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Oxidized or reduced cytochrome c and axial ligand variants all form the apoptosome *in vitro*

Deanna L. Mendez[†], Ildikó V. Akey[‡], Christopher W. Akey[‡], and Robert G. Kranz^{*,†}

[†]Department of Biology, Washington University in St. Louis, 1 Brookings Dr., St. Louis, MO 63130, USA

[‡]Department of Physiology and Biophysics, Boston University School of Medicine, 700 Albany Street, Boston, MA 02118, USA

Abstract

Cytochrome c (cyt *c*) has two important roles in vertebrates: mitochondrial electron transport and activating the intrinsic cell death pathway (apoptosis). To initiate cell death, cyt *c* dissociates from the inner mitochondrial membrane and migrates to the cytosol. In the cytosol, cyt *c* interacts stoichiometrically with Apaf-1, and upon ATP binding induces formation of the heptameric apoptosome. It is not clear however what the redox state of cyt *c* is when it functions as the “active signal” for apoptosis. Some reports have indicated that only ferri (ie. oxidized Fe³⁺ heme) but not ferro (reduced, Fe²⁺ heme) cyt *c* forms the apoptosome. Facilitated by our recently described recombinant system to synthesize novel human cyt *c* proteins, we use a panel of cyt *c* axial ligand variants that exhibit a broad range of redox potentials. These variants exist in different redox states. Here we show that cyt *c* WT and cyt *c* H1-M (reduced state) and cyt *c* M81A and cyt *c* M81H (oxidized state) all bind to Apaf-1 and form the apoptosome.

Graphical abstract

*Corresponding Author: kranz@wustl.edu.

Supporting Information

Methodology for protein purification and assembly assay and a figure showing the HPLC profiles of cyt *c* variants in HEPES buffer. (PDF)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Keywords

Cytochrome *c*; redox state; apoptosis

Cytochrome *c* (cyt *c*) is an alpha helical protein that folds around a covalently attached heme group. Its positive redox potential, approximately +250 mV, is necessary for its function in the mitochondrial electron transport chain. Under certain conditions (e.g. cellular stress), cytochrome *c* interacts with cardiolipin, dissociates from the transport chain at the inner membrane, and subsequently migrates to the cytoplasm to signal apoptosis (Fig. 1). In the cytosol, cyt *c* binds to apoptotic protease activating factor 1 (Apaf-1)^{1–4}. Apaf-1 exists in the cytoplasm in an autoinhibited state (Fig. 1). Upon cyt *c* and dATP binding, Apaf-1 oligomerizes to form the heptameric apoptosome which can bind to and cleave procaspase-9, triggering downstream apoptotic events. Cyt *c* binding between the two C-terminal WD domains triggers a series of conformational changes that transform Apaf-1 from a compact to an extended form that is able to undergo nucleotide exchange and assemble^{1–2}. Thus cyt *c* binding to Apaf-1 is a critical step in oligomerization to form the apoptosome and thus initiates the intrinsic cell death pathway.

The redox state of cyt *c*, ferri (Fe³⁺) or ferro (Fe²⁺), that is necessary to signal apoptosis has been a controversial topic. In 1998, Hampton et al.⁵ found that when cyt *c* was exogenously added to cytoplasmic extracts, it was rapidly reduced (within 5 min), but both the reduced and oxidized species could activate caspases. Pan et al.⁶ found that in cytoplasmic extracts from a mouse lymphoma cell line, exogenous oxidized cyt *c* activates apoptosis 7-fold more effectively than ferro cyt *c*, as assessed by DNA fragmentation. This enhanced activity of ferri cyt *c* is lost in the presence of reductants such as cyt *c* reductase, glutathione, L-cysteine, ascorbate, or N-acetyl-cysteine. The authors conclude that “downstream apoptotic processes depended on the redox state of iron in the heme group of cytochrome *c*.” In 2005 Suto et al.⁷ revisited the impact of the redox state of cyt *c* on apoptosis by analyzing caspase-3 and -9 activation in cell free extracts of HeLa cells. Ferri cyt *c* had 4- and 22-fold more potency than ferro cyt *c* in activating caspase-9 and -3 respectively. Reductants such as glutathione and cysteine inhibited ferri cyt *c* activity in a dose dependent manner. They concluded “It is thus likely that cyt *c* with oxidized haem is in a conformation capable of interaction with Apaf-1 and forming apoptosomes.” Borutaite and Brown⁸ analyzed caspase-3 activation in cell free extracts from murine macrophage J774 cells. The oxidation state of cyt *c* was monitored using UV-vis spectroscopy. In the presence of cyt *c* oxidase (COX), cyt *c* was maintained in the oxidized state, and exhibited the highest caspase-3 activity. The activity remained at baseline levels in the presence of the cyt *c* reductant N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). The authors suggested “one possibility is that the reduced form of cytochrome *c* has a lower affinity for Apaf-1 (or lower ability to activate) than the oxidized form”⁸. The Brittain group has since shown by *in vitro* reconstitution that purified ferri cyt *c*, Apaf-1, and dATP assemble the apoptosome, while ferro cyt *c*, Apaf-1, and dATP do not⁹. Implications that reduced cyt *c* (Fe²⁺) does not form the apoptosome have also surfaced in reference to clinical applications. Skemiene et al.¹⁰ tested the efficacy of anthocyanins as treatment for ischemia and ischemia reperfusion. The

anthocyanins, delphinidin-3-glucoside (Dp3G) and cyanidin-3-glucoside (Cy3G), were found to directly and rapidly (5 min) reduce cyt *c* and prevent apoptosis.

A number of theories have emerged regarding the role of cyt *c* redox properties as a modulator of the cell death signal in the cytoplasm. Brown and Borutaite¹¹ suggested three possible mechanisms to explain how ferri cyt *c* might be a more potent activator of apoptosis compared to ferro cyt *c*, (a) differential affinity for Apaf-1, (b) differential potency to catalyze the assembly of Apaf-1 (i.e. reduced cyt *c* might serve as a competitive inhibitor preventing apoptosome assembly), or (c) differential affinities for other cellular components (i.e. DNA etc) lowering its effective concentration near Apaf-1¹¹. Once cyt *c* is in the cytosol, Brittain and Hancock and colleagues have hypothesized that ferri cyt *c* is the active signal for apoptosis and reductants like glutathione or neuroglobin might serve to regulate the redox state of cyt *c*^{9, 12–15}. Indeed, models of apoptosis are now proposed whereby only oxidized cyt *c* binds to Apaf-1 to form the apoptosome^{9, 11–13, 16}.

Testing whether oxidized and reduced cyt *c* trigger apoptosome assembly can be technically challenging¹¹. Including oxidants or reductants in the cyt *c* and Apaf-1 assembly reaction might change more than just the redox state of cyt *c*, while using crude cytosolic extracts also confounds our understanding of the direct role of cyt *c*. We have recently published the biophysical characteristics of recombinant human cyt *c* variants that have different intrinsic redox potentials¹⁷. The WT and cyt *c* variants (H1-M, M81A, M81H) used here are >95% pure (Fig 2A, B), while cyt *c* M81H has a tendency to oligomerize in HEPES buffer, with some remaining dimeric upon SDS PAGE (Fig 2A, B). For each variant a single amino acid is changed which alters an axial ligand to the iron center in the heme group. Wild-type cyt *c* axial ligands are His19 (proximal) and Met 81 (distal), with a redox potential of +252 mV (all redox potentials are vs. standard hydrogen electrode). Cyt *c* H1-M has bis-Met axial ligation with a redox potential of +349 mV. Recombinant WT cyt *c* and H1-M cyt *c* are both purified in the reduced state. Changing the distal Met81 ligand to Ala (–19mV) or His(–69 mV) lowers the redox potential, and these cyt *c* variants are purified in the oxidized state from *E. coli*¹⁷.

To test the ability of cyt *c* to bind to Apaf-1, 10 Lg of recombinant human Apaf-1 was mixed with 0.4 Lg of each cyt *c* protein for a mol ratio of 1:0.5 (Apaf-1: cyt *c* variant) and incubated at 30°C for 5 min in Buffer A (10 mM HEPES/KOH pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). Control reactions were carried out with 10 Lg of Apaf-1 only, as well as reactions with commercial bovine cyt *c*, which is in the oxidized state. Reactions were electrophoresed on 4–12% native gradient gels. Fig 2C shows that bovine cyt *c* (oxidized, lane 2), human (reduced, lane 3), and human variants H1-M Met/Met (reduced, lane 4) and M81A His/OH⁹ (oxidized, lane 5) bind to Apaf-1, shifting the ladder of Apaf-1 species to higher molecular weights (compare with lane 1). Cyt *c* M81H His/His (oxidized) does not alter the Apaf-1 ladder indicating that it doesn't bind Apaf-1 at this concentration (see below). The initial results indicate that reduced and oxidized cyt *c* molecules can both bind to Apaf-1. To confirm this and address the possibility that reduced cyt *c* is a competitive inhibitor of apoptosome assembly, we added dATP to the assembly reaction. As shown in Fig 2D (lanes 2–5), the apoptosome assembles in the presence of bovine cyt *c* (oxidized), human cyt *c* variants WT (reduced), H1-M (reduced), and M81A

(oxidized). Thus, apoptosomes form and reduced cyt *c* does not inhibit apoptosome assembly. Cyt *c* M81H does not form the apoptosome under these conditions (Fig 2D, lane 6). We conclude that reduced (cyt *c* WT, cyt *c* H1-M) and oxidized (bovine cyt *c*, cyt *c* M81A) variants of cyt *c* bind to Apaf-1 and form the heptameric apoptosome.

To investigate the levels of cyt *c* variants that are capable of binding Apaf-1, a titration was performed (Fig 3). The amount of Apaf-1 was held constant at 10 Lg while cyt *c* (WT, H1-M, M81A) was diluted (0.5X, 0.2X, 0.1X, and 0.06X). All three cyt *c* proteins bind equally well to Apaf-1 which is not detectable at 0.1X or below. Cyt *c* M81H had a constant amount of Apaf-1 but was subjected to an increasing titration of cyt *c*, 0.5X, 1X, 2X, and 5X. At a fold concentration of 1X and above, cyt *c* M81H also forms the apoptosome (Fig 3). We conclude that cyt *c* M81H is required at 2–4 fold higher levels than cyt *c* WT, H1-M and M81A to bind to Apaf-1. With these titrations it is confirmed that reduced cyt *c* species (WT, H1-M) interact with Apaf-1 as well as oxidized species (M81A), and that the oxidized M81H variant also interacts with Apaf-1, although it binds more weakly than the others.

We suggest that the weaker affinity of cyt *c* M81H for Apaf-1 may be due to its propensity to aggregate in the apoptosome buffer (HEPES), as shown on SDS polyacrylamide gels with Coomassie and heme stains (Fig 2 A,B). We also note that cyt *c* M81H shows a broad peak on an HPLC sizing column in HEPES buffer (Figure S1, HPLC). At higher concentrations cyt *c* M81H is still competent for binding (Figure 3 lanes 16–18). An alternative explanation may have to do with the environment near cyt *c* residue 81. The neighboring residue Ile82 is critical for the interaction of cyt *c* with Apaf-1¹⁸. A cyt *c* I82E variant does not bind to Apaf-1 and form the apoptosome¹⁸. Since His81 is a stronger ligand to iron than Met81 or OH⁹, it is possible that His81 does not allow for optimal contacts between cyt *c* Ile82 and Apaf-1 and thus weakens the interaction, requiring a greater concentration of cyt *c* M81H to achieve the same oligomerization as found in WT.

We attempted to observe the redox states of cyt *c* variants using the characteristic heme *c* absorbance in the complexes with Apaf-1. Unfortunately WT cyt *c* in complex with Apaf-1 is not detectable due to light scattering upon assembly of the apoptosome, which was not observed with Apaf-1 alone. To ensure that the reduced species remained reduced in solution prior to binding Apaf-1, we added the reductant TMPD (Sigma-Aldrich) and ascorbate (MP Biochemicals LLC) to recycle the TMPD. Thus, TMPD reduces WT cyt *c* and cyt *c* H1-M but not cyt *c* M81A or cyt *c* M81H, as demonstrated in Fig 4A. When the cyt *c* proteins were reacted with Apaf-1 at a 0.5:1 ratio, in the presence of the reductants, cyt *c* WT, H1-M, M81A bound to Apaf-1 and formed the apoptosome (Fig 4B). Not surprisingly, at this concentration (as shown in Fig 2C and 2D), cyt *c* M81H showed no binding, since higher concentrations are required (Fig 3). Thus, cyt *c* in its reduced state can trigger Apaf-1 assembly.

Patriarca et al.¹⁶ hypothesized that oxidized cyt *c* has two states, one where Met81 ligates oxidized iron and one where it does not. They suggested that only the oxidized, ligated (Fe³⁺-Met 81) state of cyt *c* induces apoptosis, while the oxidized, unligated state does not¹⁶. We suggest that the cyt *c* M81A variant (with no distal amino acid ligand to iron) mimics the oxidized, unligated state hypothesized by Patriarca et al. Cyt *c* M81A binds to

Apaf-1 and induces apoptosome formation like WT. Thus oxidized cyt *c* does not require Met 81 ligation to signal apoptosis.

We conclude that the active form of cyt *c* binding to Apaf-1 and formation of the apoptosome in a purified system is independent of redox state. We rule out that reduced cyt *c* might be a competitive inhibitor of Apaf-1, limiting apoptosis signaling, since the reduced species also assembles the apoptosome. Each of four different axial ligand combinations were able to form the apoptosome (His/Met, Met/Met, Met/OH⁹, His/His).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
cyt <i>c</i>	cytochrome <i>c</i>
COX	cyt <i>c</i> oxidase.

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Figure 1.
Intrinsic apoptosis activation via cytochrome c.

Figure 2.

Both reduced and oxidized forms of cyt *c* bind to Apaf-1 and assemble the apoptosome. A. Coomassie stained SDS-page gel of 2.5 ug of cyt *c* samples. B. Heme stain of 2.5 ug of cyt *c* samples. Band shift native gels show the ability of cyt *c* proteins to bind to Apaf-1 (C, D). C: Lane 1 is Apaf-1 alone which self assembles to form a ladder of oligomers. Lanes 2–5 show the indicated cyt *c* proteins, from bovine (bov., lane 2) and human (lanes 3–6) when incubated with Apaf-1. The bis-Met cyt *c* H1-M (lane 4), His/Hydroxyl cyt *c* and M81A (lane 5) all bind to Apaf-1 and cause both a shift in the ladder sizes and an accumulation of Apaf-1 oligomers near the top of the gel. H/M refers to His/Met ligands of wild type; M/M is bis Met ligation; H/OH⁹ ligands and H/H bis His ligands. “red” and “ox” refer to the redox state of the cyt *c* when added to the reaction. Cyt *c* variant M81H does not bind to Apaf-1 at these concentrations. In D. dATP was included in the reaction mixtures. This promotes the assembly of the heptameric apoptosome which is composed of seven 1:1 complexes of Apaf-1 and cyt *c*. Lanes are as indicated for “C”.

Figure 3.

Titration of cyt *c* reveals that cyt *c* M81H binds to Apaf-1 approximately 2–4 fold more weakly than the other cyt *c* proteins. Cyt *c* proteins were in same redox states indicated in Figure 2. A constant amount of Apaf-1 (74 nmoles) (lanes 1 and 10) was combined with a variable amount of cyt *c* (4–330 nmoles) to determine the effectiveness of binding. Cyt *c* (lanes 2–5), cyt *c* H1-M (lanes 6–9), and cyt *c* M81A (lanes 11–14) are 0.5X, 0.2X, 0.1X, and 0.06X cyt *c* to Apaf-1 in molar ratio. Cyt *c* M81H was titrated in the reverse order to determine if it could drive apoptosome assembly, lanes 15–18 are 0.5X, 1X, 2X, 5X cyt *c* to Apaf-1 respectively.

Figure 4.

A. Reductants, TMPD and ascorbate ensure that cyt *c* and cyt *c* H1-M are in the reduced state but do not reduce cyt *c* M81A or M81H. B. Including these reductants in the native gel apoptosome assay confirms that reduced cyt *c* binds to Apaf-1. A. The UV-vis spectra is shown for the various cyt *c* molecules in their reduced (in red) and oxidized (in black) forms, as well as the proteins in the presence of TMPD (50 mM) and ascorbate (200 mM) (in blue). B. Cyt *c*'s at 0.5X with Apaf-1 on a native gel show that cyt *c*, cyt *c* H1-M and cyt *c* M81A bind to Apaf-1 and form the apoptosome. At this ratio, cyt *c* M81H does not bind to Apaf-1 as was previously observed in Fig 2.