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Effect of a K72A Mutation on the Structure, Stability, Dynamics and Peroxidase Activity of Human Cytochrome *c*

Shiloh M. Nold^{†,§}, Haotian Lei^{†,§}, Tung-Chung Mou^{‡,§}, and Bruce E. Bowler^{†,§,*}

[†]Department of Chemistry and Biochemistry, University of Montana, Missoula, Montana 59812

[‡]Division of Biological Sciences, University of Montana, Missoula, Montana 59812

[§]Center for Bimolecular Structure and Dynamics, University of Montana, Missoula, Montana 59812

Abstract

We test the hypothesis that Lys72 suppresses the intrinsic peroxidase activity of human cytochrome *c*, as observed previously for yeast iso-1-cytochrome *c* [McClelland et al. PNAS 111, 6648–6653 (2014)]. A 1.25 Å X-ray structure of K72A human cytochrome *c* shows that the mutation minimally affects structure. Guanidine hydrochloride denaturation demonstrates that the K72A mutation increases global stability by 0.5 kcal/mol. The K72A mutation also increases the apparent pK_a of the alkaline transition, a measure of the stability of the heme crevice, by 0.5 units. Consistent with the increase in the apparent pK_a , the rate of formation of the dominant alkaline conformer decreases and this conformer is no longer stabilized by proline isomerization. Peroxidase activity measurements show that the K72A mutation increases k_{cat} by 1.6– to 4–fold at pH values from 7 to 10, a larger effect than for the yeast protein. X-ray structures of wild type and K72A human cytochrome *c* indicate that direct interactions of Lys72 with the far side of Ω -loop D, which are seen in X-ray structures of horse and yeast cytochrome *c* and could suppress peroxidase activity, are lacking. Instead, we propose that the larger effect of the K72A mutation on peroxidase activity of human versus yeast cytochrome *c* results from relief of steric interactions between the side chains at positions 72 and 81 (Ile in human versus Ala in yeast), which suppress the dynamics of Ω -loop D necessary for the intrinsic peroxidase activity of cytochrome *c*.

Graphical abstract

*Corresponding Author: bruce.bowler@umontana.edu.

ORCID

Bruce E. Bowler: 0000-0003-1543-2466

Notes

The authors declare no competing financial interest.

Supporting Information

Figures S1 and S2 show water-mediated hydrogen bond networks at the surface of WT and K72A Hu Cyt c . Figure S3 shows typical pH jump stopped-flow data. Tables S1 to S4 provide rate constants and amplitudes obtained from pH jump stopped-flow data. Table S5 provides K_M and k_{cat} for peroxidation of guaiacol from pH 6 to 10.

INTRODUCTION

Cytochrome *c* (Cyt*c*) was first recognized for its role as an intermediary in the electron transport chain.¹ More recently, the range of its functions has expanded to include scavenging of reactive oxygen species, redox-mediated protein import and most notably apoptosis.² Initial work on the role of Cyt*c* in apoptosis³ demonstrated that Cyt*c* was released into the cytoplasm, where it interacts with apoptosis protease activating factor 1, Apaf-1, forming the apoptosome. The apoptosome activates caspase-9 ultimately leading to cell death. More recently, it has been shown that Cyt*c* acts as a peroxidase when bound to the mitochondrial lipid cardiolipin (CL).^{4, 5} Oxidation of cardiolipin leads to release of Cyt*c* from the inner mitochondrial membrane and appears to aid in permeabilization of the outer mitochondrial membrane. Thus, the peroxidase activity is believed to be the earliest signal in the intrinsic pathway of apoptosis.⁵

When Cyt*c* binds to CL-containing membranes it is known to lead to loss of heme-Met80 ligation.^{5–7} Thus, it seems likely that the highly conserved surface loop, Ω -loop D, that encompasses residues 70 to 85^{8, 9} may be involved in mediating peroxidase activity. To act as a signaling agent, it is essential that the basal peroxidase activity of Cyt*c* be minimal so that it can act as a true on/off switch. In the current work, we aim to understand how the sequence of the highly-conserved Ω -loop D is designed to suppress peroxidase activity. In particular, we investigate the role of the absolutely conserved lysine at position 72 in modulating the basal peroxidase activity of human Cyt*c* (Hu Cyt*c*). In the X-ray structures of yeast iso-1-cytochrome *c* (iso-1-Cyt*c*)¹⁰ and horse Cyt*c*,¹¹ this residue lies across Ω -loop D making steric or hydrogen-bonding contacts with residues 80 to 82 on the opposite side of the loop. These interactions might stabilize Ω -loop D, which positions Met80 for heme binding, limiting the basal peroxidase activity of Cyt*c*.

Previous work with iso-1-Cyt*c*, which contains a trimethylated lysine at position 72 (tmK72),¹⁰ showed that when an alanine is introduced at position 72 (tmK72A variant), an alternate conformer of the protein with Met80 replaced by hydroxide in the 6th coordination site of the heme can form.¹² An increase in peroxidase activity was also observed for this variant of iso-1-Cyt*c*. Studies on the kinetics and thermodynamics of a His79-mediated alkaline transition with iso-1-Cyt*c* showed that replacement of tmK72 with alanine destabilizes the native conformer relative to the alkaline conformer and increases the rate of formation of the alkaline conformer.^{13, 14} These kinetic and thermodynamic results are consistent with the enhanced peroxidase activity of the tmK72A variant of iso-1-Cyt*c*. However, a K72G variant of horse Cyt*c* changes neither the midpoint pH for the alkaline

conformational transition nor the stability of Ω -loop D,¹⁵ indicating that this residue may not affect the peroxidase activity of mammalian cytochromes *c*.

In the current work, we probe the effect of a K72A mutation on the properties of Hu Cyt*c*. We show that the native structure is unaffected by this mutation. However, we observe significant perturbation of the kinetics and thermodynamics of the alkaline transition. We also show a significant enhancement in peroxidase activity of K72A Hu Cyt*c* relative to wild type (WT) Hu Cyt*c*.

EXPERIMENTAL PROCEDURES

Preparation of Human K72A Cytochrome *c*

The K72A variant of Hu Cyt*c* was prepared with site-directed mutagenesis using the pBTR(HumanCc) plasmid provided by the laboratory of Gary Pielak at the University of North Carolina.¹⁶ pBTR(HumanCc) is derived from the pBTR1 plasmid^{17, 18} through replacement of the yeast iso-1-Cyt*c* gene (*CYCI*) with a synthetic human cytochrome *c* gene.^{16, 19} pBTR(HumanCc) like pBTR1 co-expresses the yeast heme lyase (*CYC3*) and uses it to covalently attach heme to the CSQCH heme recognition sequence of Hu Cyt*c*. Double-stranded pBTR(HumanCc) DNA was prepared using BL21-Gold(DE3) (Agilent Technologies) competent *Escherichia coli* cells and used as a template for site-directed mutagenesis with the Agilent Technologies QuikChange Lightning Site-Directed Mutagenesis Kit. The oligonucleotide, K72A: d(GGAATACCTCGAGAACCCGGCGAAATACATCCCGGGCACG) and its reverse complement, K72A-r were used for mutagenesis. The mutated DNA was transformed into TG-1 *E. coli* cells. Individual colonies were then grown in L-broth with Ampicillin (10 mL L-broth with 10 μ L of 100 mg/mL Ampicillin) at 37 °C overnight. The DNA was extracted and purified with the Promega Wizard® Plus Miniprep DNA Purification System. The sequence was confirmed with an ABI 3130 Genetic Analyzer at the Genomics Core Facility at the University of Montana.

Expression of Human Cytochrome *c*

WT and K72A Hu Cyt*c* were expressed from the pBTR(HumanCc) plasmid after transformation into Ultra BL21 (DE3) *E. coli* competent cells (EdgeBio, Gaithersburg, MD) using the manufacturers protocol. Transformed cells were grown on 2xYT bacterial media, as described previously,²⁰ except with the addition of 100 μ L of antifoam per liter of culture. On average, a 1 L culture yielded 4.7 g of pelleted cells.

Protein extraction and purification were carried out as described, previously.^{14, 20–22} In brief, cells were lysed with French Pressure Cell followed by precipitation of contaminating proteins with 50% ammonium sulfate. After CM-sepharose cation exchange chromatography, protein was exchanged into 50 mM sodium phosphate, pH 7 by centrifuge ultrafiltration, flash frozen in liquid N₂ and stored at –80 °C until used. Prior to experiments, WT and K72A Hu Cyt*c* were purified by HPLC using a BioRad UNO S6 column, as previously described.²⁰

Purified proteins were characterized by MALDI-ToF mass spectrometry using a Bruker microflex mass spectrometer. For WT Hu Cyt_c, 12,234.78 m/z (expected: 12,234.03 m/z). For K72A Hu Cyt_c, 12175.17 m/z (expected: 12,176.94 m/z)

Crystallization and Structure Determination of Human K72A Cyt_c

K72A Hu Cyt_c was oxidized with K₃[Fe(CN)₆], followed by separation from the oxidizing agent and exchanged into 50 mM Tris pH 7 using Sephadex G-25 chromatography. It was then concentrated to ~16.5 mg/ml using centrifuge ultrafiltration (Amicon Ultra-4 10,000 MWCO). Screening for crystallization conditions was carried out using the JCSG core I, JCSG core II, JCSG core III, JCSG core IV, PEGS, PEGS II, Wizard classic 1&2, Wizard classic 3&4, Classic, Classic II, and Classic Lite commercial screening suites at 20 °C. The ratio of protein to reservoir solution in the drops in the 96 well sitting drop screening plates was 1:1. We obtained initial crystals from JCSG core I, well A2, which has a 0.1 M Bicine pH 8.5, 20%(w/v) PEG 6000 reservoir solution. Additional vapor diffusion crystallization experiments were set up in a 24-well VDX plate by expanding upon the pH and precipitant concentration of this initial condition. After 4 days to 2 weeks of equilibration at 20 °C, crystals were obtained from a drop containing 1 µL of protein and 1 µL of 25% PEG 6000, 0.1 M Tris pH 8, which diffracted to 1.8 Å at SSRL beamline 14-1. Further trials in the presence of sodium dodecyl sarcosine as an additive at 4.16 mM final concentration (60 mM stock) with a reservoir solution containing 30% PEG 3350 and 0.1 M sodium citrate, pH 6.4 and protein at 17.2 mg/mL (mixed 0.14:0.93:0.93) yielded crystals diffracting to 1.25 Å resolution in the P 2₁ space group. All crystals were cryoprotected with 20% glycerol and flash frozen in liquid N₂ for data collection.

The X-ray diffraction dataset was collected under cryogenic conditions at 100 K at the SMB beamline 9-2 of the Stanford Synchrotron Radiation Lightsource (SSRL) using a Pilatus 6 M detector. Diffraction data were processed using HKL2000.²³ The initial electron density map was determined using phases obtained with the molecular replacement method using Phaser, incorporated in the PHENIX software suite,²⁴ and the coordinates of the wild-type human Cyt_c structure (PDB ID: 3ZCF)²⁵ as a search model. The initial model was built into a continuous electron density map and subsequently refined using PHENIX. The structure model was further refined to 1.25 Å resolution by multiple rounds of manual model rebuilding with COOT²⁶ and restrained refinement with PHENIX using 5 % of reflections for calculation of R_{free}. Data collection and refinement statistics of the final model are summarized in Table 1. The coordinates and structure factors for K72A Hu Cyt_c have been deposited in the Protein Data Bank (PDB ID: 5TY3).

Global Unfolding by Guanidine Hydrochloride Denaturation

Global unfolding thermodynamics of WT and K72A Hu Cyt_c were determined by guanidine hydrochloride (GdnHCl) denaturation monitored by circular dichroism (CD) spectroscopy. Protein was oxidized with potassium ferricyanide and separated from oxidant by G-25 size exclusion chromatography in CD buffer (20 mM Tris, pH 7.5, 40 mM NaCl). Concentration of GdnHCl stock solutions were determined using the empirical equation of Nozaki for the change in refractive index relative to the background buffer.²⁷

GdnHCl unfolding titrations were carried out with an Applied Photophysics Chirascan CD Spectrophotometer coupled to a Hamilton Microlab 500 Titrator. The change in ellipticity was monitored at 222 nm using 250 nm as background, $\theta_{222\text{corr}}$ ($\theta_{222\text{nm}} - \theta_{250\text{nm}}$). The protein concentration was 4 μM . $\theta_{222\text{corr}}$ versus GdnHCl concentration denaturation curves were fit to eq 1 with SigmaPlot v. 13 (Systat Software, Inc.), which assumes two state unfolding and a linear dependence of the free energy of unfolding, G_u , on GdnHCl concentration.^{29, 30} Native and denatured state baselines were also varied linearly with GdnHCl concentration. In eq 1, θ_N and

$$\theta_{222\text{corr}} = \frac{(\theta_N + m_N [\text{GdnHCl}]) + (\theta_D + m_D [\text{GdnHCl}]) \cdot \exp \left\{ \frac{m [\text{GdnHCl}] - \Delta G_u^{\circ'} (\text{H}_2\text{O})}{RT} \right\}}{1 + \exp \left[\frac{m [\text{GdnHCl}] - \Delta G_u^{\circ'} (\text{H}_2\text{O})}{RT} \right]} \quad (1)$$

m_N are the intercept and the slope of the native state baseline, θ_D and m_D are the intercept and the slope of the denatured state baseline, m is slope of G_u with respect to GdnHCl concentration and $G_u^{\circ'} (\text{H}_2\text{O})$ is the free energy of unfolding extrapolated to 0 M GdnHCl.

Measurement of the Alkaline Transition by pH Titration

The alkaline conformational transition of WT and K72A Hu Cyt c was monitored with a Beckman DU800 spectrophotometer using absorbance at 695 nm, A_{695} , a weak absorbance band which reports on the Met80-heme ligation of the native state of oxidized Cyt c .³¹ The absorbance data were corrected for baseline drift using absorbance at 750 nm, A_{750} , as baseline ($A_{695\text{corr}} = A_{695} - A_{750}$) and then converted to extinction coefficient, $\epsilon_{695\text{corr}}$, by dividing by concentration. Titrations were monitored at room temperature (22 ± 3 °C) at 175 – 200 μM protein concentration in 100 mM NaCl. Titrations were carried out by adding equal amounts of an appropriate concentration of NaOH and a 2x protein stock (350 – 400 μM protein in 200 mM NaCl) to keep protein concentration constant throughout the experiment.^{32, 33} Plots of $\epsilon_{695\text{corr}}$ versus pH were used to determine the apparent pK_a (pK_{app}) by fitting the data to eq 2 (SigmaPlot v. 13), a modified form of the Henderson-Hasselbalch

$$\epsilon_{695\text{corr}} = \frac{\epsilon_N + \epsilon_{\text{Alk}} \times 10^{n[pK_{\text{app}} - \text{pH}]}}{1 + 10^{n[pK_{\text{app}} - \text{pH}]}} \quad (2)$$

equation that evaluates the number of protons, n , linked to the alkaline conformational transition. In eq 2, ϵ_N and ϵ_{Alk} are the corrected extinction coefficients at 695 nm of the native and alkaline conformers, respectively.

pH Jump Stopped-flow Kinetic Measurements

Kinetics of the alkaline conformation transition were monitored via pH jump stopped-flow experiments carried out with an Applied Photophysics SX20 stopped-flow apparatus at

25 °C. The conformational transition was monitored at the 406 nm in the heme Soret region. Oxidized protein (Fe(III)-heme) was separated from ferricyanide using a G25 column equilibrated to and run with 0.1 M NaCl. The solution was adjusted to 20 µM protein concentration and pH 6 for upward pH jumps and to near pH 10 for downward pH jumps. 1:1 mixing with 0.1 M NaCl containing 20 mM buffer yields a final protein concentration of 10 µM in 10 mM buffer, 0.1 M NaCl. Buffers used for pH jump stopped-flow were: MES, pH 5.5 – 6.5; NaH₂PO₄, pH 6.75 – 7.5; Tris, pH 7.75 – 8.75; Boric Acid, pH 9.0 – 10.0; CAPS, pH 10.25 – 11.0. Samples were taken from the stopped-flow waste line and pH was measured directly after mixing experiments at each pH. Data were fit to a one to four exponential equation, as appropriate.

Peroxidase Activity Measurements

Peroxidase activity was measured with the colorimetric reagent, guaiacol, using previously reported conditions and procedures.^{12, 34} The reaction was monitored using an Applied Photophysics SX20 stopped-flow apparatus at 25 °C. The formation of tetraguaiacol from guaiacol and H₂O₂ in the presence of Cyt_c is monitored at 470 nm. A 4 µM Cyt_c solution in 50 mM buffer was mixed with guaiacol in 50 mM buffer at 4-fold the desired final concentration to produce a 2 µM Cyt_c and 2-fold concentrated guaiacol stock in 50 mM buffer. This solution was mixed 1:1 with 100 mM H₂O₂ in 50 mM buffer yielding a final solution containing 1 µM Cyt_c, 50 mM H₂O₂ and guaiacol at the desired concentration in 50 mM buffer. Concentration was determined using the extinction coefficients of H₂O₂ ($\epsilon_{240} = 41.5 \text{ M}^{-1} \text{ cm}^{-1}$, the average of published values)^{35, 36} and guaiacol ($\epsilon_{274} = 2,150 \text{ M}^{-1} \text{ cm}^{-1}$).³⁷ Buffers used for peroxidase experiments were the same as those used in pH-Jump experiments. Final guaiacol concentrations used were 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 150 and 200 µM.

Data were fit using SigmaPlot v. 13. The segment of the A₄₇₀ versus time data with the greatest slope following the initial lag phase was used to obtain initial velocity, v , at each guaiacol concentration. The data were fit to a linear equation and the slope from five repeats was averaged. The slope (dA_{470}/dt) was divided by the extinction coefficient of tetraguaiacol at 470 nm ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$)³⁸ and multiplied by 4 to produce the initial rate of guaiacol consumption, v . The initial rate, v , was divided by cytochrome *c* concentration, plotted against guaiacol concentration and fit with eq 3 to obtain K_m and k_{cat} values.

$$\frac{v}{[\text{Cyt}c]} = \frac{k_{cat} \cdot [\text{Guaiacol}]}{K_m + [\text{Guaiacol}]} \quad (3)$$

RESULTS

Structure of K72A Human Cyt_c

Crystals of K72A Hu Cyt_c were prepared in the absence and presence of sodium dodecyl sarcosine as an additive. In both cases, the K72A Hu Cyt_c crystallized in the P 2₁ space group. The crystal obtained in the presence of the additive diffracted to a resolution of 1.25 Å (Table 1). A high quality structural model with R_{work}/R_{free} of 0.15/0.17 (Table 1) was

obtained. Figure 1 shows an overlay of the K72A Hu Cyt c structure with that of WT Hu Cyt c . The structures are very similar. An all atom alignment of the K72A Hu Cyt c structure with the WT Hu Cyt c crystal structure (PDB ID: 3ZCF;²⁵ PyMol align function using chain A of each structure) yields an RMSD of 0.18 Å. The methyl side chain of Ala72 of K72A Hu Cyt c is in the same position as the β -carbon of Lys72 of WT Hu Cyt c (Figure 1).

Lys72 of horse Cyt c ¹¹ and trimethyllysine72 (tmK72) of yeast iso-1-Cyt c ¹⁰ both lie across Ω -loop D making contacts with residues 80 to 82 on the far side of the loop. In the case of horse Cyt c , Lys72 makes three hydrogen bonds to backbone atoms of Met80 and Phe82 (Figure 2A). In our previous work on a K72A variant of iso-1-Cyt c ,¹² we proposed that removal of this cross loop interaction by replacement of tmK72 with alanine labilized Ω -loop D permitting enhanced peroxidase activity. Thus, it seemed likely that a similar phenomenon might be operative for Hu Cyt c . However, in the structure of WT Hu Cyt c ,²⁵ Lys72 does not make direct contacts with the backbone atoms of Met80 and Phe82 as seen for horse Cyt c . In chain A of the WT Hu Cyt c crystal structure, Lys72 makes water-mediated contacts with the backbone atoms of Thr78, Met80 and Phe82 (Figure 2B). In our K72A Hu Cyt c crystal structure, the position of the ϵ -NH₂ group of Lys72 in chain A of the WT structure is occupied by a water molecule (Figure 2C). However, in the other three molecules in the asymmetric unit of the WT Hu Cyt c crystal structure, the hydrogen bond network around Lys72 does not lead to cross loop contacts (Figure S1). Instead, the water-mediated cross loop interactions in these molecules of WT Hu Cyt c are similar to those in the two molecules in the asymmetric units of our K72A Hu Cyt c structure (Figure 2C and Figure S2). In particular, irrespective of the presence of lysine at position 72, there is always a hydrogen bond between the carbonyl of Leu68 and the amide NH of Ile 85 at the neck of Ω -loop D (Figures 2, S1 and S2). Similarly, there is always a water-mediated hydrogen bond between the amide NH of residue 72 and the carbonyl of Phe82, which is often further supported by a water-mediated hydrogen bond to the side chain of Asn70 (Figures 2, S1 and S2). In many cases, the water-mediated hydrogen bond network extends the entire length of Ω -loop D.

Global Unfolding of Human Cyt c Variants

Global unfolding thermodynamics of WT²⁰ and K72A Hu Cyt c were monitored by CD at 25 °C and pH 7.5 with the use of GdnHCl as denaturant. Figure 3 compares the denaturation curves, $\theta_{222\text{corr}}$ versus GdnHCl concentration, for the two proteins. The unfolding curve for the K72A variant is shifted to slightly higher GdnHCl concentration indicating a small stabilization relative to WT Hu Cyt c . To fit the K72A Hu Cyt c data, a sloping native state baseline was necessary (eq 1, Experimental Procedures). For consistency, we refit our previously published WT Hu Cyt c data,²⁰ which had originally been fit with a constant native state baseline. Thermodynamic parameters from fits to a two-state model are given in Table 2. Within error, the denaturant m -value is unchanged by the K72A mutation. However, the K72A mutation increases the unfolding midpoint, C_m , by ~ 0.2 M and the stability, $G_u^{\circ'}$ (H₂O), by ~ 0.5 kcal/mol. Our previously reported stability parameters for Hu WT Cyt c are similar to those in Table 2.²⁰ C_m is unchanged, however, m increases by ~ 0.2 kcal mol⁻¹ M⁻¹ when a sloping native state baseline is used, which leads to an increase of ~ 0.5 kcal/mol in the stability of WT Hu Cyt c .

Local Unfolding via the Alkaline Conformational Transition

The local unfolding thermodynamics for the alkaline conformational transition of WT and K72A Hu Cyt c were determined by pH titration, monitored at 695 nm to observe the loss of Met80-heme iron ligation³¹ when a lysine from Ω -loop D binds to the heme. A schematic representation of the human alkaline conformational transition is shown in Figure 4A. Lysines 72, 73 and 79 could all potentially bind to the heme. However, while all three lysines contribute to the alkaline conformer of yeast iso-1-Cyt c expressed from *E. coli*,¹⁸ NMR studies suggest only two distinguishable alkaline conformers exist for horse Cyt c .³⁹ Mutational analysis indicates that lysines 73 and 79 contribute to the alkaline transition of horse Cyt c , but that Lys72 does not.¹⁵ Representative pH titration data for K72A Hu Cyt c are compared to previously reported data for WT Hu Cyt c ²⁰ in Figure 4B. Within error, $\epsilon_{695\text{corr}}$ of the native form of Hu Cyt c (below pH 8) is unaffected by the K72A mutation indicating minimal mutation-induced perturbation to Met80-heme ligation in the native state.^{40, 41} The K72A mutation, however, does cause a shift of the alkaline transition of Hu Cyt c to higher pH. Fits of the K72A Hu Cyt c data to eq 2 (Experimental Procedures) yield $pK_{\text{app}} = 10.0 \pm 0.1$ for the K72A variant, which is ~ 0.5 units higher than $pK_{\text{app}} = 9.54 \pm 0.03$ for WT Hu Cyt c measured under the same conditions.²⁰ The number of protons linked to the alkaline transition is near 1 for both WT Hu Cyt c ($n = 1.03 \pm 0.02$)²⁰ and K72A Hu Cyt c ($n = 0.98 \pm 0.06$).

Kinetics of the Alkaline Conformational Transition

For WT Hu Cyt c , the upward pH jump kinetic traces are best fit to a two exponential equation below pH 9.5, a three exponential equation from pH 9.6 to pH 10 and a four exponential equation above pH 10 (Figure S3 and Table S1 in the Supporting Information). The rate constant for the phase which emerges above pH 9.5, $k_{\text{obs},3}$, is near 0.1 s^{-1} , typical for proline isomerization (Figure 5A). The amplitude of this phase, A_3 , is largest at the pH where it can first be observed near the midpoint of the alkaline conformational transition of WT Hu Cyt c (Figure 5B). As pH increases and the alkaline conformer fully populates, the amplitude of this phase decreases. This amplitude behavior is characteristic of proline isomerization.⁴² Above pH 10, a kinetic phase, $k_{\text{obs},4}$, with a rate constant of $20 - 30 \text{ s}^{-1}$ begins to be observable. Fast phases in the $20 - 100 \text{ s}^{-1}$ range are usually observed above pH 10 in the kinetics of the alkaline transition of Cyt c .⁴³⁻⁴⁸ This fast phase has been assigned to a transient intermediate, which is thought to have either a weakened heme-Met80 bond or a hydroxide replacing Met80 as a heme ligand.^{45, 47} The intermediate has also been suggested to be due to a conformational change involving opening of the least stable substructure of Cyt c .⁴⁴

For K72A Hu Cyt c a two exponential fit is sufficient to fit the upward pH jump kinetics traces up to pH 10 (Figure S3 and Table S3 in the Supporting Information). No slow proline isomerization phase is evident as for WT Hu Cyt c . However, as with WT Hu Cyt c , a fast phase with a rate constant near 20 s^{-1} is observed above pH 10 (Table S3 of the Supporting Information).

Rate constants and amplitudes for both upward and downward pH jump experiments are shown in Figure 5 for WT and K72A Hu Cyt c . The pH dependencies of both $k_{\text{obs},1}$ and

$k_{\text{obs},2}$ are qualitatively consistent with the behavior expected for the standard mechanism for the alkaline conformational transition for both WT and K72A Hu Cyt c . This mechanism involves a rapid deprotonation equilibrium (K_{H}) followed by a conformational change with forward and backward rate constants, k_{f} and k_{b} , respectively (eq 4).⁴⁹ Thus, at low pH k_{obs} is equal to k_{b} . k_{obs} then

$$k_{\text{obs}} = k_{\text{b}} + k_{\text{f}} \times \left(\frac{K_{\text{H}}}{K_{\text{H}} + [\text{H}^+]} \right) \quad (4)$$

increases when an ionizable group is deprotonated (K_{H} or $\text{p}K_{\text{H}}$) ultimately leveling off at $k_{\text{b}} + k_{\text{f}}$, if sufficiently high pH is achieved. While the pH dependence of $k_{\text{obs},1}$ and $k_{\text{obs},2}$ are qualitatively consistent with eq 4, the upper limit of neither $k_{\text{obs},1}$ nor $k_{\text{obs},2}$ is sufficiently well-defined to allow reliable fits to eq 4 to extract k_{f} and K_{H} .

The amplitude data show that the phase corresponding to $k_{\text{obs},2}$ is the dominant alkaline conformer for both WT and K72A Hu Cyt c (Figure 5B). The pH dependence of A_2 is similar for both proteins. At low pH, where $k_{\text{obs},2}$ should equal k_{b} , $k_{\text{obs},2}$ obtained from downward pH jump experiments is near $0.02 - 0.03 \text{ s}^{-1}$ for both WT and K72A Hu Cyt c (Tables S2 and S4). The approximately 50 second timescale of $k_{\text{obs},2}$ at low pH for both WT and K72A Hu Cyt c is typical for lysine ligation in the alkaline state.⁵⁰ As pH increases, $k_{\text{obs},2}$ increases more rapidly for WT than for K72A Hu Cyt c . The magnitude of $k_{\text{obs},1}$ is $\sim 0.3 \text{ s}^{-1}$ near pH 8.5 (Tables S1 and S3), about 10-fold larger than $k_{\text{obs},2}$. The magnitude of $k_{\text{obs},1}$ increases slowly as pH increases (Figure 5A inset), but the phase is a minor contributor to overall amplitude at all pH values for both WT and K72A Hu Cyt c (Figure 5B inset) and is not observable in downward pH jump experiments for either protein. At pH 10.7, $k_{\text{obs},1}$ and $k_{\text{obs},2}$ become indistinguishable for WT Hu Cyt c (Table S1 of the Supporting Information). For WT Hu Cyt c , $k_{\text{obs},3}$ is also observable in downward pH jump experiments. As in upwards pH jump experiments, it has a value near 0.1 s^{-1} , consistent with proline isomerization.

Peroxidase Activity

In the early stages of apoptosis, Cyt c acts as a peroxidase in conjunction with binding to the mitochondrial membrane lipid cardiolipin.⁵ In previous work, we have shown that a K72A mutation to yeast iso-1-Cyt c enhances peroxidase activity and provides access to alternate conformers of Cyt c .¹² Thus, it is of interest to determine if a similar effect occurs for Hu Cyt c . Peroxidase activity of WT and K72A Hu Cyt c was measured by monitoring the formation of tetraguaiacol from guaiacol at 470 nm in the presence of H_2O_2 . Michealis-Menten plots with respect to guaiacol concentration were generated to permit extraction of k_{cat} and K_{m} across the pH range 6 to 10. It is evident in Figure 6A that the K72A mutation causes a significant increase in k_{cat} at pH 7. Similar behavior is observed at pH 8 – 10, while k_{cat} is within error the same at pH 6 for WT and K72A Hu Cyt c (Figure 6B, Table S5). The K_{m} values with respect to guaiacol concentration are not strongly affected by the mutation (Table S5).

DISCUSSION

Effect of the K72A Mutation on Structure and Stability of Hu Cyt_c

The native state structure of Hu Cyt_c is essentially unaffected by the K72A mutation (Figure 1). Given that Lys72 is located on the surface of Hu Cyt_c only modest effects on stability would be expected.⁵¹ We observe a small shift in the midpoint for GdnHCl unfolding, C_m , from 2.55 ± 0.01 M for WT to 2.75 ± 0.03 M for K72A Hu Cyt_c. Given that the denaturant m -value is unchanged by the K72A mutation, the result is a small increase in global stability, $\Delta G^\circ(\text{H}_2\text{O})$, of ~ 0.5 kcal/mol.

Effect of the K72A mutation on the Alkaline Transition of Hu Cyt_c

Our data show that the K72A mutation causes the pK_{app} for the alkaline transition to shift ~ 0.5 units to higher pH, consistent with a destabilization of the alkaline state relative to the native state of Hu Cyt_c. The ~ 0.5 kcal/mol stabilization of the native state of HuCyt_c by the K72A mutation suggests that stabilization of the native state by this mutation could contribute to the higher pK_{app} .

In previous work on the kinetics of the alkaline transition of WT Hu Cyt_c, a single exponential equation was used to fit the pH jump kinetic traces.⁵² In our hands, we observe significantly improved fits of the pH jump kinetic traces for the alkaline transition to a double exponential function at mildly alkaline pH and 3 and 4 exponential equations above pH 9.5 (Figure S3). The $k_{obs,1}$ and $k_{obs,2}$ phases show behavior consistent with the standard model for the alkaline transition (eq 4) for both WT and K72A Hu Cyt_c. Both these phases have similar amplitude behavior for both WT and K72A Hu Cyt_c, too. Thus, these data indicate that Lys72 is not an important ligand in the alkaline transition of Hu Cyt_c as observed in previous studies on a K72G variant of horse Cyt_c.³¹ There are two effects of the K72A mutation on the kinetics of the alkaline transition. The rate of formation of the dominant Lys-mediated alkaline conformer increases more slowly above pH 9.5 and the proline isomerization phase coupled to formation of the dominant alkaline conformer is eliminated. Given our previous observation that a proline isomerization phase is coupled to formation of a His73-heme alkaline conformer of yeast iso-1-Cyt_c,⁵³ we tentatively assign the dominant alkaline conformer (A_2 , $k_{obs,2}$) to the Lys73-mediated alkaline conformer of Hu Cyt_c.

Given that the K72A mutation increases the global stability of Hu Cyt_c (Table 2), increases the pK_{app} of the alkaline transition (Figure 4) and decreases the rate of formation of the dominant alkaline conformer ($k_{obs,2}$), the simplest explanation is that this mutation stabilizes the native state relative to the alkaline state. However, the loss of proline isomerization in the alkaline state may cause some destabilization of this state.

Effect of K72A Mutation on Peroxidase Activity of Hu Cyt_c

The primary impetus for this work was to determine if the enhancement of peroxidase activity observed for yeast iso-1-Cyt_c upon introduction of the K72A mutation also occurs with Hu Cyt_c. The pH dependence of k_{cat} for WT versus K72A Hu Cyt_c is similar to our previous observations for yeast iso-1-Cyt_c. At pH 6, k_{cat} is identical for the WT proteins and

the K72A variants of both yeast iso-1-Cytc and Hu Cytc. However, at pH 7 and above k_{cat} is significantly higher for the K72A variants of yeast and human Cytc. At pH 7, the K72A mutation leads to a 30% increase in k_{cat} for yeast iso-1-Cytc, whereas for Hu Cytc a 60% increase is observed. At pH 8, a two-fold increase in k_{cat} results from the K72A mutation for yeast iso-1-Cytc, whereas a three-fold increase is observed for Hu Cytc. Thus, Lys72 is also important in suppressing the basal peroxidase activity of Hu Cytc. However, the percent increase in activity is larger for Hu Cytc than for yeast iso-1-Cytc.

The decrease in k_{cat} as pH increases (Figure 6B) is also observed for horse Cytc^{38, 54} and has been attributed to replacement of Met80 by a lysine, which is a stronger ligand for Fe(III) than methionine. At pH 7 and 8, the alkaline conformer for both WT and K72A Hu Cytc is poorly populated (Figure 4) and thus is not a significant factor in the pH regime where the largest enhancement of peroxidase activity is observed for the K72A variant relative to WT Hu Cytc (Figure 6). Based on the horse Cytc (Figure 2A) and yeast iso-1-Cytc structure, we have suggested that Lys72 may act like a brace across Ω -loop D, stabilizing it against opening and allowing reactive oxygen species like hydrogen peroxide to bind to the heme iron.¹² The human Cytc structure indicates that the direct interaction of Lys72 with the other side of Ω -loop D may not be the primary reason that Lys72 inhibits the intrinsic peroxidase activity of Hu Cytc. In fact, the water-mediated hydrogen bond network that connects one-side of Ω -loop D with the other is similar in WT and K72A Hu Cytc (Figures 2, S1 and S2). In our structure of K72A iso-1-Cytc,¹² where Met80 ligation to the heme is replaced by a hydroxide, the smaller side chain at position 72 appears to be essential for allowing the backbone movement necessary for expulsion of Met80 from the heme crevice. Thus, the primary function of Lys72 may be to sterically inhibit this backbone motion, suggesting that any amino acid of sufficient steric size at position 72 would reduce the intrinsic peroxidase activity of Cytc. Our K72A iso-1-Cytc structure shows that the side chain of residue 81 must move toward Lys72 to allow expulsion of Met80 from the heme crevice.¹² Yeast iso-1-Cytc has alanine at this position and Hu Cytc has isoleucine at this position. The larger percent increase in k_{cat} for the K72A mutation for Hu Cytc versus yeast iso-1-Cytc may result from the greater steric congestion of isoleucine at position 81 of Hu Cytc versus alanine at this position for yeast iso-1-Cytc.

The increased peroxidase activity of K72A Hu Cytc is perhaps surprising given that the K72A mutation increases the stability of the native state relative to the alkaline state (Figure 4) and appears to slow the formation of the alkaline conformer (Figure 5A). The structural fluctuation that is enhanced by the K72A mutation and allows access of peroxide does not appear to require the full cooperative structural rearrangement of the alkaline transition. Our X-ray structure of K72A iso-1-Cytc shows that a very modest structural rearrangement is sufficient to allow loss of Met80 ligation to the heme.¹² Segments of all of the substructures of horse Cytc are known to undergo hydrogen-deuterium (HD) exchange by local structural fluctuations that are more rapid than the full cooperative unfolding of the substructure. Available data indicate that Lys72 and Ile81 are prone to non-cooperative fluctuations that allow exchange at lower free energy than the full cooperative unfolding of the Ω -loop, which positions the Met80 heme ligand.^{55, 56} Thus, it seems plausible that the K72A mutation might increase local flexibility enhancing access of peroxide to the heme and thus increasing k_{cat} . At the same time, this increase in local flexibility could entropically stabilize the native

state of the protein leading to the observed effects on both global (Figure 3) and local (Figure 4) stability.

Comparison with Peroxidase Activity in other Variants and other Species

To keep the current results in context, we note that the basal peroxidase activity of WT Hu Cyt c is about 15% less than horse Cyt c at pH 7 under the same conditions. The K72A mutation leads to a 1.6-fold increase in k_{cat} , whereas formation of a domain-swapped dimer of horse Cyt c produces a 5-fold increase in k_{cat} .³⁴ Both mammalian proteins have a k_{cat} that is 20-fold less than that of yeast iso-1-Cyt c . Thus, while Lys72 plays a role in modulating peroxidase activity in both yeast and mammalian cytochromes c , evolution of other segments of the primary structure of cytochrome c are responsible for the substantial suppression of the basal peroxidase activity of Cyt c in higher eukaryotes.

There has also been considerable interest in naturally occurring variants of cytochrome c , G41S,⁵⁷ Y48H⁵⁸ and A51V,⁵⁹ which have enhanced apoptotic activity and are implicated in thrombocytopenia. The physical and biochemical properties of the G41S, G41A and G41T variants have been extensively studied.^{25, 60–64} All three variants show increased peroxidase activity⁶¹ that correlates with the stability of the heme crevice as measured by the accessibility of the alkaline conformer and the strength of the heme-Met80 bond.⁶² However, introduction of the G41S mutation into mouse Cyt c shows that the correlation between a more accessible alkaline state and higher peroxidase activity is not absolute,⁶¹ consistent with our results for K72A Hu Cyt c . HD exchange and backbone dynamics measured by NMR show that the G41S mutation in Hu Cyt c enhances the dynamics of both the 40–57 and 70–85 Ω -loops of Hu Cyt c , suggesting that increased dynamics near the heme are primarily responsible for the enhanced peroxidase activity.⁶⁴ As noted in the studies on the peroxidase activity of G41S Cyt c , the effects of the intrinsic peroxidase activity of Cyt c when it is not bound to cardiolipin remain unclear.⁶¹ However, the much lower intrinsic peroxidase activity of Cyt c from higher eukaryotes, like horse^{12, 34} and human, compared to lower eukaryotes like yeast,¹² show that Cyt c has evolved to limit intrinsic peroxidase activity. Our work shows that Lys72 contributes to limiting the intrinsic peroxidase activity of Cyt c .

Relevance to Peroxidase Activity in the Presence of CL

Both compact and extended conformers of Cyt c form when it binds to CL, with the latter being more important at higher lipid to protein ratios.^{7, 65, 66} Both the compact and extended conformers are competent for peroxidase activity.^{7, 67} However, peroxidase activity is higher for the extended conformer, suggesting that it could be more effective at CL oxidation.⁷ Which CL-bound conformer functions *in vivo* remains a matter of debate.⁷ The peroxidase activity of the position 41 variants, which is higher in aqueous solution, is not enhanced in the presence of CL vesicles, nor is release of Cyt c from mitochondria enhanced by position 41 mutations after stimulating apoptosis.⁶¹ Mutations that effect intrinsic peroxidase activity in aqueous solution would seem more likely to affect peroxidase activity of compact conformers than extended conformers. Thus, it may be important to study the effect of lipid to protein ratio on the peroxidase activity of Cyt c variants permissive for peroxidase activity in aqueous buffer to understand whether these variants also affect peroxidation of CL.

The Many Roles of Lys72

Position 72 of Cyt c is absolutely conserved as lysine (or trimethyllysine in fungi).^{9, 31} Lys72 has been implicated as essential for interaction with redox partners⁶⁸ for being a component of site A for the interaction with CL,⁶⁹ and for the interaction of Cyt c with Apaf-1 to form the apoptosome.^{16, 70, 71} Our current work indicates that Lys72 may also be important in limiting the intrinsic peroxidase activity of Cyt c , so that this activity is more effectively turned off when Cyt c is not bound to CL. The K72A mutation increases basal peroxidase activity, so it should not inhibit release of Cyt c from mitochondria. Studies with K72A Cyt c knockin mice show that Cyt c is still released from the mitochondria after pro-apoptotic stimuli.⁷² However, as expected, the apoptosome does not form and apoptosis does not proceed. Given the involvement of Lys72 in so many of the functions of Cyt c , it is not surprising that it is evolutionally conserved.

CONCLUSIONS

Lys72 is a significant suppressor of peroxidase activity in both yeast and human cytochromes c . The percent effect of Lys72 is somewhat larger in human cytochrome c . The K72A mutation causes a small increase in global stability, thus the intrinsic peroxidase activity of cytochrome c must not be solely controlled by global stability. Structural data for horse and yeast cytochromes c suggested that Lys72 stabilizes Ω -loop D via direct interactions with the far side of Ω -loop D. The X-ray structures of WT Hu Cyt c and of the K72A variant indicate that Lys72 may not be the primary mediator of interactions between opposite sides of Ω -loop D. Instead, it appears that steric clashes between the side chains of positions 72 and 81 may be of primary importance in suppressing the Ω -loop D dynamics, which support peroxidase activity. This interpretation is supported by our crystal structure of K72A iso-1-Cyt c with Met80-heme ligation replaced by heme-hydroxide ligation which requires the side chain of position 81 to move against that of residue 72.¹² Our observation that the percent enhancement of peroxidase activity by the K72A mutation is larger for Hu Cyt c , which has isoleucine at position 81, than for yeast iso-1-Cyt c , which has alanine at position 81, supports this interpretation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Cytc	cytochrome <i>c</i>
Hu Cytc	human cytochrome <i>c</i> , iso-1-Cytc, yeast iso-1-cytochrome <i>c</i> , WT, wild type
CD	circular dichroism
GdnHCl	guanidine hydrochloride
tmK	trimethyllysine

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Figure 1. Overlay of the structures of K72A Hu Cyt c (PDB ID: 5TY3) and WT Hu Cyt c (PDB ID: 3ZCF).²⁵ The K72A variant is shown in light gray and WT Hu Cyt c is shown in dark gray. The heme-crevice loop (Ω -loop D, residues 70–85) is highlighted in salmon for the K72A variant and tan for WT Hu Cyt c . The heme and its environment, Met80, His18, and Tyr 67, are shown as stick models. The three lysine residues in Ω -loop D (Lys/Ala72, Lys73 and Lys79) are also shown as stick models.

Figure 2. Hydrogen bond network across Ω -loop D. (A) Horse Cytc (PDB ID: 1HRC) (B) WT Hu Cytc (PDB ID: 3ZCF, chain A) and (C) K72A Hu Cytc (PDB ID: 5TY3, chain A). Ω -loop D is colored salmon. Residues in the H-bond network are labeled. Waters are shown as red spheres and hydrogen bonds as yellow dashed lines. In part (C) the $2mF_o - F_c$ electron density map for Ω -loop D (light blue) and for the waters (dark blue) is contoured at 1.2σ .

Figure 3.

Denaturation curves of WT HuCyt c (black) and K72A Hu Cyt c (red) are shown as plots of corrected ellipticity at 222 nm, $\theta_{222\text{corr}}$, versus GdnHCl concentration. Solid curves are fits to eq 1 in Experimental Procedures. Parameters obtained from the fits are given in Table 2. Experiments were performed at 25 °C in CD Buffer (20 mM Tris, 40 mM NaCl, pH 7.5) at 47 μM protein concentration. Previously reported data for WT Hu Cyt c²⁰ is refit here.

Figure 4.

(A) Schematic representation of the alkaline conformational transition for Hu Cyt *c* showing all three possible alkaline conformers with lysines from Ω -loop D as alkaline state ligands. (B) Plots of $\epsilon_{695\text{corr}}$ versus pH for the alkaline transition for WT (black)²⁰ and K72A (red) Hu Cyt *c*. Data were collected at room temperature ($22 \pm 3^\circ\text{C}$) in 0.1 M NaCl solution at protein concentrations of 200 μM and 175.5 μM for the WT Hu Cyt *c* and K72A Hu Cyt *c*, respectively. Solid lines are fits to eq 2 in Experimental Procedures.

Figure 5.

(A) Rate constants as a function of pH for the alkaline conformational transition of WT (black symbols) and K72A Hu Cyt c (red symbols). Data for a fast phase, $k_{\text{obs},1}$, are shown in the inset. Data for two slower phases, $k_{\text{obs},2}$ and $k_{\text{obs},3}$, are shown as circles and triangles, respectively, in the main panel. (B) Amplitudes as a function of pH for the alkaline conformational transition of WT (black symbols) and K72A Hu Cyt c (red symbols). Data for the fast phase amplitude, A_1 , are shown in the inset. Two slower phases, A_2 and A_3 , are shown as circles and triangles, respectively, in the main panel. The solid curves are fits of the A_2 versus pH data for WT (black) and K72A (red) Hu Cyt c to the Henderson-Hasselbalch equation (eq 2 in Experimental Procedures). Rate constants and amplitudes obtained from upward pH jump experiments are shown as solid symbols and for downward pH jump experiments as open symbols. Upward jumps had an initial pH of 6.0 and downward jumps had an initial pH of near 10. Data were acquired at 25 °C. Final buffers after mixing were 10 mM with 100 mM NaCl. Final protein concentration was 10 μM . Error bars are standard deviations. Rates constants are collected in Tables S1 to S4 of the Supporting Information.

Figure 6.

Peroxidase activity of WT (black) and K72A (red) Hu Cyt c. (A) Michaelis-Menten plots for WT (solid black circles) and K72A (solid red circles) Hu Cyt c at pH 7. The solid curves are fits to eq 3 (Experimental Procedures). Data were acquired at 25 °C in 10 mM buffer and 100 mM NaCl. (B) k_{cat} versus pH for WT (white bars black outline) and K72A (red outlined bars with slashes) Hu Cyt c. Error bars are the standard deviation from three independent experiments.

Table 1

X-ray crystallography and data collection and refinement statistics.

PDB ID	5TY3
Beamline	SSRL SMB 9-2
Wavelength (Å)	1.07
Resolution range (Å)	25.00-1.25 (1.29-1.25) *
Space group	P 2 ₁
Unit cell dimensions	
a, b, c (Å)	56.8, 37.9, 60.3
α, β, γ (°)	90, 116.8, 90
Total reflections	1539904
Unique reflections	62478 (5985) *
Redundancy	6.5 (5.3) *
Completeness (%)	97.8 (93.6) *
Mean I/σ(I)	24.5 (6.1) *
Wilson B-factor	10.85
R _{sym} [†]	0.07 (0.27) *
Refinement	
R _{work} [§]	0.147 (0.194) *
R _{free} [§]	0.166 (0.201) *
Number of total atoms	2204
protein	1660
ligands	96
solvent	448
Total protein residues	210
RMS (bonds, Å)	0.017
RMS (angles, °)	1.38
Ramachandran favored (%) ^{††}	98
Rotamer outliers (%) ^{††}	0.2
Average B-factor	17.69
macromolecules	14.89
ligands	11.44
solvent	29.40

* Data for highest resolution shell are given in brackets.

[†] $R_{sym} = \sum_{hkl} \sum_i |I_i(hkl) - \bar{I}(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$ where $I_i(hkl)$ is the i th observation of the intensity of the reflection hkl .

[§] $R_{work} = \sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes for each reflection hkl . R_{free} was calculated with 5% of the diffraction data that were selected randomly and excluded from refinement.

^{††} Calculated using MolProbity.²⁸

Table 2

Thermodynamic parameters for global unfolding of WT and K72A Hu Cyt c at 25 °C and pH 7.5.

Variant	m , kcal mol ⁻¹ M ⁻¹	$G_u^{\circ'}(\text{H}_2\text{O})$, kcal mol ⁻¹	C_m , M
WT	3.71 ± 0.05	9.46 ± 0.11	2.55 ± 0.01
K72A	3.64 ± 0.15	10.01 ± 0.30	2.75 ± 0.03