Stoichiometry, Free Energy, and Kinetic Aspects of Cytochrome *c*: Apaf-1 Binding in Apoptosis

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A few years ago, what was called a "bizarre and unexpected"[1] discovery was made that cytochrome *c*, the paradigm for a small redox protein, also plays a major role in apoptosis. It has been shown that cytochrome *c* (Cc) along with ATP or dATP are necessary cofactors with apoptosis protease activating factor-1 (Apaf-1) for the activation of caspase-9.[2−4] Caspases, a family of genetically conserved cysteine proteases, are key mediators of the execution phase of apoptosis.[5] It has been indirectly inferred that cytochrome *c* forms a complex with Apaf-1, but until now the direct binding of cytochrome *c* has not been demonstrated, and neither the binding constant nor even the stoichiometry of binding were known.[4,6]

The proposed mechanism for caspase-9 activation is that cytochrome *c*, upon its release from the mitochondria, binds to Apaf-1. When Apaf-1 binds to cytochrome *c*, the Apaf-1 caspase recruitment domain (CARD) is exposed, which is able to bind and activate caspase-9.[3] We have taken the first step in characterizing this mechanism by showing that cytochrome *c* does, in fact, bind strongly to Apaf-1, apparently with a 2:1 Cc:Apaf-1 stoichiometry. Using fluorescence polarization methods we have measured the binding constant for Apaf-1 and horse heart ZnCc, a fluorescent derivative of Cc in which the heme iron is replaced by zinc. This species is known to be structurally equivalent to wild-type FeCc in binding to physiological protein partners of Cc.[7]

Cytochrome *c* is a small (∼12 kDa) protein. Consequently, its rotational correlation time is relatively fast on the time scale of fluorescence. Therefore, when its fluorescent derivative, ZnCc, is excited with a polarized light source, most of the molecules will rotate before fluorescence occurs and the measured polarization will be small.[8] Apaf-1, on the other hand, is much larger (∼130 kDa). Thus, when ZnCc is bound to Apaf-1, the complex rotates more slowly than does ZnCc alone, leading to a higher measured polarization ratio.

In our experiments, we performed fluorescence polarization titrations in which Apaf-1 was added to nanomolar concentrations of ZnCc and the change in polarization was monitored. Cc and Apaf-1 concentrations were measured by UV/visible absorbance. The extinction coefficient for ZnCc is $\epsilon_{585} = 7.9 \times 10^3$ M^{-1} cm^{-1}.[11] The extinction coefficient for Apaf-1 was calculated to be $\epsilon_{280} = 1.85 \times 10^5$ M^{-1} cm^{-1}.[12] The data are best fit to a stoichiometric binding model, i.e., assuming all of the Cc is bound at the concentrations used (Figure 1). This plot indicates a stoichiometry of 2:1 Cc:Apaf-1 and that the binding constant is much larger than 10^8 M^{-1}. However, scatter in the data makes it difficult to rule out 1:1 binding with lower affinity (∼10^8 M^{-1}). Kinetic data, however, suggest that the strong binding model is correct (Figure 2).

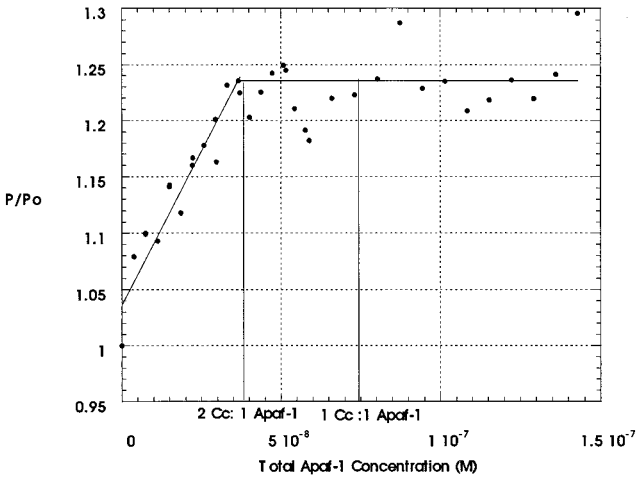

Figure 1. Binding curve for ZnCc with Apaf-1. ZnCc concentration is 75 nM, and Apaf-1 was titrated in to a final concentration of ∼150 nM. Fluorescence conditions: excitation = 550 nm, emission = 587 nm, polarization *G* value = 0.53. The sample was in 2 mL Buffer A[9] at 30 °C.[10] Free Apaf-1 concentration of 3.75×10^{-8} M corresponds to 2:1 binding, and 7.50×10^{-8} M corresponds to 1:1 binding.

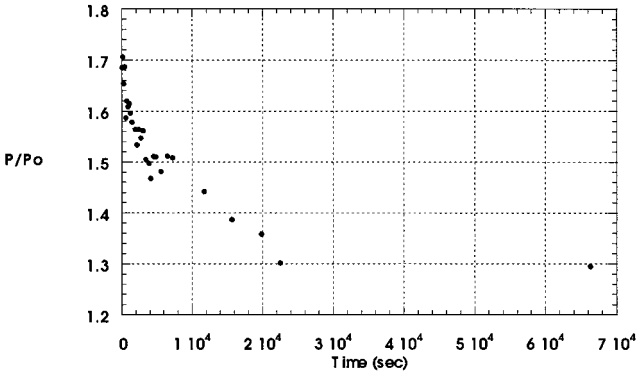

Figure 2. Change in P/P_o versus time after a 1000-fold excess of FeCc was added to ZnCc bound to Apaf-1.

After the fluorescence polarization titration was complete, a large excess (∼1000-fold) of horse heart FeCc was added to compete with ZnCc for Apaf-1 binding. The FeCc displaced the bound ZnCc and a decrease in polarization was observed. However, the rate of dissociation of ZnCc from Apaf-1 is quite slow. The polarization ratio, P/P_o, where P_o is the initial ZnCc polarization and P is the polarization at each point, decreased for a time period of several hours as the ZnCc dissociated and was replaced by FeCc. From these data, we estimate the half-life

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(9) Buffer A is 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, at pH 7.4
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for the competition reaction as approximately 1.5 h, and the dissociation rate constant, k_{off}, as approximately 10^{-4} s^{-1}. Furthermore, we observe from the polarization titrations that binding is complete within seconds, so that $k_{on} \geq 10^7$ M^{-1} s^{-1}. Stopped-flow experiments are underway to better refine this value. Since $K = k_{on}/k_{off}$, $K \geq 10^7$ M^{-1} $s^{-1}/10^{-4}$ $s^{-1} = 10^{11}$ M^{-1}. These kinetic results preclude data modeling for a 1:1 Cc:Apaf-1 stoichiometry, which requires weaker ($K \sim 10^8$ M^{-1}) binding. We conclude from all the data that Apaf-1 binds two Cc molecules with high affinity ($K \geq 10^{11}$ M^{-1}). Such strong binding is unusual. Known binding constants for Cc with its mitochondrial binding partners are substantially weaker. For example, the binding constants for Cc binding with both cytochrome *c* peroxidase and cytochrome b_5 are on the order of 10^7 M^{-1}.[13]

A 2:1 Cc:Apaf-1 stoichiometry has interesting structural implications. Cc is believed to bind to the WD-40[12] domain of Apaf-1.[4] This domain consists of 14 WD-40 repeats.[4] The sequence of this region suggests that two 7-strand domains may exist, and each may be capable of independent Cc binding.[4] The possibility of cooperative Cc binding has potentially interesting implications for signaling, and is currently under investigation.

The observation that binding is as strong as 10^{11} M^{-1} has interesting implications for the cellular control of apoptosis. If the cytosolic volume of a typical human cell is taken to be approximately 10^{-11} L,[14] then the amount of cytochrome *c* that must be released from the mitochondria for Apaf-1 binding to take place involves only individual molecules. If so, then Cc:Apaf-1 binding is less well described as an equilibrium phenomenon, but rather as a cellular signaling event. In essence every Cc molecule released from the mitochondria could bind to Apaf-1 and presumably trigger caspase-9 activation. The detailed dependence of K_B on factors such as pH, ionic strength, and ATP concentration may serve to further modulate this response.

The quantitative determination of a Cc:Apaf-1 binding constant paves the way for future experiments to map out the binding interaction between these two proteins using a library of cytochrome *c* point mutants, which will be reported in due course.

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