Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 733–740
BBRC
www.elsevier.com/locate/ybbrc

Stability and apoptotic activity of recombinant human cytochrome *c*

Alina Olteanu,[a,1] Chetan N. Patel,[a,1] Matthew M. Dedmon,[a] Scott Kennedy,[a] Michael W. Linhoff,[c] Camille M. Minder,[a] Patrick R. Potts,[c] Mohanish Deshmukh,[c] and Gary J. Pielak[a,b,*]

[a] *Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, USA*
[b] *Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA*
[c] *Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599, USA*

Received 17 October 2003

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*.

Keywords: Cytochrome *c*; Apoptosis; Circular dichroism; Protein stability

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 apoptosome 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].

* Corresponding author. Fax: 1-919-966-3675.
E-mail address: gary_pielak@unc.edu (G.J. Pielak).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.182

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 H_2O_2-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 apaf-1 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 (Stratagene). 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. The

```
                          G   D   V   E   K   G   K   K   I   F       10
CGA CGC TCA GCC ATG GGC GAC GTG GAA AAA GGC AAA AAG ATC TTC      45
GCT GCG AGT CGG TAC CCG CTG CTC CTT TTT CCG TTT TTC TAG AAG
          NcoI                                          BglII

 I   M   K   C   S   Q   C   H   T   V   E   K   G   G   K       25
ATC ATG AAA TGC TCG CAG TGC CAC ACG GTG GAA AAA GGC GGC AAA      90
TAG TAC TTT ACG AGC GTC ACG GTG TGC CAC CTT TTT CCG CCG TTT

 H   K   T   G   P   N   L   H   G   L   F   G   R   K   T       40
CAC AAA ACC GGT CCC AAC CTG CAC GGC CTG TTC GGC CGC AAA ACG     135
GTG TTT TGG CCA GGG TTC GAC GTG CCG GAC AAG CCG GCG TTT TGC
         AgeI                                SfiI

 G   Q   A   P   G   Y   S   Y   T   A   A   N   K   N   K       55
GGC CAG GCG CCG GGC TAC AGC TAC ACG GCG GCG AAC AAA AAC AAA     180
CCG GTC CGC GGC CCG ATG TCG ATG TGC CGC CGC TTG TTT TTG TTT
       NarI

 G   I   I   W   G   E   D   T   L   M   E   Y   L   E   N       70
GGC ATC ATC TGG GGC GAA GAC ACG CTG ATG GAA TAC CTC GAG AAC     225
CCG TAG TAG ACC CCG CTT CTG TGC GAC TAC CTT ATG GAG CTC TTG
                                          XhoI

 P   K   K   Y   I   P   G   T   K   M   I   F   V   G   I       85
CCG AAA AAA TAC ATC CCG GGC ACG AAA ATG ATC TTC GTG GGC ATC     270
GGC TTT TTT ATG TAG GGC CCG TGC TTT TAC TAG AAG CAC CCG TAG

 K   K   K   E   E   R   A   D   L   I   A   Y   L   K   K      100
AAA AAA AAA GAA GAA CGC GCG GAC CTG ATC GCG TAC CTG AAA AAG     315
TTT TTT TTT CTT CTT GCG CGC CTG GAC TAG CGC ATG GAC TTT TTC
                                              EcoNI

 A   T   N   E STOP STOP                                      104
GCG ACG AAC GAA TGA TAA GGT ACC CCG AGG ACC G                  349
CGC TGC TTG CTT ACT ATT CCA TGG GGC TCC TGG C
                          KpnI
```

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.

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 QuikChange Kit. The forward and reverse oligonucleotides used for producing the K13A, K27A, K39A, K72A, and K86A mutants are

GCAAAAAGATCTTCATCATGCCGTGCTCCGAGTGCCAC ACGG,
CCGTGTGGCACTGCGAGCACGCCATGATGAAGATCTTT TTGC,
GGAAAAAGGCGGCAAACACGCGACCGGTCCCAACCTG CAC,
GTGCAGGTTGGGACCGGTCGCGTGTTTGCCGCCTTTTT CC,
CCTGCACGGCCTGTTCGGCCGCGCGACGGGCCAGGCG CCGGGCTA,
TAGCCCGGCGCCTGGCCCGTCGCGCGGCCGAACAGGC CGTGCAGG,
GGAATACCTCGAGAACCCGAAAGCGTACATCCCGGGC ACG,
CGTGCCCGGGATGTACGCTTTCGGGTTCTCGAGGTATT CC,
GAAAATGATCTTCGTGGGCATCGCGAAAAAAGAAGAAC GCGCGGAC, and
GTCCGCGCGTTCTTCTTTTTTCGCGATGCCCACGAAGAT CATTTTC, respectively.

Expression and purification of recombinant human cytochrome c. Twenty milliliters of overnight cultures were shaken at 37 °C in rich media (12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 8 ml L^{-1} glycerol, 2.3 g L^{-1} KH_2PO_4, and 12.5 g L^{-1} K_2HPO_4) supplemented with 100 mg L^{-1} ampicillin. A single, 20-mL culture was used to inoculate 2 L of the same media in a 6-L Erlenmeyer flask. The culture was shaken at 37 °C for 24 h, after which the cells were harvested at 4000 rpm for 20 min using a Sorvall RC-3B refrigerated centrifuge. One liter of saturated culture yields 12 g of cell paste. The purification scheme for recombinant human cytochrome *c* and its variants is similar to that for recombinant horse and yeast cytochromes *c* [24,26]. Three milliliters of lysis buffer (50 mM Tris–HCl, pH 6.8, 1 mM EDTA, pH 8.0, 3 g L^{-1} lysozyme, a few crystals of DNase I and RNase A, and 1 mM phenylmethylsulfonyl fluoride) was added per gram of cell paste and the suspension was stirred for 4 h at room temperature. The lysis solution was sonicated for 5 min and then centrifuged at 10,000 rpm for 30 min (Sorval RC-5B, S-12 rotor). $(NH_4)_2SO_4$ was slowly added to the lysate to a final concentration of 350 g L^{-1}, while stirring on ice. After a second centrifugation step (10,000 rpm for 30 min), the supernatant was dialyzed overnight in a 7000 MWCO Snake-skin membrane (Pierce) against 10 L of distilled and deionized water at 4 °C. 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} NaH_2PO_4, 7.31 g L^{-1} Na_2HPO_4) at a flow rate of 2 mL min^{-1}. Cytochrome *c* was eluted with high salt buffer (0.652 g L^{-1} NaH_2PO_4, 4.10 g L^{-1} Na_2HPO_4, and 58.4 g L^{-1} NaCl) by using a linear, 10-column volume (~200 mL) gradient from 100% low salt buffer to 100% high salt buffer. Ten milliliters of fractions were collected and analyzed by monitoring the ratio of the absorbance at 410 nm to the absorbance at 280 nm. Fractions with a ratio of >4.0 were considered pure, as supported by SDS–PAGE analysis. Cytochrome *c* was stored in low salt buffer at 20 °C. The final concentration was determined by using an extinction coefficient of 106.1 mM^{-1} cm^{-1} at 410 nm, an isosbestic point for the ferri and ferro forms [27]. Protein sequence analysis was performed on an Applied Biosystems Model 4777 Protein Sequencer in the UNC Protein Chemistry Laboratory. Authentic horse cytochrome *c* (Sigma, C-7752) was used without further purification. Mass spectrometry analysis was carried out at the UNC Proteomics Facility. Recombinant horse cytochrome *c* was expressed and purified as previously described [24].

Cell-free caspase activation assay. The ability of cytochrome *c* to bind apaf-1 and activate caspase-3 was determined by using a cell-free caspase activation assay. Briefly, a 100 μL reaction containing 100 μg of cell extract from cultured 293 human embryonic kidney cells was incubated with 10 μM cytochrome *c* in buffer A (25 mM Hepes, pH 7.5, 50 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, and 1 mM DTT) [28] at 37 °C with a final dATP concentration of 1 mM. After 30 min, the fluorogenic caspase-3 substrate, acetyl-asp-glu-val-asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC, 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 five-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 heme lyase 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

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 ($\varepsilon = 9.6 \times 10^2\,\mathrm{cm^{-1}\,M^{-1}}$) in the absorbance spectrum of the ferri form of the protein, and a strong resonance at 24 ppm in the ^{1}H 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 ^{1}H 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 (T_m) and the van't Hoff enthalpy of denaturation (ΔH_m) were determined by fitting the data to a two-state model [30]. The change in heat capacity upon denaturation (ΔC_p) was determined by measuring ΔH_m and T_m values over a range of pH values. Stability was calculated as the free energy of denaturation (ΔG_D) by using the integrated Gibb–Helmholtz equation [30]. Thermodynamic parameters from these experiments are summarized in Table 1. The uncertainties in ΔH_m and T_m for recombinant human cytochrome *c* are the standard deviations from three independent experiments. The uncertainty in ΔC_p is the standard deviation of the slope obtained from linear least

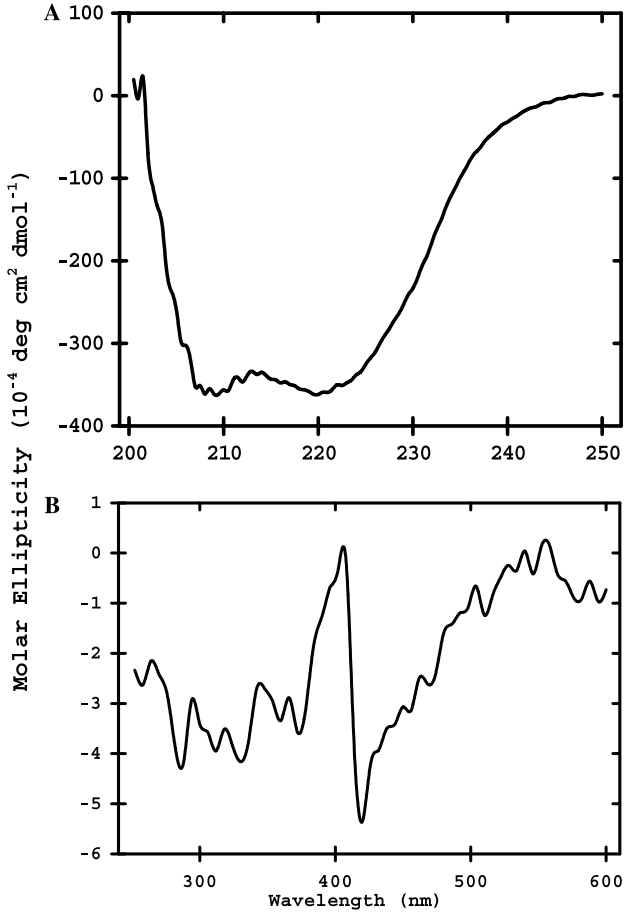

Fig. 2. Far-ultraviolet (A) and near-ultraviolet/Soret (B) circular dichroic spectra of recombinant human ferricytochrome *c*. The experiments were carried out at 25 °C in 50 mM sodium acetate, pH 4.6.

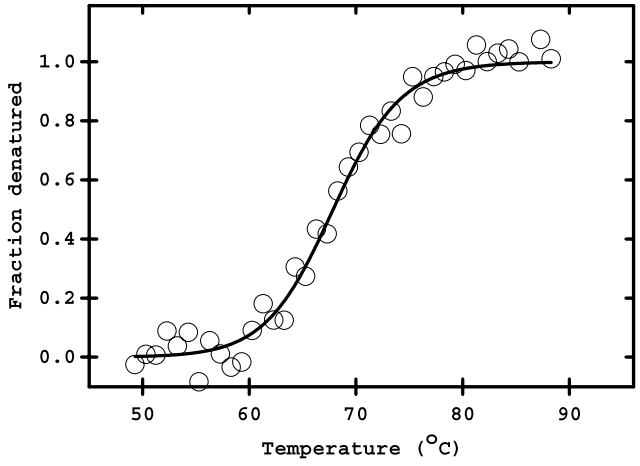

Fig. 3. Typical circular-dichroism detected thermal-denaturation profile of recombinant human ferricytochrome *c*. Experiments were carried out in 50 mM citric acid, pH 3.8, 30 μM protein. Ellipticity was monitored at 222 nm. The curve obtained by fitting the data to a two-state model [30] is also shown.

Table 1
Thermal denaturation parameters from circular dichroism studies of recombinant ferricytochrome *c*

Cytochrome *c*	T_m[a] (K)	ΔH_m[a] (kcal mol^{-1})	ΔC_p[b] (kcal mol^{-1} K^{-1})	ΔG_D[a,c] (kcal mol^{-1})
Human	344.3 ± 2.6[d]	73.9 ± 1.7[d]	1.6 ± 0.5	8.7 ± 1.7[d]
Horse[e]	336.8 ± 1.0	79.8 ± 3.7	1.1 ± 0.3	6.6 ± 0.8
Yeast[f]	315.8 ± 1.1	71.9 ± 3.9	1.4 ± 0.1	3.4 ± 0.3

[a] At pH 3.8.
[b] Uncertainty is from least squares fit of T_m versus ΔH_m.
[c] At 298 K.
[d] Uncertainties are the standard deviations calculated from the results of three independent measurements.
[e] Extrapolated from Ref. [24].
[f] Extrapolated from Ref. [30].

squares fitting. Comparing the ΔG_D values shows that recombinant human ferricytochrome *c* is ~2 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-alanine 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

$$r/P = C_s/(K_d + C_s), \quad (1)$$

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

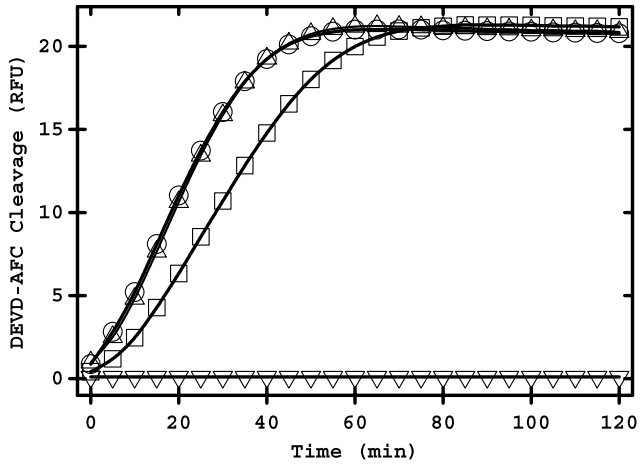

Fig. 4. Cell-free caspase activation assay. The relative fluorescence intensity for recombinant human (□), recombinant horse (○), and authentic horse cytochrome *c* (△) compared to the buffer control (▽).

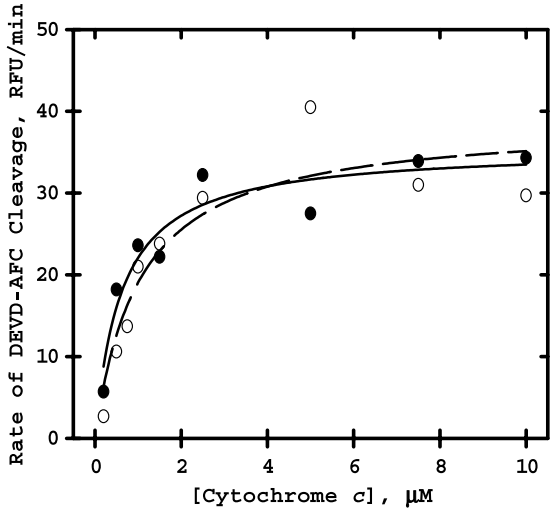

Fig. 5. Effect of varying cytochrome *c* concentration on the rate of Ac-DEVD-AFC cleavage. Varying concentrations of either horse heart cytochrome *c* (open circles) or recombinant human cytochrome *c* (closed circles) were added to 293HEK cell extracts. Initial rates of DEVD-AFC cleavage were determined 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).

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_{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_{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 μM) 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 μM. The V_{max} for this batch of extracts was 28 ± 7 RFU min^{-1} for recombinant horse cytochrome *c* and 33 ± 7 RFU min^{-1} for recombinant human cytochrome *c*. The concentration of cytochrome *c* at half maximum velocity, K_d, was 0.9 ± 0.2 μM and 0.7 ± 0.2 μM 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.

Table 2
Parameters for human cytochrome *c*-induced caspase-3 activation

Cytochrome *c*	K_d (μM)	Relative K_d[a]	Relative V_{max}[b]
Wild-type	0.7 ± 0.2[c]	–	–
K13A	3.7 ± 0.8	0.19	0.9
K27A	1.1 ± 0.3	0.64	0.9
K39A	3.9 ± 1.4	0.18	0.8
K72A	4.1 ± 0.9	0.17	0.6
K86A	4.6 ± 0.5	0.15	0.6

[a] Relative K_d is the ratio of wild-type K_d to variant K_d.
[b] Relative V_{max} is the ratio of the variant V_{max} to the velocity induced by 10 μM human cytochrome *c*.
[c] Uncertainty is the standard deviation from three independent experiments; other uncertainties are fitting errors.

Relative V_{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_{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

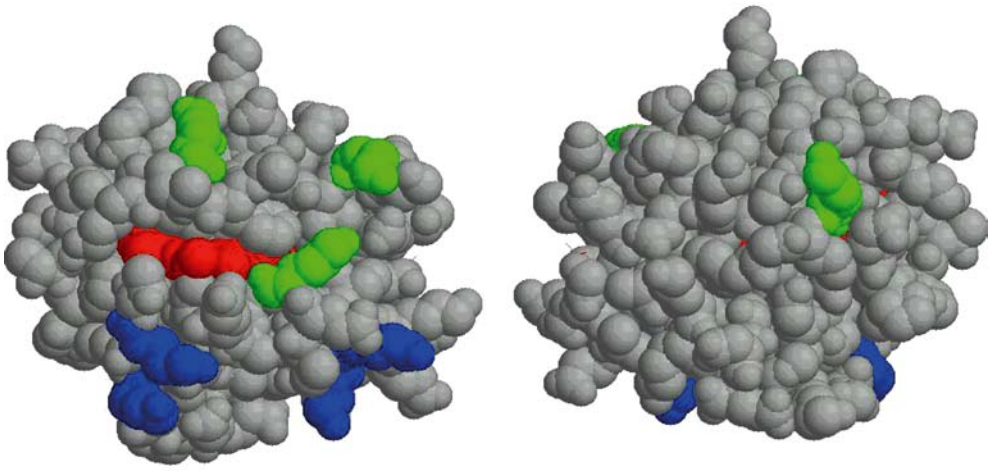

Fig. 6. The apaf-1 binding site of cytochrome *c*. Lysines are colored green if a residue change has a major effect on apaf-1 binding or caspase activation and blue if a change has a minor effect. The left panel shows the heme-exposed side of the protein. The heme is shown in red. The colored lysine 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].

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 caspases. 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].

Acknowledgments

We thank Grant Mauk for providing the original yeast iso-1-cytochrome *c* construct, the Pielak group for helpful discussions, and Julie Bryant and Gregory Young for collecting the NMR spectrum. This work was funded by the NIH (R21 ES10774) and the NSF (MCB-0109366 and 0212939).

References

[1] G.R. Moore, G.W. Pettigrew, Cytochromes *c*: Evolutionary, Structural, and Physicochemical Aspects, Springer-Verlag, Berlin, 1990.

[2] M.E. Dumont, T.S. Cardillo, M.K. Hayes, F. Sherman, Role of cytochrome *c* heme lyase in mitochondrial import and accumulation of cytochrome *c* in *Saccharomyces cerevisiae*, Mol. Cell Biol. 11 (1991) 5487–5496.

[3] M.E. Dumont, A.F. Corin, G.A. Campbell, Noncovalent binding of heme induces a compact apocytochrome *c* structure, Biochemistry 33 (1994) 7368–7378.

[4] H. Pelletier, J. Kraut, Crystal structure of a complex between electron transfer partners, cytochrome *c* peroxidase and cytochrome *c*, Science 258 (1992) 1748–1755.

[5] X. Wang, G.J. Pielak, Equilibrium thermodynamics of a physiologically relevant heme-protein complex, Biochemistry 38 (1999) 16876–16881.

[6] D.L. Brautigan, S. Ferguson-Miller, E. Margoliash, Definition of cytochrome *c* binding domains by chemical modification I. Reaction with 4-chloro-3,5-dinitrobenzoate and chromatographic separation of singly substituted derivates, J. Biol. Chem. 253 (1978) 130–139.

[7] P. Li, D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, X. Wang, Cytochrome *c* and dATP dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade, Cell 91 (1997) 627–637.

[8] M. Hashimoto, A. Takeda, L.J. Hsu, T. Takenouchi, E. Masliah, Role of cytochrome *c* as a stimulator of α-synuclein aggregation in Lewy Body disease, J. Biol. Chem. 274 (1999) 28849–28852.

[9] D.R. Green, G.I. Evan, A matter of life and death, Cancer Cell 1 (2002) 19–30.

[10] R.M. Friedlander, Apoptosis and caspases in neurodegenerative diseases, N. Engl. J. Med. 348 (2003) 1365–1375.

[11] S. Shimizu, M. Narita, Y. Tsujimoto, Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC, Nature 399 (1999) 483–487.

[12] C. Adrian, E.A. Slee, M.T. Harte, S.J. Martin, Regulation of apoptotic protease activating factor-1 oligomerization and apoptosis by the WD-40 repeat region, J. Biol. Chem. 274 (1999) 20855–20860.

[13] J. Rodriguez, Y. Lazebnik, Caspase-9 and apaf-1 form an active holoenzyme, Genes Dev. 13 (1999) 3179–3184.

[14] H. Zou, L. Yuchen, X. Liu, X. Wang, An apaf-1:cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9, J. Biol. Chem. 274 (1999) 11549–11556.

[15] R.M. Kluck, S.J. Martin, B.M. Hoffman, J.S. Zhou, D.R. Green, D.D. Newmeyer, Cytochrome *c* activation of CPP32 like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system, EMBO J. 16 (1999) 4639–4649.

[16] T. Yu, X. Wang, C. Purring, Y. Wei, G.L. McLendon, A mutational epitope for cytochrome *c* binding to the apoptosis protease activation factor-1, J. Biol. Chem. 276 (2001) 13034–13038.

[17] C. Purring, H. Zou, X. Wang, G. McLendon, Stoichiometry, free energy, and kinetic aspects of cytochrome *c*: apaf-1 binding in apoptosis, J. Am. Chem. Soc. 121 (1999) 7435–7436.

[18] C. Purring, G. McLendon, Cytochrome *c* binding to apaf-1: the effects of dATP and ionic strength, Proc. Natl. Acad. Sci. USA 97 (2000) 11928–11931.

[19] W. Jeng, C. Chen, H. Chang, W. Chuang, Expression and characterization of recombinant human cytochrome *c*, J. Bioenerg. Biomembr. 34 (2002) 423–431.

[20] A.G. Martin, H.O. Fearnhead, Apocytochrome *c* blocks caspase-9 activation and bax-induced apoptosis, J. Biol. Chem. 277 (2002) 50834–50841.

[21] Y. Tanaka, T. Ashikari, Y. Shibano, T. Amachi, H. Yoshizumi, H. Matsubara, Construction of a human cytochrome *c* gene and its functional expression in *Saccharomyces cerevisiae*, J. Biochem. (Tokyo) 103 (1988) 954–961.

[22] W.B. Pollock, F.I. Rosell, M.B. Twitchett, M.E. Dumont, A.G. Mauk, Bacterial expression of a mitochondrial cytochrome *c*. Trimethylation of Lys-72 in yeast iso-1-cytochrome *c* and the alkaline conformational transition, Biochemistry 37 (1998) 6124–6131.

[23] D.A. Dolgikh, R.F. Latypov, Z.K. Abdullaev, V. Kolov, H. Roder, M.P. Kirpichnikov, Expression of mutant horse cytochrome *c* genes in *Escherichia coli*, Bioorg. Khim. 24 (1998) 756–759.

[24] C. Patel, M. Lind, G.J. Pielak, Characterization of horse cytochrome *c* expressed in *Escherichia coli*, Protein Expr. Purif. 22 (2001) 220–224.

[25] J.N. Rumbley, L. Hoang, S.W. Englander, Recombinant equine cytochrome *c* in *Escherichia coli*: high-level expression, characterization, and folding and assembly mutants, Biochemistry 41 (2002) 13894–13901.

[26] A.S. Morar, D. Kakouras, G.B. Young, J. Boyd, G.J. Pielak, Expression of ^{15}N-labeled eukaryotic cytochrome *c* in *Escherichia coli*, J. Biol. Inorg. Chem. 4 (1999) 220–222.

[27] E. Margoliash, N. Frohwirt, E. Weiner, A study of the cytochrome *c* heamochromogen, Biochem. J. 71 (1959) 550–572.

[28] A. Saleh, S.M. Srinivasula, S. Acharya, R. Fishel, E.S. Alnemri, Cytochrome *c* and dATP-mediated oligomerization of apaf-1 is a prerequisite for procaspase-9 activation, J. Biol. Chem. 274 (1999) 17941–17945.

[29] G.J. Pielak, D.S. Auld, J.R. Beasley, S.F. Betz, D.S. Cohen, D.F. Doyle, S.A. Finger, Z.L. Fredericks, S. Hilgen-Willis, A.J. Saunders, S.K. Trojack, Protein thermal denaturation, side-chain models, and evolution: amino acid substitutions at a conserved helix–helix interface, Biochemistry 34 (1995) 3268–3276.

[30] D.S. Cohen, G.J. Pielak, The stability of yeast iso-1-cytochrome *c* as a function of pH and temperature, Protein Sci. 3 (1994) 1253–1260.

[31] P.E. Thomas, D. Ryan, W. Levin, An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl polyacrylamide gels, Anal. Biochem. 75 (1976) 168–176.

[32] L. Banci, I. Bertini, A. Rosato, G. Varani, Mitochondrial cytochromes *c*: a comparative analysis, J. Biol. Inorg. Chem. 4 (1999) 824–837.
[33] G.W. Bushnell, G.V. Louie, G.D. Brayer, High resolution three-dimensional structure of horse heart cytochrome *c*, J. Mol. Biol. 214 (1990) 585–595.
[34] Y.P. Myer, Circular dichroism studies of electron-transport components, Curr. Topics Bioenerg. 14 (1985) 149–188.
[35] G.J. Pielak, K. Oikawa, A.G. Mauk, M. Smith, C.M. Kay, Elimination of the negative Soret Cotton effect of eukaryotic cytochromes *c* by replacement of an invariant phenylalanine residue by site-directed mutagenesis, J. Am. Chem. Soc. 108 (1986) 2724–2727.
[36] H.A. Harbury, J.R. Cronin, M.W. Fanger, T.P. Hettinger, A.J. Murphy, Y.P. Myer, S.N. Vinogradov, Complex formation between methionine and a heme peptide from cytochrome *c*, Proc. Natl. Acad. Sci. USA 54 (1965) 1658–1664.
[37] G.J. Pielak, D.S. Auld, S.F. Betz, S.E. Hilgen-Willis, L.L. Garcia, Nuclear magnetic resonance studies of class I cytochromes *c*, in: R.A. Scott, A.G. Mauk (Eds.), Cytochromes *c*: A Multidisciplinary Approach, University Science Books, Sausalito, 1996, pp. 203–284.
[38] K. Wang, H. Mei, L. Geren, M.A. Miller, A. Saunders, X. Wang, J.L. Waldner, G.J. Pielak, B. Durham, F. Millett, Design of a ruthenium-cytochrome *c* derivative to measure electron transfer to the radical cation and oxyferryl heme in cytochrome *c* peroxidase, Biochemistry 35 (1996) 15107–15119.
[39] D.J. Winzor, W.H. Sawyer, Quantitative Characterization of Ligand Binding, Wiley-Liss, New York, 1995.
[40] Z.K. Abdullaev, M.E. Bodrova, B.V. Chernyak, D.A. Dolgikh, R.M. Kluck, M.O. Perezeverzev, A.S. Areeniev, R.G. Efremov, Kirpichnikon, E.N. Mokhova, D.D. Newmeyer, H. Roder, V.P. Skulachev, A cytochrome *c* mutant with high electron transfer and antioxidant activities but devoid of apoptogenic effect, Biochem. J. 362 (2002) 749–754.
[41] R. Sayle, E.J. Milner-White, Rasmol: biomolecular graphics for all, Trends Biochem. Sci. 20 (1995) 374–376.