Physiological Concentrations of K⁺ Inhibit Cytochrome c-dependent Formation of the Apoptosome*

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In many forms of apoptosis, cytochrome c released from mitochondria induces the oligomerization of Apaf-1 to form a caspase-activating apoptosome complex. Activation of caspases in vitro with dATP and cytochrome c results in the formation of an active caspase-processing ~700-kDa apoptosome complex, which predominates in apoptotic cells, and a relatively inactive ~1.4-MDa complex. We now demonstrate that assembly of the active complex is suppressed by normal intracellular concentrations of K⁺. Using a defined apoptosome reconstitution system with recombinant Apaf-1 and cytochrome c, K⁺ also inhibits caspase activation by abrogating Apaf-1 oligomerization and apoptosome assembly. Once assembled, the apoptosome is relatively insensitive to the effects of ionic strength and processes/activates effector caspases. The inhibitory effects of K⁺ on apoptosome formation are antagonized in a concentration-dependent manner by cytochrome c. These studies support the hypothesis that the normal intracellular concentrations of K⁺ act to safeguard the cell against inappropriate formation of the apoptosome complex, caused by the inadvertent release of small amounts of cytochrome c. Thus, the assembly and activation of the apoptosome complex in the cell requires the rapid and extensive release of cytochrome c to overcome the inhibitory effects of normal intracellular concentrations of K⁺.

Apoptotic cell death is characterized by morphological and biochemical changes, which are the consequence of the activation of a family of cysteine proteases known as caspases (for review, see Refs. 1 and 2). The activation of caspases is a central and key event in apoptotic cell death. During both death receptor- and nonreceptor-mediated apoptosis, large caspase-activating complexes are formed, which recruit and activate the effector caspases (3). In receptor-mediated apoptosis, such as CD95/Fas/APO-1, receptor ligation and trimerization induce the formation of a death-inducing signaling complex, located at the cell membrane, which recruits procaspase-8, via the FADD/MORT1 adaptor molecule (4). This causes a conformational change in procaspase-8, which results in caspase-8 cleavage and activation (4–7). Stress-induced apoptotic cell death is triggered by the release of mitochondrial cytochrome c (for a review, see Ref. 8), which, together with Apaf-1 and dATP/ATP, induces the processing of caspase-9 and initiates the caspase cascade. Apaf-1, the first identified mammalian homologue of CED-4, is an ~130-kDa protein that contains an N-terminal caspase recruitment domain, a CED-4 homology region, and a C-terminal domain containing multiple WD-40 repeats, which are involved in protein-protein interactions (9). Studies in lysates (10, 11) and with recombinant proteins (12, 13) have shown that in the presence of cytochrome c and dATP/ATP, Apaf-1 undergoes oligomerization to form large apoptosome complexes with molecular masses of between ~700 and 1400 kDa. The oligomerized Apaf-1 apoptosome complex recruits and activates procaspase-9, forming a holoenzyme complex (11, 14, 15), which then recruits, processes, and activates the effector caspases.

Studies in simplified reconstitution systems, using recombinant proteins (12, 13), have shown that cytochrome c and dATP or ATP are all required for the necessary conformational changes that allow Apaf-1 oligomerization and recruitment of caspase-9. The kinetics, mechanism, and regulation of apoptosome formation are still not fully understood. Initial studies suggested a requirement for dATP/ATP hydrolysis, since non-hydrolyzable analogues, such as ATPγS, were incapable of activating Apaf-1 (9, 13). However, more recent studies using highly purified Apaf-1 have shown that dATP is not hydrolyzed and that nonhydrolyzable β,γ-methylene adenosine 5’-triphosphate can support apoptosome formation (16). Cytochrome c facilitates dATP binding to Apaf-1, possibly by inducing a conformational change, which exposes dATP binding sites (16).

Initial studies showed that cytochrome c binds to Apaf-1 in a 2:1 stoichiometry with high affinity (K₁ = 10¹¹ M⁻¹) that is markedly reduced (K₂ = 4 × 10⁷ M⁻¹) in the presence of normal intracellular K⁺ concentrations [K⁺], (17, 18). These findings imply that the ionic conditions in the cell may regulate cytochrome c binding to Apaf-1, thereby modulating apoptosome formation. Interestingly, [K⁺], has been proposed to regulate the apoptotic process, since normal levels suppress caspase activation and DNA fragmentation (19). Furthermore, during apoptotic cell death [K⁺], decreases significantly, accompanied by a corresponding increase in caspase activity (19, 20), suggesting that a threshold level must be reached before caspase activation and associated events can be activated.

Since cytochrome c-dependent caspase activation is antagonized by increasing ionic strength (21), it is possible that the regulatory control point is the formation of the apoptosome complex. Previously, we had shown in THP-1 cell lysates that dATP activation produces two apoptosome complexes with approximate molecular masses of ~1.4 MDa and 700 kDa (11). The smaller ~700-kDa complex is the most active caspase-

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1 The abbreviations used are: ATPγS, adenosine 5’-O-(thiotriphosphate); CHAPS, 3-[3-cholamidopropyl]-dimethylammonio-1-propane-sulfonic acid; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; TLCK, N-tosyl-L-lysyl chloromethyl ketone; DTT, dithiothreitol; [K⁺], intracellular potassium concentration.
processing complex and predominates in apoptotic cells (11, 22). We now show that normal [K\(^+\)], strongly inhibits the formation of this apoptosome complex and that high levels of cytochrome c overcome this inhibition. We propose that the normal ionic environment of the cell protects against apoptosome formation unless a critical threshold of cytochrome c is exceeded, which then allows assembly and activation of the ~700-kDa complex. In effect, this provides a safety mechanism, which guards against inappropriate activation of the caspase cascade.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Apoptosis Assays, and Preparation of Control and Caspase-activated Cell Lysates**—Human monocytic tumor cells (THP.1) were grown in RPMI 1640 media with 10% heat-inactivated fetal bovine serum in 5% CO\(_2\) at 37 °C. Cell lysates prepared (100,000 × g supernatant) from control and apoptotic cells were prepared as described previously (10, 11). Apoptotic cell death was assessed by increased annexin V binding and flow cytometry in THP.1 cells treated for 6 h with various stimuli including etoposide (20 μM) (a DNA topoisomerase II inhibitor) or TPCK (75 μM) (a chymotrypsin-like serine protease inhibitor), TLCK (200 μM) (a trypsin-like serine protease inhibitor) plus cycloheximide (100 μM), and staurosporine (0.5 μM) (a protein kinase c inhibitor) as described previously (23, 24). Caspase activation in control lysates was induced by incubation (10 mg/ml) at 37 °C with 2 μM dATP and 2 mM MgCl\(_2\) (10). The [K\(^+\)] and [Na\(^+\)] of lysates and assay buffers were determined using an ion-selective electrode and an Abbot Aeroset Clinical Analyzer (Abbots Diagnostic Division, Maidenhead, UK).

**Fluorometric Assay of Casapse Activity—DEVDEase activity (i.e. primarily caspase-3 and -7) of lysates or column fractions was measured fluorometrically (λ\(_{\text{ex}}\)/λ\(_{\text{em}}\) = 405/510 nm) at 37 °C in 96-well plates in 200 μM assay buffer (20 μM benzoyloxycarbonyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin, 0.1% CHAPS, 10 mM DTT, 100 mM HEPES, and 10% sucrose, pH 7.0) using a Wallac Victor 1420 Multilabel counter. The 96-well plates were assayed for 10 cycles, and cleavage rates were determined by linear regression. The protease activities were expressed as either pmol/min/mg of protein or pmol/min/fraction.

**Chromatographic Methods**—Lysates were fractionated by size exclusion chromatography on Superose 6 columns using either an FPLC (HR 10/30 column) or SMART (PC 3.2/20 column) protein purification system (Amersham Pharmacia Biotech). The columns were eluted at 4 °C with 5% (v/v) sucrose, 0.1% (v/v) CHAPS, 20 mM HEPES/NaOH, 5 mM DTT, and 0.5 mM NaCl, pH 7.0. Column fractions and fraction volumes are shown in the appropriate figures. The columns were calibrated with protein standards (Amersham Pharmacia Biotech), including blue dextran, thyroglobulin, ferritin, catalase, bovine serum albumin, ovalbumin, and bovine heart cytochrome c (Sigma) as indicated in the figures.

**Assay of Apoptosome Effector Caspase-activating Activity**—The biological activity of the apoptosome complexes as assessed by their ability to process and activate effector procaspases was assayed as previously described (11). Briefly, aliquots were incubated with procaspases (25 μg of protein) for 30 min before assaying for DEVDEase activity. The procaspases (“free caspases”) were obtained following fractionation of control lysates by gel filtration on a preparative (26/60) Hi-Prep S300 chromatography column (Amersham Pharmacia Biotech). The column was eluted sequentially with buffer A (20 mM HEPES, 0.1 mM DTT, 1.5 mM MgCl\(_2\), 1 mM KCl, 1 mM phenylmethylsulfonyl fluoride, pH 8.0); A plus 1 mM NaCl and 20 mM imidazole, followed by stepwise elution with buffer A supplemented with 40 mM, 160 mM, and 500 mM imidazole. Individual fractions were analyzed for protein and Apaf-1 by SDS-polyacrylamide gel electrophoresis/Western blotting. Apaf-1-containing fractions were also assayed for their ability to activate effector procaspases by incubating with 2 μM dATP, 2 mM MgCl\(_2\), 10 μM cytochrome c, and 25 μg of procaspases. Staining with Coomassie Blue confirmed that the Apaf-1 was pure. The recombinant protein was supplemented with 20% glycerol and stored at –80 °C in 100-μl aliquots at a concentration of 80 μg/ml.

**Reagents and Western Blot Analysis**—Most reagents, including antibodies to caspase-3, -7, and -9, were obtained from the indicated sources (10, 11). Protein samples (~20 μg) were resolved on 10 or 12.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia Biotech), and antibody binding was detected as described previously (11). In addition, a monoclonal antibody specific for holocytochrome c was obtained from R & D systems (Oxford, UK). This antibody is specific for amino acid sequence 60–63, which surrounds the heme group of the human, rat, and mouse native cytochrome, and it does not cross-react with apocytochrome c.

**RESULTS**

**Normal Intracellular Concentrations of K\(^+\) Prevent Formation of a Functional Apoptosome Complex**—Caspase activation has been modeled in vitro by incubating HeLa or THP.1 cell lysates with dATP or ATP in the presence of cytochrome c (10, 25). In the present study, we have examined the effects of K\(^+\) and Na\(^+\) on dATP-dependent caspase activation. Control lysates were routinely activated with 2 μM dATP at a concentration of 10 mg/ml. Under these conditions, the Na\(^+\) and K\(^+\) concentrations of the reaction medium were 38 and 26 mM, respectively. Supplementing the lysate with increasing [Na\(^+\)] or [K\(^+\)] markedly inhibited caspase activation, and final concentrations of 80–90 mM K\(^+\) or Na\(^+\) totally blocked caspase activation (Fig. 1A), in agreement with previous studies (19, 21). This inhibition was not a direct effect on the caspases, because studies on recombinant caspase-3 and -7 have shown that their activity is not markedly affected by ionic strength (26). Also, once activated, the DEVDEase activity of dATP activated lysates was essentially unaffected by NaCl (data not shown). Thus, the inhibitory effect of K\(^+\) and Na\(^+\) on caspase activation is not on the activity of the caspases per se but rather on an upstream event, preceding caspase-3 and -7 activation.

We speculated that this inhibition was due either to an inhibition of apoptosome function (i.e. the ability to recruit, cleave, and activate caspase-9 and the effector caspases) or an inhibition of apoptosome formation. As previously reported following dATP activation of THP.1 cell lysates (11), Apaf-1 forms predominantly two large apoptosome complexes with masses of ~1.4 MDa and ~700 kDa. The latter complex is a functional apoptosome complex, since it is considerably more active at processing procaspase-9 and -3. We therefore prepared active ~700-kDa apoptosome complexes from dATP activated lysates following Superose 6 chromatography and assayed their caspase activating activity in the presence of increasing concentrations of NaCl (Fig. 1B). The caspase-activating activity of the ~700-kDa apoptosome complex was relatively insensitive to Na\(^+\) inhibition and even exhibited maximal activity at 90–100 mM NaCl (Fig. 1B), a concentration that totally inhibited dATP-dependent caspase activation in lysates (Fig. 1A). Thus, the ability of Na\(^+\)/K\(^+\) to inhibit effector caspase processing is not due to the inhibition of apoptosome function.

We then investigated the possibility that inhibition was due to disruption of Apaf-1 oligomerization by using Superose 6 gel filtration chromatography to detect apoptosome formation. In unactivated control lysates, Apaf-1 eluted as a monomeric pro-
tein (Fig. 1C, fractions 18–23), whereas after dATP treatment most of the Apaf-1 was associated with the ~1.4-MDa (Fig. 1C, fractions 5–8) and ~700-kDa complexes (Fig. 1C, fractions 8–14). In the presence of K" or Na" , the formation of the ~700-kDa complex was completely abrogated, whereas the assembly of the ~1.4-MDa complex was much less affected. We have also confirmed this effect in Jurkat cell lysates, demonstrating that this is not a cell type-specific phenomenon (27).

A significant proportion of the Apaf-1 remained as the monomeric protein (Fig. 1C, fractions 17–22) when caspase activation was inhibited by K" or Na" . This may be explained by our earlier observations of the different kinetics of formation of the two Apaf-1 complexes (11). The ~700-kDa complex is formed rapidly, exhibiting saturation kinetics within ~5–10 min, while the ~1.4-MDa complex is formed more slowly not reaching a maximum until 30 min after initiation of oligomerization. Assembly of the two apoptosome complexes appears to be mediated by two separate and independent processes, since abrogation of formation of the ~700-kDa complex did not markedly affect formation of the ~1.4-MDa complex. The slower rate of formation of the larger complex means that in the time frame used not all of the Apaf-1 was oligomerized, thereby resulting in some Apaf-1 remaining as free monomeric Apaf-1 (Fig. 1C, fractions 17–22).

Cytochrome c Reverses the Inhibitory Effects of K" by Restoring Formation of the Functional ~700-kDa Apoptosome Complex—Previously, we had demonstrated the formation of caspase-activating apoptosome complexes in apoptotic cells (11, 22). Therefore, in order for active apoptosome complexes to form intracellularly, it was apparent that some cellular factors must overcome the inhibitory effects of the [K"]. As activation of caspases requires dATP/ATP and cytochrome c, we varied these parameters to try and overcome the inhibitory effects of K". Varying the dATP/ATP (0.1–8 mM) concentration did not abrogate the inhibitory effects of K" (data not shown). THP-1 lysates usually do not need exogenous cytochrome c to undergo dATP-dependent caspase activation, presumably because the cell lysis technique releases sufficient mitochondrial cytochrome c to support Apaf-1 oligomerization (10). This conclusion was supported by the observations that the addition of exogenous cytochrome c produced only a very small increase in caspase activation (Fig. 2A) and also because significant amounts of cytochrome c were detected in control and apoptotic lysates using antibodies, which recognize either apo and holocytochrome c.

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Cytochrome c is synthesized on cytoplasmic ribosomes as the apo (non-heme-containing) form and transported into mitochondria, where heme is inserted to form holocytochrome c, which is retained in the intermembrane space (28). Since only holocytochrome c and not the apo form supports dATP-dependent activation of caspases in cell lysates (21, 29), we analyzed control lysates with a holocytochrome c-specific antibody. Little or no holocytochrome c was detected in control cell lysates (Fig. 2B, lane 2) either because the concentration of the protein was below the detection limits of the antibody or the antibody failed to recognize the holocytochrome c. To resolve this, we analyzed lysates from cells undergoing chemically induced apoptosis where cytochrome c release is an integral feature of cell death (8). All of these apoptotic stimuli induced apoptosis in THP-1 cells as previously described (24), and this was accompanied by the release of significant quantities of holocytochrome c from the mitochondria (Fig. 2B, lanes 1 and 3–5).

These results showed that the antibody was detecting the release of holocytochrome c in apoptotic cells and suggested that the concentration of holo form in the control lysates was very low. These low levels of holocytochrome c may only support caspase activation if the [K+] is also low. Higher concentrations of K+ would be predicted to antagonize cytochrome c binding to Apaf-1, thereby preventing the conformational changes necessary for formation of the apoptosome. Conversely, by increasing the concentration of cytochrome c, it should be possible to reverse the inhibitory effects of K+. Consequently, we found that varying the concentration of added cytochrome c from 0 to 32 μM significantly but not completely reversed the inhibition of caspase activation by 100 mM KCl (Fig. 3A). Cytochrome c (≥8 μM) caused a maximum reversal of inhibition, restoring caspase activation to ~70–80% of the uninhibited control reaction. Suppression of caspase activation with higher concentrations of K+ (150 mM) required even higher concentrations of cytochrome c, which partially (~50%) overcame the inhibition. To determine whether these higher concentrations of cytochrome c also restored formation of the ~700-kDa apoptosome complex, lysates were again fractionated before and after dATP activation. In control unactivated lysate, almost all of the Apaf-1 eluted as a monomer (Fig. 3B, fractions 18–22), whereas, after dATP activation, Apaf-1 oligomerized to produce the ~700-kDa apoptosome complex (Fig. 3B, fractions 8–14), which was markedly inhibited in the presence of K+. However, in the absence of cytochrome c, Apaf-1 oligomerization to form the ~700-kDa complex was largely restored (Fig. 3B, fractions 8–14, bottom panel). To confirm that the restored ~700-kDa apoptosome complex was functionally active, we analyzed the column fractions for caspase-activating activity (Fig. 3C). Column fractions from control lysates had negligible caspase-activating activity, whereas those from the dATP-activated lysate exhibited significant caspase-activating activity, which predominantly eluted with the ~700-kDa complex (Fig. 3C, fractions 5–7) in agreement with our previous studies (13). In KCl-treated lysates, none of the column fractions contained caspase-processing activity (Fig. 3C). However, the addition of cytochrome c to KCl-inhibited lysate restored caspase-activating activity, which again eluted exclusively with the ~700-kDa apoptosome complex (Fig. 3C, fractions 8–14). These results demonstrated that normal [K+] inhibited the formation of the functional ~700-kDa apoptosome and that this inhibition could be reversed with increasing concentrations of cytochrome c.

**K+ Inhibits Cytochrome c-dependent Formation of the Reconstituted Recombinant ~700-kDa Apoptosome Complex**—To determine whether KCl directly inhibited the interactions of cytochrome c and Apaf-1, we utilized a more defined system, namely recombinant Apaf-1 and cytochrome c. Recombinant Apaf-1 in the absence of dATP and cytochrome c eluted predominantly as a monomer (Fig. 4A, fractions 18–23). However, after Apaf-1 was incubated with dATP and cytochrome c, it eluted predominantly as an ~600–700-kDa complex (Fig. 4A, fractions 10–14) together with much smaller amounts of a larger sized complex (~1.4–2 MDa). The size of the recombinant apoptosome complex was smaller than the ~700-kDa complex isolated from dATP-activated lysates by Superose-6 gel filtration chromatography. Fractions were then analyzed either for Apaf-1 (Fig. 2B) or their ability to activate effector caspases (Fig. 2C) as described under “Experimental Procedures.”

![Fig. 3. Increasing concentrations of cytochrome c reverse K+ inhibition of apoptosome formation.](http://www.jbc.org/)

**A)** Formation of the recombinant active apoptosome complex was almost totally inhibited by 150 mM KCl (Fig. 4, A and C),
and the recombinant Apaf-1 inhibited by KCl eluted as a complex of about 300–500 kDa (Fig. 4A, fractions 14–19) and also as the monomeric protein (Fig. 4A, fractions 20–25). This concentration of KCl completely inhibited caspase activation, which could not be reversed even with high concentrations of cytochrome c (40 μM). Lower KCl concentrations (50 mM) also inhibited caspase-activating activity, but this was partially reversed by increased concentrations of cytochrome c (Fig. 4C).

**DISCUSSION**

Previous studies have established that the normal [K$^+$], inhibits apoptosis and cytochrome c-dependent caspase activation (19, 21). We now provide an insight into the mechanism of this inhibition. First, in cell lysates, where the concentration of holocytocrome c is low, normal [K$^+$], completely blocks formation of the ∼700-kDa apoptosome (Fig. 1C). In cell lysates, this inhibition is abrogated by increasing concentrations of cytochrome c (Fig. 2). In this respect, recent studies have shown that the release of mitochondrial cytochrome c from apoptotic cells is both rapid and complete, resulting in cytosolic concentrations of ∼10 μM (30, 31). Since the intermembrane space occupies between 1 and 3% of the total cell volume and contains 0.5–5 mM cytochrome c (32), the cytochrome c concentration of an apoptotic cell could range from 5 to 150 μM (31). Taken together, these data demonstrate that the potential cytosolic concentrations of cytochrome c, which are achievable in an apoptotic cell, are capable of overcoming the inhibitory effects of [K$^+$], thus allowing formation of the active apoptosome complex.

Our data are compatible with K$^+$ competing for the same binding sites on Apaf-1 as cytochrome c, although the kinetics of inhibition are not truly competitive, since both the $V_{\text{max}}$ and $K_m$ appear to be altered (Fig. 3A). This may in part be due to the formation of a larger complex, which comprises inappropriately oligomerized Apaf-1 (33). When measured at physiological [K$^+$], the binding affinity of cytochrome c to Apaf-1 is only $4 \times 10^7$ M$^{-1}$ (18). Thus, in the normal intracellular milieu, high concentrations of cytochrome c are required to bind to Apaf-1 and form the active apoptosome complex. Taken together, these data support the hypothesis that during apoptosis significant amounts of cytochrome c have to be released from the intermembrane space in order to overcome [K$^+$], inhibition of apoptosome formation. These data suggest that there must be a threshold level for cytochrome c that has to be exceeded before the apoptosome is formed. This would provide a mechanism to safeguard against inappropriate activation of the caspases caused by the inadvertent release of small amounts of cytochrome c. Alternatively, dATP activation of cell lysates may not fully reflect the mechanism of caspase-9 activation in cells undergoing apoptosis. Thus, Apaf-1 might oligomerize in apoptotic cells at concentrations of potassium that prevent apoptosome formation in vitro, and this may be facilitated by molecules derived from other cellular organelles, such as mitochondria, which are perturbed during the initiation of apoptosis.

Using recombinant Apaf-1, cytochrome c, and dATP, we have shown formation of a large (∼600–700-kDa) caspase-activating apoptosome complex in agreement with earlier studies (12, 13). This complex is smaller than the ∼700-kDa complex isolated from dATP-activated lysates (Fig. 1C; Ref. 11), which is to be expected given that it has been assembled in the absence of caspase-9 and other potential apoptosome-binding proteins, which may be present in the normal lysate. The apoptosome complex has been estimated to be between ∼700 and 1400 kDa (11–13), and while gel filtration chromatography is capable of separating two large complexes from monomeric Apaf-1 (see Fig. 3; Ref. 11), it does not give totally precise values for the $M_r$ of the complex. Invariably, calibration is carried out with large globular standard proteins, and exact sizing depends on the assumption that the apoptosome is also a globular protein. Gel filtration chromatography gives fairly broad peaks, and the ∼700-kDa apoptosome complex we have observed in cell lysates essentially spans a molecular mass range from around 600 to 900 kDa, with the apex of the peak eluting slightly earlier than the thyroglobulin (669 kDa) protein marker. This is ∼700 kDa, and interestingly we have found that the 20 S (∼700-kDa) proteasome, which is not globular, co-migrates with the most active apoptosome complex (data not shown).
Thus, the exact size and stoichiometry of the apoptosis complex(es) remain to be determined.

The binding site for cytochrome c on Apaf-1 has not been determined. Expression and functional analysis of Apaf-1 isoforms show that only those with an extra WD-40 repeat can bind cytochrome c, self-associate, and activate caspase-9 (34). These results suggest that the binding site on Apaf-1 for cytochrome c involves this WD-40 repeat region or at the very least is conformationally regulated by these domains. Mutational epite studies show that the cytochrome c binding site on Apaf-1 is different from that observed with various electron transfer proteins (35). In these proteins, the lysine-rich interface close to the heme pocket is the main determinant for binding, whereas cytochrome c binding to Apaf-1 not only involves this interface but also requires an opposite surface of the molecule. This suggests that Apaf-1 essentially wraps around cytochrome c, and presumably this causes major conformational changes in Apaf-1, allowing it to oligomerize in the correct manner. However, it is clear from our data that cytochrome c-dependent oligomerization of Apaf-1 is inhibited markedly by KCl and only partially reversible with excess cytochrome c. In addition, the recombinant apoptosis complex does not appear as active as a caspase-processing complex as that formed in lysates, although the Apaf-1 concentrations were approximately equivalent. This suggests that the reconstituted apoptosis system does not fully mimic apoptosis formation, which is observed in cell lysates. Taken together, this suggests that other as yet unidentified proteins, such as chaperones or heat shock proteins (36), may be required for folding/oligomerization of the complex for optimal activity.

In conclusion, our data suggest that the [K+]<sup>+</sup> acts to safeguard the cell against inappropriate caspase activation by the accidental release of small quantities of cytochrome c. The [K+]<sup>+</sup> antagonizes cytochrome c binding to Apaf-1, and it is only when cytochrome c reaches a critical threshold level that this inhibition is overcome and Apaf-1 oligomerization initiated. During apoptotic cell death, the [K+]<sup>+</sup> decreases to around 70 mM (19), when lower levels of cytochrome c would initiate apoptosis formation. This suggests that controlling the concentration of [K+] is an important regulatory mechanism for ensuring the appropriate activation of the apoptosis complex. This may act in concert with additional cellular defense mechanisms such as the X-linked inhibitor of apoptosis protein (15) or heat shock proteins, which limit the activation of caspases once the apoptosis is formed (36–38).

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