipin induces its own peroxidation via its interactions with cyt c, and the resulting hydroperoxides induce the release of pro-apoptotic factors. Peroxidase activity would result from His26-triggered β -sheet conversion, which displaces the heme, breaking the Met80–Fe bond, and allowing access of peroxide to the heme Fe. We previously showed that heating cyt c at pH 3 induces peroxidase activity with the same T_m as is observed for β -sheet formation.¹⁶

In its complex with cyt c, cardiolipin has been shown to insert its acyl chains into hydrophobic regions of the protein. Two hydrophobic sites have been suggested, one in the region of the Met80 loop,⁹ and the other a channel extending into the interior from Asn52, an invariant residue.¹⁰ Sinibaldi et al.¹³ have proposed that both sites are occupied. Rytomaa et al.⁷⁹ proposed that Asn52 forms an H-bond with the phosphate headgroup at the end of the acyl chain that is inserted in the Asn52 channel. Asn52 is on the 50s helix, which is part of the 40s Ω loop. We suggest that the geometry of the complex with cardiolipin brings Pro44, at the end of the 40s Ω loop, into contact with the membrane surface, and that its H-bond with His26 is broken because His26 H-bonds instead with a phosphate headgroup of the membrane. Alternatively, His26 may actually become protonated by proximity to the surface, given the lowered pH at the surface of anionic membranes.⁸⁰

His26-triggered β -sheet conversion, and the associated melting of the 60s helix may also loosen the C-terminal helix; one of its residues, the invariant Arg91, which has a significant effect on cardiolipin-induced redox modulation,^{81,82} is H-bonded to Met65, on the 60s helix. FRET measurements by Hanske et al.⁵ indicate that cardiolipin induces unfolding of the C-terminal helix. Groves and co-workers have proposed insertion of this helix into the lipid membrane to explain their important discovery of cardiolipin-promoted membrane pore formation by cyt c,⁸³ permitting its own penetration through the membrane. This finding can explain the phenomenon of cyt c migration from the mitochondrion to the cytosol, where it binds and activates the apoptosis protease activation factor, APAf-1.^{8,14,15} The conformation change induced by breaking the Pro44–His26 H-bond, by destabilizing the 60s helix, may lower the barrier for unmooring the C-terminal helix, allowing it to penetrate the lipid membrane.

5. CONCLUSIONS

At pH 3, cyt c undergoes a reversible unfolding process, which differs from the thermal unfolding seen at neutral pH. UVRR analysis shows that instead of a simple loss of secondary structure, there is a conformation change to β -sheet. This change occurs in two phases, involving first the loss of β -turns, and then the loss of helix structure. T-jump studies indicate that these phases occur with 3.9 and 67 μ s time constants, consistent with intramolecular unfolding dynamics in small proteins. UVRR analysis also shows reorientation of the Trp59

indole ring on this time scale, following a faster, solvent penetration phase.

UVRR/pH titrations reveal a depressed pK_a for His26, reflecting its H-bond interaction with Pro44. Loss of this interaction labilizes the 40s Ω loop, one of two low-energy foldons in cyt c. It is proposed that, in the first phase of thermally induced β -sheet formation, a short native β -sheet segment at the 40s Ω loop neck is extended into the rest of the loop, while in the second phase, the sheet is further extended into the adjacent 60s and 70s helices. The conformation change alters the orientation of the Trp59 and Tyr48 side chains, which are H-bonded to a heme propionate substituent. This reorientation is suggested to reflect displacement of the heme as a result of the 40s Ω loop conformation change. Heme displacement accounts for the observed loss of the Met80–Fe bond, and the induction of peroxidase activity. T_m 's for these events coincide with that of β -sheet formation and of Trp59 and Tyr48 reorientation.

These observations point to a mechanism for the initiation of apoptosis by cardiolipin binding to cyt c, inasmuch as the binding event appears to labilize the 40s Ω loop, and induces β -sheet formation as well as peroxidase activity. Insertion of cardiolipin acyl chains into cyt c is suggested to disrupt the Pro44/His26 H-bond through interaction of His26 with phosphate head groups in the lipid bilayer.

■ ASSOCIATED CONTENT

● Supporting Information

Temperature dependent UVRR spectra of cyt c at pH 3, excited at 197 and 229 nm; illustration of the subtraction procedure used to enhance the amide bands at 197 nm excitation; and the spectrum of cyt c in D₂O excited at 220 nm. 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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