Stability and apoptotic activity of recombinant human cytochrome c

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Abstract

An efficient system for producing human cytochrome c variants is important to help us understand the roles of this protein in biological processes relevant to human diseases including apoptosis and oxidative stress. Here, we describe an Escherichia coli expression system for producing recombinant human cytochrome c. We also characterize the structure, stability, and function of the protein and show its utility for studying apoptosis. Yields of greater than 8 mg of pure protein per liter culture were attained. Circular dichroism spectropolarimetry studies show that the secondary and tertiary structures of the human protein are nearly identical to those of the horse protein, but the human protein is more stable than other eukaryotic cytochromes c. Furthermore, recombinant human cytochrome c is capable of inducing caspase-3 activity in a cell-free caspase activation assay. We use data from this assay along with data from the literature to define the apaf-1 binding site on human cytochrome c.

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Cytochrome c is well known as the penultimate electron transport protein of the eukaryotic respiratory chain [1]. The protein is synthesized in the cytoplasm. Post-translational addition of its heme moiety is catalyzed by heme lyase in the inner-membrane space of the mitochondrion [2]. The heme is necessary for the protein to attain its proper three-dimensional fold [3]. In the mitochondria, cytochrome c transfers an electron from cytochrome c reductase to cytochrome c oxidase. These electron transfer reactions are mediated by a conserved cytochrome c binding epitope [4–6]. This epitope consists of surface lysine and arginine residues that are involved in electrostatic interactions with aspartic acid and glutamic acid side chains on the surface of cytochrome c’s electron transfer partners.

Recent discoveries implicate this protein in two other biological processes, apoptosis and oxidative stress [7,8]. Apoptosis, also called programmed cell death, is key to development and is linked to human diseases, including cancer and neurodegeneration [9,10]. The release of cytochrome c from mitochondria initiates an apoptotic protease cascade [11]. Specifically, it has been suggested that cytochrome c binds the WD domain of the cytosolic protein apoptotic protease activating factor-1 (apaf-1) [12]. This protein–protein interaction, along with the binding of dATP and the protease, caspase-9, results in the formation of a holoenzyme called the apoptosome [13]. The apoptosome targets and activates the protease caspase-3 which is largely responsible for cellular degradation [7,14].

Clearly, a proper understanding of how cytochrome c binds apaf-1 is important for understanding apoptosis formation. Only cytochromes c from higher-eukaryotes are capable of binding apaf-1 [15]. Specifically, horse cytochrome c, but not yeast cytochrome c, can bind apaf-1 and subsequently activate caspase-3 [16]. To date, most studies of cytochrome c binding to apaf-1 have been carried out with horse cytochrome c [16–18].
Cytochrome c has also been implicated in oxidative stress, which results from the run-away production of reactive oxygen species. The cellular damage associated with oxidative stress has been associated with several diseases, including Parkinson’s disease. Specifically, it has been shown that cytochrome c is co-localized with α-synuclein aggregates in Lewy Bodies which are the pathological hallmarks of Parkinson’s disease [8], and that cytochrome c catalyzes the H₂O₂-induced aggregation of α-synuclein [8].

A recombinant expression system for human cytochrome c would be more physiologically relevant for studying apoptosis and human disease. Until recently, there has not been a convenient source of human cytochrome c [19,20], let alone human cytochrome c variants, although the recombinant protein was expressed in low yields in yeast [21]. To overcome this limitation, we made a high-yield Escherichia coli expression system for recombinant human holocytochrome c. The system utilizes the co-expression of human cytochrome c and yeast heme lyase from a single plasmid [22]. This strategy has been used successfully to express recombinant yeast and horse cytochromes c in E. coli [22–26]. We also characterize the structure and stability of our recombinant human cytochrome c, show that the protein activates caspase-3 in a cell-free caspase activation assay, and investigate the apafl binding site on cytochrome c by using variant human cytochromes c. We envision that production of human cytochrome c variants will be important not only for understanding apoptosis, but also for elucidating the roles of this protein in cellular respiration and oxidative stress.

Materials and methods

Recombinant human cytochrome c gene. We converted the E. coli expression system for horse cytochrome c, pBTR(hCc) [24], to a human cytochrome c expression system. The changes were made by using the QuikChange Site-Directed Mutagenesis Kit (Strategene). The conversion was performed in seven steps: (F46Y, T47S, D50A), (K60G), (V11I, Q12M), (A15S), (T58I, E62D), (A83V), and (T89E, E92A) to yield the plasmid pBTR (HumanCc) as shown in Fig. 1.

![Fig. 1. Nucleotide and amino acid sequences of the recombinant human cytochrome c gene. The amino acid sequence is labeled with the standard one-letter designation above the coding strand. The nucleotide and amino acid numbers are written on the right-hand side and unique restriction sites are labeled below the DNA sequence. The differences between the recombinant horse cytochrome c gene [24] and the recombinant human cytochrome c gene are highlighted in bold.](image-url)
oligonucleotides used for mutagenesis were synthesized at the Nucleic Acid Core Facility in the UNC Lineberger Cancer Center. The nucleotide sequence of the recombinant human cytochrome \( c \) gene was confirmed by the UNC Automated Sequencing Facility. The new plasmid, pBTR (HumanCc), was transformed into \( E. coli \) strain BL21(DE3).

Lysine-to-alanine mutants were also produced with the Quik-Change Kit. The forward and reverse oligonucleotides used for producing the K13A, K27A, K39A, and K86A mutants are 

\[
GCAAAAAAGATCTTCATCATCGCGGTCCCTGCGTGGCAGCCG
\]

ACGG, 

\[
CGGTGGGCACCTGGGCCAGCCGTCATGAAGAATTTCCTG
\]

GC, and 

\[
GGCTGCGGCGCCGGCGCCGAGCCGCCGAGCCG
\]

CCGGC. The dialysate was loaded onto a FPLC S-Sepharose cation-exchange column (Amersham–Pharmacia Biotechnology) equilibrated with low salt buffer (1.76 g L\(^{-1}\) NaCl, 4.0 g L\(^{-1}\) KH\(_2\)PO\(_4\), 7.31 g L\(^{-1}\) Na\(_2\)HPO\(_4\)) at 37 °C with a final dATP concentration of 1 mM. After 30 min, the fluorescent caspase-3 substrate, acetyl-asp-glu-val-asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-APC, Biomol Research Laboratories), was added to a final concentration of 50 μM. Fluorescence was measured by using a Fluoroskan Ascent FL plate reader (Thermo LabSystems). Data were acquired every 5 min for a total of 150 min and are reported in relative fluorescence units (RFU).

Titration experiments were performed with cytochrome \( c \) concentrations from 0.1 to 50 μM. For each concentration, the initial rate of substrate cleavage was determined from the slope of the linear region of the relative-fluorescence-versus-time curve by using SigmaPlot 2000 (SPSS Science).

Circular dichroism spectropolarimetry. Studies were carried out on an Aviv Model 202-01 spectropolarimeter equipped with a thermostatted, fixed-position sample changer. Spectra were acquired in a 0.1 cm quartz cuvette at 25 °C in 50 mM sodium acetate buffer, pH 4.6, with a resolution of 1 nm and an averaging time of 3 s/point. The near-ultraviolet/Soret spectrum was collected by using a 50 μM protein solution, and the far-ultraviolet spectrum was acquired with a 15 μM solution. Circular-dichroism detected thermal-denaturation profiles were acquired in a 50 mM citric acid buffer by monitoring the ellipticity at 222 nm as a function of temperature [29]. Data were acquired with a protein concentration of 30 μM, at pH 3.0, 3.3, 3.5, 3.8, and 4.0, between 5 and 95 °C, in 1°C increments. \( T_m \), ΔH\(_m\), ΔC\( _p \), and ΔG\(_D\) were determined as described previously [30].

Results and discussion

Recombinant human cytochrome \( c \) expression system

We have produced an \( E. coli \) expression system, pBTR (HumanCc), that yields >8 mg of pure recombinant human holocytochrome \( c \) per L of culture. The expression of both the recombinant human cytochrome \( c \) (Fig. 1) and yeast hemoglobin genes in pBTR (HumanCc) is constitutive. Heme-staining [31] and protein staining are coincident on SDS–PAGE (data not shown). The coincidence proves the heme is covalently attached to the recombinant protein. Amino acid sequence analysis of the first ten residues confirms the sequence and shows that the N-terminus of the recombinant human cytochrome \( c \) is not acetylated. Two forms of recombinant human cytochrome \( c \) were detected by MALDI mass spectrometry analysis. The minor form (~10%), with a molecular weight of 12,372 ± 10 Da, is consistent with the calculated molecular weight of 12,362 Da. The vast majority of the protein (~90%) had a molecular weight of 12,236 ± 10 Da, consistent with the post-translational removal of the N-terminal methionine. The expression system described here differs from other recently described systems [19,20]. Our human cytochrome \( c \) gene contains

\[
\begin{align*}
\text{TAGCCCGGCGCCTGGCCCGTCGCGCGGCCGAACAGGC} \\
\text{AGCGCGGAC} & \quad \text{ACGG,} \\
\text{CCGGGGCTA,} & \quad \text{GGATATCTCGAGAAACGCGAAAGCTACATCCCGGGC} \\
\text{CGTGCAGG,} & \quad \text{GGAATACCTCGAGAACCCGAAAGCGTACATCCCGGGC} \\
\text{CGGCCGCGCGTTCTTCTTTTCGCGATGCCCACGAAGAT} & \quad \text{GTCCCGCGGTTCCTTCTTTCGGATGCCACAGAT} \\
\text{CATTTC,} & \quad \text{respectively.}
\end{align*}
\]
several useful unique restriction sites (Fig. 1) to facilitate cassette mutagenesis and screening for site-directed mutations. Unlike one of the systems [20], which uses the human heme lysase, our system and the other system [19] use the yeast lysase. Additionally, in our system the protein is expressed constitutively and without a fused purification tag. As described below, we have used our system to produce protein variants and assess their biological activity.

**Characterization of structure and stability**

Although the high-resolution, three-dimensional structure of human cytochrome c is unknown, the high degree of sequence identity between human cytochrome c and its structurally defined homologs [32,33] suggests that the human and horse proteins have similar folds. We used circular dichroism spectropolarimetry to characterize the secondary structure of the recombinant human protein in the far-UV region (Fig. 2A). The spectrum shows an α-helical pattern that is comparable to the spectra of other cytochromes c [24]. Specifically, we observe the minima at 208 and 222 nm that are characteristic of α-helix. We also assessed the integrity of the heme environment as a measure of tertiary structure. The near-UV/Soret spectrum (Fig. 2B) shows the characteristic negative Soret Cotton effect at ~410 nm [34]. We also observe the 695 nm maximum (ε = 9.6 × 10^4 M⁻¹ cm⁻¹) in the absorbance spectrum of the ferri form of the protein, and a strong resonance at 24 ppm in the ¹H NMR spectrum of the ferri form (data not shown). In summary, heme staining proves the heme is covalently bound, the negative Soret Cotton effect at 410 nm proves the heme environment is like that of other cytochromes c [35], and the 695 nm absorbance maximum [36] of the ferri form and the upfield shifted resonance in the ¹H NMR of the ferri form [37] prove the sulfur of methionine 80 is coordinated to the iron. These observations show that the yeast heme lyase correctly inserts the heme into recombinant human cytochrome c.

As shown in Fig. 3, we also characterized the stability of recombinant human cytochrome c by monitoring the ellipticity at 222 nm as a function of temperature [29]. The recombinant human protein was shown to denature reversibly by using previously described criteria [30]. The midpoint of thermal denaturation (Tm) and the van’t Hoff enthalpy of denaturation (∆H m) were determined by fitting the data to a two-state model [30]. The change in heat capacity upon denaturation (∆Cp) was determined by measuring ∆H m and Tm values over a range of pH values. Stability was calculated as the free energy of denaturation (∆GD) by using the integrated Gibb–Helmholtz equation [30]. Thermodynamic parameters from these experiments are summarized in Table 1. The uncertainties in ∆H m and Tm for recombinant human cytochrome c are the standard deviations from three independent experiments. The uncertainty in ∆Cp is the standard deviation of the slope obtained from linear least

![Fig. 2. Far-ultraviolet (A) and near-ultraviolet/Soret (B) circular dichroic spectra of recombinant human ferricytochrome c. The experiments were carried out at 25 °C in 50 mM sodium acetate, pH 4.6.](image)

![Fig. 3. Typical circular-dichroism detected thermal-denaturation profile of recombinant human ferricytochrome c. Experiments were carried out in 50 mM citric acid, pH 3.8, 30 μM protein. Ellipticity was monitored at 222 nm. The curve obtained by fitting the data to a two-state model [30] is also shown.](image)
Comparing the $\Delta G_D$ values shows that recombinant human ferricytochrome $c$ is 2.6 kcal mol$^{-1}$ more stable than recombinant horse ferricytochrome $c$. This observation suggests that human cytochrome $c$ may be even better than horse cytochrome $c$ as a model protein for biophysical analysis.

**Recombinant human cytochrome $c$ activates caspases in vitro**

We assessed the biological activity of recombinant human cytochrome $c$ by using a cell-free caspase activation assay that indirectly measures its ability to bind apaf-1. This binding allows assembly of the apoptosome with subsequent activation of caspase-3 [14]. Recombinant human cytochrome $c$ shows significant activity compared to the buffer control (Fig. 4). Moreover, the rate is similar to those of both authentic and recombinant horse cytochromes $c$. The absolute rates vary slightly because different cell extracts are used.

**Cytochrome binds apaf-1 at a conserved binding epitope**

Previous studies show that cytochrome $c$ interacts with its electron transfer partners at a conserved binding epitope comprising surface lysine and arginine residues [4–6]. We hypothesized that this epitope is conserved in its interaction with the non-electron transfer partner, apaf-1. Specifically, we hypothesized that lysine-to-arginine variants of human cytochrome $c$ at positions 13, 27, 72, and 86 would decrease apaf-1 binding. As a control, we also studied the K39A variant because it is not part of the electron transfer binding epitope [4,38].

Titration experiments of both wild-type and variant proteins were carried out to obtain quantitative information about binding. We used a single site binding model [39] to fit the data

$$r/P = C_s / (K_d + C_s),$$

where $r$ is the binding function, $P$ is the number of sites, $C_s$ is the concentration of free cytochrome $c$, and $K_d$ is the dissociation constant. We assumed that the concentration of bound cytochrome $c$ is small compared to the total concentration of cytochrome $c$, making $C_s$ equal to

![Fig. 4. Cell-free caspase activation assay. The relative fluorescence intensity for recombinant human (■), recombinant horse (○), and authentic horse cytochrome $c$ (△) compared to the buffer control (▼).](image)

![Fig. 5. Effect of varying cytochrome $c$ concentration on the rate of Ac-DEVD-AFC cleavage. Varying concentrations of either horse heart cytochrome $c$ (open circles) or recombinant human cytochrome $c$ (closed circles) were added to 293HEK cell extracts. Initial rates of DEVD-AFC cleavage were determined and plotted against the cytochrome $c$ concentration. Fits to Eq. (1) are shown for horse heart (dashed line) and recombinant human cytochromes $c$ (solid line).](image)
the total cytochrome c concentration. For enzyme-catalyzed reactions like the one studied here, \( r/P \) is equivalent to the initial velocity \( (V_0) \) divided by the maximum velocity \( (V_{\text{max}}) \) making Eq. (1) equivalent to the Michaelis–Menten equation \([39]\). The constant \( K_d \) should not be considered a microscopic binding constant because of the complexity of the system, but comparison of \( K_d \) values should give information about relative affinities of the variants. However, \( V_{\text{max}} \) can change from one batch of cell extracts to another. To circumvent this problem, we always run a control with a saturating amount (10 \( \mu \)M) of wild-type human cytochrome c.

The initial rate of substrate cleavage for the wild-type and variant proteins was plotted against the cytochrome c concentration and the data were fit to Eq. (1). As shown in Fig. 5, the rate of Ac-DEVD-AFC cleavage approaches a maximum at wild-type cytochrome c concentrations greater than 4 \( \mu \)M. The \( V_{\text{max}} \) for this batch of extracts was 28 \( \pm \) 7 RFU min\(^{-1}\) for recombinant horse cytochrome c and 33 \( \pm \) 7 RFU min\(^{-1}\) for recombinant human cytochrome c. The concentration of cytochrome c at half maximum velocity, \( K_d \), was 0.9 \( \pm \) 0.2 \( \mu \)M and 0.7 \( \pm \) 0.2 \( \mu \)M for recombinant horse and human cytochromes c, respectively. Based on their similar \( K_d \) and maximum velocity values, we concluded recombinant horse and human cytochromes c interact similarly with apaf-1. Our \( K_d \) values are about 1000 times greater than those obtained from studies that monitored changes in fluorescence of Zn-substituted horse cytochrome c upon binding apaf-1 \([17]\). As discussed above, it may not be useful to compare our values to those in the literature because of the complexity and indirect nature of our assay. For instance, the difference might be explained by the binding of cytochrome c to other components of the cell extract. Nevertheless, comparing our \( K_d \) value for the wild-type protein to our \( K_d \) values for variants provides information on the importance of the specific residues to apaf-1 binding because any binding to cellular components is expected to affect all the variants equally.

Relative \( V_{\text{max}} \) and \( K_d \) values for the lysine-to-alanine variants are listed in Table 2. The variants exhibit an increase in \( K_d \) compared to the wild-type protein. The increase in \( K_d \) is between 5- and 7-fold, with one exception, lysine 27. However, the relative \( V_{\text{max}} \) values are within a factor of 2 for all the variants. Taken together, the data show that lysines 13, 72, 86, and surprisingly 39 are most important for apaf-1 binding, whereas lysine 27 is less important.

We have combined the data in Table 2 with information from other studies \([16,40]\) to produce a map of the binding epitope for apaf-1 on cytochrome c (Fig. 6). The map shows that lysine residues on both the front (heme-exposed) side and the backside of cytochrome c are

![Fig. 6. The apaf-1 binding site of cytochrome c. Lysines are colored green if a residue change has a major effect on apaf-1 binding or caspase activation and blue if a change has a minor effect. The left panel shows the heme-exposed side of the protein. The heme is shown in red. The colored lysine residues are starting from the top, 72, 86, and 13 (green), and 7, 8, 25, and 27 (blue). The right panel is a 180° rotation about the vertical axis and shows the location of lysine 39. Data for lysines 13, 27, 39, and 86 are from this study (Table 2). Other data are from previous mutagenesis studies \([16,40]\). The structure of horse cytochrome c ([33], PDB entry 1HRC) was rendered with RASMOL [33,41].](image)
important for apaf-1 binding. This situation is quite different from the interaction between cytochrome \( c \) and its redox partners, which only involves the front side. This “expanded” binding site is consistent with the conclusion from other studies where activation was measured by assessing cytochrome \( c \)-induced cleavage of caspase-9 [16].

In summary, we have characterized the structure and stability of human cytochrome \( c \) and identified residues that are important for binding apaf-1 and activating caspasps. The site involves not only residues near the solvent-exposed heme edge but also residues on the backside of cytochrome \( c \) indicating a large binding surface [16].

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References


