with cytochrome c. However, the concentration of cytochrome c in the solution (>1.5 μM) needed to achieve a complete titration titrate was found to quench the intrinsic fluorescence of Bcl-xL. The quenching of Bcl-xL fluorescence due to cytochrome c in the bulk solution obscures the actual change in fluorescence due to the interaction of the two proteins. A control experiment where a lysozyme solution was titrated with cytochrome c showed exactly the same behavior, suggesting that fluorescence is not preferred to probe the Bcl-xL-cytochrome c interaction.

Next, optical absorption of a fixed concentration of cytochrome c in the Soret heme region was measured as a function of variable concentration of Bcl-xL. Figure 2a shows that the absorbance due to cytochrome c increases with increments of Bcl-xL; for the 3 μM solution of cytochrome c, the change in absorbance across the complete titration is 33 milliOD. The difference spectra generated by subtracting the spectrum of cytochrome c alone from spectra in the presence of Bcl-xL (Figure 2a, inset) show that Bcl-xL binding causes the absorption peak to shift from 415 to 413.2 nm. These observations suggested that heme optical absorption of cytochrome c could be used as a reliable marker to follow its interaction with Bcl-xL.

Figure 2b shows the plot used for binding analysis as described under Materials and Methods. The x-intercept of the linear fit of the data yields the association constant, $K_{\text{ass}}=8.4(\pm4)\times10^6$ M$^{-1}$, indicating tight affinity of cytochrome c for Bcl-xL. The slope of the plot (n=0.6) indicates a 1:1 interaction. The Gibbs free energy for Bcl-xL-cytochrome c interaction ($\Delta G^o=-RT \ln K_{\text{ass}}$) calculated by using this value of $K_{\text{ass}}$ is ~9.3 kcal mol$^{-1}$ at 22°C.
Cytochrome c is lysine-rich, and hence highly basic. It is therefore desirable to address as to how its interaction with Bcl-xL varies with ionic strength and pH. Fig. 2c shows that $K_{ass}$ is maximum at ~80 mM NaCl, and declines by nearly 12-fold in the presence of 600 mM NaCl, suggesting that the physiological ionic strength supports the tightest interaction between the two proteins.

**Figure 2.** (a) Absorption spectra of cytochrome c in the presence of different concentrations of Bcl-xL, pH 7, 22°C, 3.5 mM DTT, 100 mM NaCl. The inset shows difference absorption spectra obtained by subtracting the spectrum of cytochrome c alone from the spectra of cytochrome c in the presence of different concentrations of Bcl-xL. (b) The final plot for analysis of binding data, as described under Materials and Methods (eq 1 and 2). The equilibrium association constant, $K_{ass}$ (8.4(±4) μM⁻¹), is calculated from the x-intercept of the straight line (pK_{ass}). The slope of the line (n=0.6) indicates a 1:1 binding interaction. (c) The variation of $K_{ass}$ as a function of NaCl concentration in 50 mM phosphate buffer, pH 7, 4 mM DTT, 22°C. Mixtures of Bcl-xL and cytochrome c were incubated for 2-8 hours. Optical absorption spectra were analyzed as described under Methods.
proteins. The dependence of $K_{ass}$ on pH could not be studied in detail due to alkaline isomerization of cytochrome $c$ in basic medium (pH >8), and heme spin change in acid solutions (pH <5). In the accessible range of pH, $K_{ass}$ was found to change little.

The kinetics of protein-protein interactions are of special significance since rates of such interactions often play important regulatory roles. To determine the rate of binding of Bcl-xL to cytochrome $c$, we performed stopped-flow experiments where the time dependence of the change in 415-nm absorbance due to cytochrome $c$ was monitored after mixing the two protein solutions. The representative traces labeled 1, 2, and 3 in Fig. 3a show the time dependence of the change in absorbance after mixing 3 μM cytochrome $c$ with 6, 4, and 2 μM Bcl-xL, respectively. The solid lines through the data are single-exponential fits, showing clearly that the rate of interaction changes with the concentration of Bcl-xL. The dependence of the observed rate on the concentration of Bcl-xL is shown in Fig. 3b, the slope of the linear fit of which yields the bimolecular association rate constant, $k_{bi}=0.24 \times 10^6 \text{M}^{-1} \text{s}^{-1}$.

Incorporation of a control experiment is necessary to show that cytochrome $c$ does not interact with other proteins indiscriminately. As a negative control, the interaction of Bcl-xL with myoglobin (the latter was chosen because it resembles cytochrome $c$ in containing a heme group and a fairly sizable number of positively charged amino acids) was checked, but no binding was detected. As a positive control, the interaction of cytochrome $c$ with Bad BH3 was looked into. Since the BH3 domain is involved in a number of interactions amongst the Bcl-2 family proteins and Bcl-xL is found to interact with cytochrome $c$; one naively expects binding of the BH3 peptide to cytochrome $c$. The optical absorbance of cytochrome $c$ ($\lambda_{max} \approx 415 \text{ nm}$) was used to assess this interaction. Advantage of the fluorescence emission of the fluorescein-labeled Bad BH3 peptide ($\lambda_{max} \approx 496 \text{ nm}$) was also taken to monitor the titration of 475 nM cytochrome $c$ with increments of the peptide up to 7
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μM. In this range of concentration of the peptide, no inner filter effect in the fluorescence of the peptide was detected. The analysis of the titration monitored by

**Figure 3.** Kinetics of binding of Bcl-x<sub>L</sub> to cytochrome c in 50 mM phosphate buffer, pH 7, 4 mM DTT, 22°C. The concentration of cytochrome c was held constant at 3 μM. (a) Representative kinetic traces: labels 1 to 3 correspond to 6, 4, and 2 μM Bcl-x<sub>L</sub>, respectively. The solid lines through the data represent single-exponential fits. (b) The rate obtained is proportional to the concentration of Bcl-x<sub>L</sub>. The slope of the linear fit through the data yields the bimolecular rate constant for binding, $k_{bi}=0.24 \, \mu M^{-1} s^{-1}$. The error bars represent standard deviations of the rate constants measured with different preparations of Bcl-x<sub>L</sub>. 
the absorbance of cytochrome c (equations 1,2) yields $K_{ass}=4.1(\pm 1)\times 10^6 \text{ M}^{-1}$, indicating rather tight affinity of the BH3 peptide for cytochrome c (Figure 4a), and the slope of 0.91 suggests 1:1 interaction. Values of $K_{ass}$ and $n$ extracted from the peptide fluorescence-monitored data (equation 3 and Figure 4b) are $1.2(\pm 0.2)\times 10^6 \text{ M}^{-1}$ and $1.3(\pm 0.1)$, respectively, fairly consistent with the values obtained from the optical absorbance data.

In another control experiment, the equilibrium binding of Bid with cytochrome c was studied. The working rationale was the same as mentioned above in the context of interaction of Bad BH3 peptide with cytochrome c. Since the Bid protein binds both pro- and antiapoptotic proteins,

Figure 4. (a) Analysis of horse cytochrome c and Bad BH3 peptide binding (equation 1,2) monitored by the optical absorbance of cytochrome c, pH 7, 22°C, 3.5 mM DTT, 100 mM NaCl. Values of $K_{ass}$ and $n$ are $4.1(\pm 1) \mu\text{M}^{-1}$ and 0.91, respectively. (b) The same binding monitored by fluorescence of the fluorescein-labeled Bad BH3 peptide (equation 3). Values of $K_{ass}$ and $n$ extracted from this analysis are $1.2(\pm 0.2) \mu\text{M}^{-1}$ and 1.3, respectively.
including Bcl-x<sub>L</sub>, one expects its interaction with cytochrome <i>c</i> as well. Indeed, by titrating 300 nM cytochrome <i>c</i> with increments of Bid, we obtained <i>K</i><sub>ass</sub>=4.05(±2.25)×10<sup>6</sup> M<sup>-1</sup> for this interaction (Table 1). These results suggest that in addition to Bcl-x<sub>L</sub> certain pro- and antiapoptotic Bcl-2 proteins may bind to the cytosolic pool of cytochrome <i>c</i> in apoptosed cells.

4.5 Discussion

4.5.1 Binding of Bcl-x<sub>L</sub> to cytochrome <i>c</i>

The major finding of this study is a tight 1:1 binding of Bcl-x<sub>L</sub> and cytochrome <i>c</i>, and it is of interest as to how this affinity compares with those for known protein-protein or protein-peptide interactions involved in survival and death. The apparent binding affinities or dissociation constants (<i>K</i><sub>diss</sub>=1/<i>K</i><sub>ass</sub>) for the interaction of Bcl-x<sub>L</sub> with other members of the Bcl-2 family have been reported in the 2-6 μM range at pH 7 [22]. In more recent studies, the apparent <i>K</i><sub>diss</sub> values for the interactions of BH3 peptides from pro-apoptotic Bcl-2 family proteins with Bcl-x<sub>L</sub> ΔCx (truncated at the C-tail by <i>x</i> residues) have been found to fall in the 1-90 nM range [23], suggesting tighter binding, while the affinity of BH3 peptides from anti-apoptotic Bcl-2 family proteins with other survival or killer proteins is relatively low, into micromolar range [24]. Thus the binding of Bcl-x<sub>L</sub> with cytochrome <i>c</i> (<i>K</i><sub>diss</sub>~120 nM at pH 7) is indeed tight, and is fairly comparable to its affinity for BH3 domains from the anti-survival proteins of the Bcl-2 family. Even the bimolecular/second order rate constants for the binding of Bcl-x<sub>L</sub> to cytochrome <i>c</i> (<i>k</i><sub>bi</sub>=0.24 μM<sup>-1</sup> s<sup>-1</sup>, Figure 3b) and Bcl-x<sub>L</sub> to other Bcl-2 family proteins (<i>k</i><sub>bi</sub> in the 0.27-0.34 μM<sup>-1</sup> s<sup>-1</sup> range [22]) are nearly identical, implying that these interprotein interactions closely match in terms of binding free energy and the activation energy barrier.
4.5.2 Cytochrome c-Bad BH3 and cytochrome c-Bid interactions

Another interesting finding is individual binding interactions of cytochrome c with Bad BH3 and Bcl-xL. The idea that such interactions could exist emanated as a corollary of the BH3-mediated interprotein interactions amongst the Bcl-2 family of proteins, and the high-affinity binding of Bcl-xL with cytochrome c observed in this study. The binding affinity \( (K_{diss}=1/K_{ass}) \) of Bad BH3 for cytochrome c, averaged from optical absorption and fluorescence data (Figure 4), is 538(±295) nm, which is numerically significantly higher than the value of 21.5 nm reported for the Bad BH3–Bcl-xL interaction [25, 26]. The numerical value for the binding affinity of Bid for cytochrome c (373(±126) nM), on the other hand, is rather smaller than the reported value of 1.9 μM for Bcl-xL-Bid interaction at neutral pH [22]. This suggests that while the affinity of Bad BH3 for cytochrome c is considerably lower, the affinity of Bid for cytochrome c is higher, both

Table 1. \( K_{ass} \) values for interactions amongst horse cytochrome c, human His6-tagged Bcl-xL, and Bad BH3 peptide

<table>
<thead>
<tr>
<th>Binding interactions</th>
<th>( K_{ass} ) (μM(^{-1}))</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-xL–cytochrome c</td>
<td>8.4 (±4)</td>
<td>this work</td>
</tr>
<tr>
<td>Bcl-xL–Bad BH3</td>
<td>46.5</td>
<td>31</td>
</tr>
<tr>
<td>Bad BH3–cytochrome c</td>
<td>2.65 (±1.45)</td>
<td>this work</td>
</tr>
</tbody>
</table>

compared with the affinity of cytochrome c for Bcl-xL (Table 1). Nonetheless, it does appear that the cytosolic pool of cytochrome c is capable of interacting with both pro- and antiapoptotic proteins with varying degrees of affinity.
**Bcl-x<sub>L</sub>-Cyt c Interaction**

**4.5.3 Possible structural factors for Bcl-x<sub>L</sub> and cytochrome c interaction**

From parametric similarity of interactions between Bcl-x<sub>L</sub> and cytochrome c, and Bcl-x<sub>L</sub> and other Bcl-2 family proteins, one may naively assume that Bcl-x<sub>L</sub> deploys the same surface to bind cytochrome c as it does for binding with other Bcl-2 family members or the BH3 sequences derived from them. The availability of NMR and X-ray structures of Bcl-x<sub>L</sub> [27, 28] augments deduction of the structural basis of these interprotein interactions. Muchmore et al. have proposed that an elongated hydrophobic cleft, the constituent residues of which are highly conserved in the Bcl-2 family of proteins, is the site of interaction of Bcl-x<sub>L</sub> with death-promoting proteins [28]. Structures of BH3 peptide-bound Bcl-x<sub>L</sub> show that the alignment of the BH3 helix along the hydrophobic cleft of Bcl-x<sub>L</sub> is stabilized by apolar interactions at the base and polar contacts along the sides of the cleft [29]. Cytochrome c is a highly charged protein with 19 lysine residues most of which are surface exposed. It is likely that the polar residues along the side of the same Bcl-x<sub>L</sub> cleft supports charged interactions with cytochrome c. The decrease of the binding affinity for the Bcl-x<sub>L</sub>-cytochrome c interaction at high NaCl concentration (Figure 2c) lends support to this view. Strategic high-resolution surface mapping experiments are necessary to test this conjecture.

**4.5.4 How relevant is the Bcl-x<sub>L</sub>-cytochrome c interaction for upstream regulation of apoptosis?**

From the results of this study together with earlier immunochemical results demonstrating that Bcl-x<sub>L</sub> interacts with cytochrome c as a part of the cellular response to ionizing radiation and other genotoxic agents [21], it might seem that this interaction is operative *in vivo* as well. If the interaction indeed exists under cellular conditions, then a regulatory role can be established on the basis of the current understanding of upstream interprotein interactions involving cytochrome c. Once translocated to cytosol in response
to an apoptotic stimulus [1, 10, 15], cytochrome \(c\) binds Apaf-1 in the presence of dATP or dADP [10]. The binary complex in turn interacts with procaspase-9, and subsequently cleaves the CED-3-like prodomain of the zymogen form of caspase-9 [30]. The ternary cyt-Apaf-1-caspase-9 complex recruits procaspase-3 and possibly procaspase-7 in quick succession to form a functional apoptosome [2]. Thus, the initial ternary complex formed of cytochrome \(c\), Apaf-1, and caspase-9 (the cyt-Apaf-1-caspase-9 complex) appears to be the hallmark of the initiation of the death cascade [31]. The quantitative and conclusive \textit{in vitro} data presented here leads one to wonder if Bcl-x\(_L\) competes with Apaf-1 in order to block the formation of the initial Apaf-1-cytochrome \(c\) complex, thereby inhibiting caspase activation.

Unfortunately, there is no conclusive evidence for this regulatory response \textit{in vivo}. If a regulatory role of Bcl-x\(_L\)-cytochrome \(c\) interaction is granted, the effectiveness of the regulation must be considered in the light of possible affinities of cytochrome \(c\) for other Bcl-2 family proteins. For example, some data here indicate variable affinity interactions of cytochrome \(c\) with Bid and with Bad BH3. In this perspective, the extent to which Bcl-x\(_L\) can arrest cytochrome \(c\) to exert a regulatory role would depend on the balance of thermodynamics of various interactions involving the pro- and antisurvival proteins, provided these interactions operate \textit{in vivo}. Clearly, more evidence must be obtained that the Bcl-x\(_L\)-cytochrome \(c\) interaction is indeed relevant for regulation of apoptosis \textit{in vivo}. 

Chapter 5
Bcl-xL-Cyt c Interaction

4.6 References


Bcl-xL-Cyt c Interaction


CHAPTER 5

Amyloid Fibrillation of Human Apaf-1 CARD

5.1 Abstract

The idea of establishing amyloid-like fibrillation tendency of pro- and anti-survival proteins of human apoptotic pathways is relevant for delineating the conditions that lead to aberrant differentiation, development, and tissue homeostasis. As the first step in this direction, we report here that the Caspase Recruitment Domain (CARD) of recombinant human Apoptotic Protease Activating Factor-1 (Apaf-1) can be induced to undergo amyloid-like fibrillation. The study was initiated with a set of biophysical investigations that served as the explorer of the possibility and in vitro conditions for fibril growth. By scanning the pH-induced conformational transitions, protein stability, and stopped-flow folding-unfolding kinetics, we have detected a molten globule (MG) transition of CARD at pH < 4. In a bid to reduce the surface-accessible hydrophobic patches in the MG state, the CARD monomer undergoes self-association to produce soluble oligomers that serve as precursor aggregates (PA) for protofibril formation. The monomer-to-oligomer self-association process is akin to the well known homophilic CARD/CARD interaction by which CARD of the same or different apoptotic proteins associate in order to transduce and regulate the apoptotic signal. The fibrillation reaction of Apaf-1 CARD was carried out at pH 2.1 and 60°C, because reduction of exposed hydrophobic surfaces in the MG state is more favored at the moderated solution condition. The Gaussian distributions of diameters of fibril population suggest values of 2.1 and 2.7 nm for the mean diameter of PA and protofibrils or elongated fibrils, respectively. We discuss the consequences of fibrillation supposing a finite probability of the process occurring under cellular conditions.
5.2 Introduction

The supramolecular assembly of a sizable number of diverse proteins and polypeptides into amyloid and amyloid-like fibrils has attracted attention of many for a number of reasons. First, the pathogenesis of a number of human conditions, including Alzheimer’s disease, Parkinson’s disease, spongiform encephalopathy, Huntington’s disease, senile amyloidosis, and type II diabetes [1-7] is fundamentally associated with amyloids. A detailed understanding of the processes leading to extracellular amyloid deposition, intracellular neurofibrillary tangles, and the development of amyloid toxicity is necessary to devise strategies for therapeutic intervention and management of these diseases. Such β-sheet-based structural assemblies are also promising for industrial application, and in material science and biotechnology [21-26]. Second, the observation that a wide variety of non-disease-related proteins and polypeptides that presumably do not undergo amyloid-like transitions in vivo, but can be induced to do so in test tubes by changing the solution conditions [5, 8-13], has added newer dimensions to the multifarious response of proteins to solvent conditions. Given the number and variety of proteins from which amyloids have been formed, it is now generally accepted that amyloid aggregates are a generic structure for all proteins.

Since the amino acid composition, sequence, and the native-state structure are not determinants of amyloidogenicity [2, 14], amyloid fibrillation could originate from anywhere in the conformational landscape, entropically as low as the native or native-like states to as high as unfolded states in a protein specific manner [2, 15, 16], implying that some combination of the initial structure, number of intramolecular contacts, chain dynamics, and surface dielectric may be required to promote fibrillation, and that these factors may also be related to kinetic mechanism of the composite fibrillation reaction. Understanding of such issues needs to be augmented.
Amyloid fibrillation of CARD

Atomic models of amyloid fibril structure based on a variety of evidences, each limited by the extent amenable to the experiment, have been discussed [17-20]. Although there is a consensus that amyloid and amyloid-like fibrils contain \( \beta \)-sheet conformations, achieving atom-level resolution of arrangements of \( \beta \)-sheet structures has been seriously hindered by the difficulties of handling fibrous supramolecular forms that are often insoluble, especially at concentrations required for structural work. Thus, the specific structural and chemical features of proteins and the solvent-dependent reactivity that promote amyloidogenesis are at the focus of current research.

Concerning the etiology of amyloid cytotoxicity at the molecular level, fewer studies conducted to date have indicated that the fibrils and the precursor \( \beta \) oligomers trigger apoptosis in cells [27-35]. These *in vitro* studies confined to cultured neurons have relied principally on the observation of cell morphology and biochemical characteristics of apoptosis in response to treatment with soluble fibrils and PA. The evidences provided are compelling that amyloid-induced activation of an apoptotic pathway is one of the reasons for neural cell death in neurodegenerative diseases.

We were contemplating on the general possibility of amyloid-like transition of the pro- and anti-survival proteins of mammalian apoptotic pathways, even though at present there is no known amyloidosis that results from aggregation of any of these proteins, wildtype or otherwise. Should any of these proteins undergo amyloid fibrillation, the normal development, tissue differentiation, and homeostasis will be critically affected. In this study, we report on the *in vitro* transformation of the recombinant CARD domain of human Apaf-1 (Figure 1) into amyloid protofibrils. Apaf-1, a large anti-survival protein (~ 130 kDa), is the key molecule for activation of procaspase-9 in mitochondrial pathway of apoptosis in neuronal and somatic cells alike [36, 37]. Structurally, the Apaf-1 CARD domain consists of six tightly packed
amphipathic $\alpha$-helices (Figure 1, ref 38). The rationale for using the CARD domain is the fact that this domain is present as the N-terminal prodomain in a sizable set of apoptotic proteins, including some caspases, and CARD domains are known to mediate apoptotic signaling through homophilic CARD/CARD interactions [38-42]. To arrive at the *in vitro* conditions for transformation of the CARD protein to fibrils, we carried out a series of biophysical experiments as the prelude. The results of pH dependent conformational transitions, protein stability, and folding-unfolding kinetics showed that Apaf-1 CARD undergoes a molten globule-like transition under acidic conditions. This low pH-denatured form of CARD then undergoes further conformational transitions to produce soluble precursor aggregates (PA) of amyloid-like protofibrils. The consequences of CARD fibrillation and possible implications for cell survival are discussed.

**5.3 Material and methods**

**5.3.1 Cloning and generation of the CARD expression construct**

Total RNA isolated from HeLa cells was used for the cDNA amplification of the 300 bp gene fragment of Apaf-1. The 300 bp region of CARD domain of Apaf-1 was amplified by standard PCR reaction using the, forward and reverse primers designed with pGEX4T-1 compatible restriction sites at their 5' ends. The forward primer has a *BamH1* site, and the reverse a *XhoI* site.
Amyloid fibrillation of CARD

**CARD-F**: 5' CGGGATCCATGGATGCAAAAGCTCGAA 3'  \( T_m = 56 \, ^\circ\text{C} \)

**BamH1**

**CARD-R**: 5' CCCTCGAGCTAAGAAGAGACACAGGAATG 3'  \( T_m = 56 \, ^\circ\text{C} \)

**Xho1**

cDNA was synthesized by reverse transcriptase, and the 300 bp CARD fragment was amplified by Taq DNA polymerase. Both steps were achieved by the use of the ‘one-step RT-PCR kit’ from ABGene Technologies. The PCR-amplified fragment was isolated by Qiagen Gel extraction kit, ligated into TA cloning vector pTZ57 R/T (MBI-Fermentas), and transformed into competent DH5\(\alpha\) E. coli CaCl\(_2\) treatment. The transformed cells were plated onto LB agar plates with 100 \(\mu\text{g/mL}\) ampicillin and 1mM IPTG and X-gal. The positive clones were selected by blue-white screening and colony PCR. The positively-screened colonies were used for isolation of plasmid DNA by standard protocols. The PCR fragment cloned into the TA cloning vector was restricted using BamH1 and Xho1, and analyzed on 1% agarose gel. The expression vector pGEX4T-1 was also restricted in the same manner. The CARD gene fragment was then ligated into the digested pGEX4T-1 by using T4 DNA ligase, and transformed into DH5\(\alpha\) cells. Cell colonies positive with the recombinant plasmid were screened by colony PCR. That the recombinant plasmids contained the expression vector carrying the insert was once again checked by restriction digestion. The sequence and orientation of CARD fragment was confirmed by sequencing. The restriction-positive plasmids were transformed into E. coli BL21 cells for expression of the CARD protein.

**5.3.2 Protein expression and purification**

500 ml LB broth with 100 \(\mu\text{g ml}^{-1}\) ampicillin was inoculated with 25 ml of overnight- grown culture (1:20 ratio) that contained the recombinant positive clone.
The protein expression was induced at OD\(_{600}\)=0.5 with 1 mM IPTG. Cells were grown for 5 hrs at 30 °C. Cells were pelleted and resuspended in 20 ml PBS and lysed by sonication for 3 min with repetitive 30 sec on and off cycles. The lysate was centrifuged at 3000 g for 30 min at 4 °C. The supernatant was collected and DTT was added to a final concentration 1 mM. This was loaded onto 1 mL GSH Sepharose 4B column that was preequilibrated with 10 volumes of PBS. The column was washed with 20 volumes of PBS. The GST-CARD protein that was bound to the column was eluted with 10 volumes of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8). The purified GST-CARD protein was dialyzed against 1X PBS to remove the glutathione, and digested with recombinant thrombin (Amersham Biosciences) by standard protocols. Following digestion, thrombin was removed by Benzamidine Sepharose, and the protein mixture was loaded on a GSH-Sephrse 4B column. The 10 kDa CARD protein was eluted in the flow through. The eluted fractions were pooled and loaded on to a 1.6 X 100 cm (diameter and height, respectively) Sephadex G75 column preequilibrated with 50 mM Tris.Hcl, pH 7.5. Fractions each of 1.0 ml size were collected at a flow rate of 20 ml/h. The fractions were pooled and concentrated by ultrafiltration.

For western blot analysis of expressed CARD protein, the thrombin digested GST-CARD was transferred onto a PVDF membrane. The membrane was probed with anti-Apaf-1 rabbit polyclonal IgG and then with ALP conjugated anti-rabbit 2° antibody. The blot was developed with BCIP-NBT solution.

5.3.3 Equilibrium measurement of CARD stability towards pH, NaCl, and GdnHCl

For these titrations, separate samples of identical protein concentration (5-7 \(\mu\)M) were employed. Protein solutions held at different values of pH or NaCl concentration were incubated for ~6 hours before taking fluorescence spectra. For
**Amyloid fibrillation of CARD**

GdnHCl unfolding, samples containing different concentrations of the denaturant were prepared by mixing two stock protein solutions, one containing 4 M GdnHCl and the other without. Buffers for various pH ranges were: 50 mM glycine (pH <3), 50 mM sodium acetate (pH 3-5), 17 mM each of HEPES-PIPES-Tris (pH 6-9), and 25 mM each of Glycine-CAPS (pH 9-11). All experiments were done at 22 °C using a Fluoromax-4 (Horiba Jobin Yvon) instrument.

**5.3.4 Stopped-flow kinetics of CARD folding and unfolding**

These experiments involved two-syringe mixing, and invariably employed 8-fold dilution of a 10 μM protein solution with the relevant buffer. For refolding, the protein initially unfolded in 5.4 M GdnHCl and equilibrated for ~1 hour was mixed with the refolding buffer containing variable amount of GdnHCl. Unfolding was initiated by diluting the native protein solution with the unfolding buffer containing desired concentrations of GdnHCl. The buffer systems were same as given above. Kinetics were recorded at 22 °C in a Bio-Logic SFM4 mixing module using a 0.8 mm square flow cell (mixing dead time, ~ 2 ms). Typically, 8-10 shots were averaged for noise reduction.

**5.3.5 Fibrillation kinetics**

CARD solutions (~15 μM) containing ~50 μM thioflavin (4-(3,6-dimethylbenzothiazol-2-yl)-N,N-dimethyl-aniline, ThT) were prepared in 50 mM glycine-HCl buffer, pH 2.1, and incubated at 60 °C in a heating block. Samples incubated up to different time extent were cooled and analyzed by 482-nm ThT fluorescence, excited at 432 nm.
5.3.6 Atomic force microscopy

For microscopy, fibrillation was allowed in the absence of thioflavin. Films of samples incubated up to variable extent of time were deposited on freshly cleaved mica plates, and allowed to dry under nitrogen for ~15 minutes. Films were then washed thoroughly by gently flowing deionized water over the mica plate, and dried under nitrogen for ~1 hr. Imaging in the semi-contact mode was performed in a NT-MDT Solver microscope using a 3-micron scanner head. Images were processed using the NOVA software supplied by the microscope manufacturer.

5.4 Results

5.4.1 The Apaf-1 CARD expression system and the recombinant protein

Initially we generated two gene constructs for *E. coli* expression of human Apaf-1 CARD, one with a N-terminal His\(_6\) tag (pET28a vector) and the other with GST fused at the N-terminus (pGEX4T-1 vector), but the latter was chosen for the production of the recombinant protein because of the ease and convenience of purifying GST-fused small proteins.

![Figure 2](image_url)

**Figure 2.** (a) Agarose gel electrophoresis showing one step RT–PCR amplification of 300bp Apaf-1 CARD from HeLa cells total RNA. Lane labels are: M, 100 bp DNA ladder; 1, amplified 300 bp CARD fragment. (b) Colony PCR analysis for recombinant pGEX4T-1 CARD. Lane labels are: M, 100 bp DNA ladder; Lanes 1-5 300 bp amplification CARD in positive clones.
Amyloid fibrillation of CARD

Figure 3. (a) SDS-PAGE analysis of Affinity purification of Apaf-1 CARD by GSH-Sepharose 4B column. Lane labels are: M, molecular weight markers; 1, whole cell lysate; 2, insoluble pellet fraction; 3, Cleared lysate loaded onto the column; 4, column flow through fraction; 5, wash fraction; 6, eluted GST-CARD. (b) SDS-PAGE analysis of thrombin digestion and purification of Apaf-1 CARD. Lane labels are: M, molecular weight markers; 1, GST-CARD purified from GSH Sepharose 4B column; 2, the thrombin-digested GST-CARD mixture; 3, the digest after removal of thrombin by Benzamidine Sepharose; 4, homogeneous and pure CARD obtained after passing the thrombin-stripped digest through a GSH-Sepharose 4B column. The CARD protein elutes in the flow through fractions. (c) Western blot analysis of thrombin digested GST-CARD. The upper ~35 kDa band is undigested GST-CARD and lower 10 kDa band is Apaf-1 CARD.

Fig.4. Sephadex G-75 purification of Apaf-1 CARD. The single peak in chromatogram corresponds to the monomeric CARD. The Lanes 1-3 in the inset SDS-PAGE picture are the purified CARD protein.
This expression vector was used in earlier studies of NMR solution structure of Apaf-1 CARD [38], although mutagenesis-based cloning and expression in pET-3d vector has also been reported [43]. As Figure 3 shows, the recovered CARD (~6 mg per liter of *E. coli* culture) is homogeneous and highly pure. The purified CARD was monomeric which was confirmed by Sephadex G-75 gel filtration chromatography (Figure 4). The western blot of thrombin digested GST-CARD shows the detected 10 kDa CARD protein by the polyclonal anti-Apaf-1 rabbit IgG.

5.4.2 *Different pH forms of CARD*

The rationale for examining CARD conformational changes as a function of pH was that partly denatured proteins at acidic and alkaline pH can often reveal structural and functional regulation of proteins. A contextual example is low-pH dimerization of apoptotic Bcl-2 family of proteins which possibly leads to ion channel formation in synthetic membranes [44]. For Apaf-1 CARD, Figure 5a shows the general trend of fluorescence decrease with increasing pH. The primary structure of CARD has no tryptophan and the observed fluorescence with the $\lambda_{\text{max}}$ of 309 nm, due likely to tyrosine, is weak. The absence of any shift in the fluorescence maximum across the pH range may be due to very similar polarity of the environment of tyrosines under native and denaturing conditions. Absence of fluorescence band shift across the folding-unfolding transition is seen for cytochrome *c* also. A closer examination of the pH dependence of the 309-nm fluorescence (figure 5b) shows a pronounced dip at ~pH 4 on either side of which the fluorescence signal increases. In the 5-8 range of pH, the fluorescence remains unchanged, but decreases gradually for pH>8. The data thus indicate three pH transitions: an alkaline transition with a pH midpoint near 10 attributable to tyrosyl side-chain ionization, a weakly acidic transition with a midpoint around pH 4.5 due likely to ionization of Glu or Asp side-
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**Figure 5.** Fluorescence monitored pH dependence of CARD conformational transitions. For all these experiments, the protein concentration was ~6 μM.

(a) Fluorescence spectra as a function of pH generally conveys increased quenching with pH increments. (b) The plot of 309-nm fluorescence with pH, however, shows three prominent transitions: an alkaline transition, a weakly acidic transition, and a more acidic transition. The interpretations and assignment of each of the transitions to specific conformational changes of CARD are described in the text. Due to the complexity of analysis of the three linked transitions, the data have not been fitted to a function. (c) pH effects on ANS binding to CARD. The protein and ANS concentrations in the solution at different pH were 7 and 15 μM, respectively. The open circles show the 492-nm fluorescence of control samples from which the protein was excluded.

... -COOH, and a molten globule-like transition when strongly acidic conditions are approached. While the molten globule-like transition involves a global denaturation transition of CARD, the other two transitions could simply be associated with side-chain ionization-linked conformational perturbation. As a simple test for the extent of structure perturbation...
in each of these transitions, we measured the ANS fluorescence of CARD solutions containing ANS (6 μM protein and 12 μM ANS) as a function of pH. As Figure 5c shows, ANS is fluorescence-silent at pH>4, but is very highly fluorescent at pH values less than 4. The ANS fluorescence indicates a major transition in the 2-4 range of pH, closely reproducing the molten globule-like transition considered above (Figure 5b). Indeed, ANS binds to solvent exposed clusters of hydrophobic groups, and its strong binding is a particularly convenient test for the molten globule state [45]. But then, the ANS fluorescence decreases when the pH value falls below 2 (Figure 5c), indicating self-association or oligomerization of acid-denatured CARD in a manner that conceals the exposed hydrophobic surfaces. The CARD monomers possibly oligomerize by hydrophobic interactions.

To understand the CARD conformational changes further, we extended the pH dependent fluorescence experiment by including NaCl concentration as another variable (Figure 6a). Clearly, all three transitions seen in Figure 5b—the alkaline, the acidic, and the molten globule-like transition—are reproduced in the presence of any concentration of NaCl used in the 0-1 M range (Figure 6a). Further, NaCl sets the fluorescence in a pH dependent manner (Figure 6b). At intermediate pH values, the fluorescence increases with NaCl, but decreases relatively more at acidic and alkaline values most likely due to electrostatic screening of protein charges by Cl\(^-\) and Na\(^+\) ions, respectively. Thus, at pH near 2, the acid-denatured state is transformed to a molten globule state. To summarize this section, CARD at acid pH (<4) undergoes a major denaturational transition accompanied by a substantial exposure of otherwise buried hydrophobic surfaces. Under strongly acidic conditions (<pH 2), the denatured monomers interact with each other possibly by hydrophobic interactions to produce soluble oligomers or aggregates. We have called them precursors aggregates (PA).
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Figure 6. CARD fluorescence as a function of pH at different concentrations of NaCl. (a) All three pH-induced conformational transitions of CARD are seen in NaCl independent manner, although some details of the transitions, including the sharpness and fluorescence amplitudes, are affected (see Results). (b) The effect of NaCl on the fluorescence amplitude depends on the pH of the medium. The substantial decrease of fluorescence at acidic and alkaline pH is likely due to electrostatic screening of protein charges by Cl\(^-\) and Na\(^+\) ions, respectively.
5.4.3 Equilibrium and kinetic aspects of folding of Apaf-1 CARD at acid and neutral pH

To learn more about the influence of pH on the stability and structure, we examined the GdnHCl-induced folding behavior of the protein by equilibrium and stopped-flow kinetic methods. Figure 7a shows the equilibrium unfolding transition at pH values 6 and 3.1. At pH 6, the initial increase of fluorescence in the pretransition region is followed by a relatively sharp drop in the unfolding transition region. The structural details associated with these changes is subject to scrutiny. For the present though, a fit of the data to the two-state \( N \leftrightarrow U \) model [46], where \( N \) and \( U \) are native and unfolded states, respectively, by assuming a second-order polynomial dependence of the pretransition fluorescence with GdnHCl yields the protein stability, \( \Delta G^\circ = 12.5 \pm 0.5 \) kcal mol\(^{-1} \), and the transition midpoint, \( C_m = 2.4 \pm 0.1 \) M. In an earlier study of CARD unfolding in the presence of urea, \( \sim 6 \) kcal mol\(^{-1} \) was reported for the value of \( \Delta G^\circ \) [47]. Generally, the \( \Delta G^\circ \) value determined by urea unfolding is lower than that extracted from GdnHCl unfolding. Part of the discrepancy also arises from the large increase in the fluorescence in the pretransition baseline (Figure 7a) which the earlier authors did not notice in their study by the use of urea [47]. At pH 3.1, a clear unfolding transition of CARD is not detected. The change in the fluorescence stretches out for GdnHCl>1.5 M, indicating lack of compactness and well defined structural elements typical of an acid-denatured state in the absence of added anions [45]. The monotony in fluorescence change might simply reflect expansion of the chain. To show that the unfolded state in the presence of 4 M GdnHCl does not contain any considerable structure, the protein was titrated in the 1-7.5 range of pH holding the denaturant concentration constant at 4 M (Figure 7b). Within the error limit, the fluorescence hardly changes in the pH range 2-7.5, suggesting that unfolding is complete at 4 M GdnHCl.
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Figure 7. pH-dependent stability and folding kinetics of Apaf-1 CARD. (a) GdnHCl-induced equilibrium unfolding at pH 6 (●) and pH 3.1 (■). At pH 6, the global unfolding transition is preceded by a pretransition zone characterized by a substantial increase in fluorescence. Since the details of CARD structural changes occurring in the pretransition region is unclear at the moment, the data were modeled with a two-state N ↔ U transition by assuming that the pretransition changes are due to solvent dependent baseline effect having a second-order polynomial dependence for fluorescence changes with GdnHCl. The fit of the data yields $\Delta G^\circ=12.5\pm0.5 \text{ kcal mol}^{-1}$ and $C_m=2.4\pm0.1 \text{ M}$ (see text). At pH 3.1, no pronounced global transition is apparent, suggesting the absence of well-defined tertiary structure. (b) pH titration of the 4 M GdnHCl-unfolded protein. (c) Representative kinetic traces for refolding of CARD in the presence of 1 M GdnHCl (pH 6) or 0.7 M GdnHCl (pH 3.1). For both experiments the protein was initially unfolded in 5.4 M GdnHCl at the respective pH. The refolding at pH 6 is described by two rising exponentials, $k_{\text{fast}}=60 \text{ s}^{-1}$ and $k_{\text{slow}}=1.8 \text{ s}^{-1}$ with fractional observed amplitudes of 0.7 and 0.3, respectively. At pH 3.1, there is a fast decaying phase ($k_{\text{fast}}=262 \text{ s}^{-1}$) followed by a slow rising phase ($k_{\text{slow}}=2 \text{ s}^{-1}$), suggesting the possible formation of an aggregate or misfolded intermediate at early times of refolding. (d) Residuals of the 2-exponential fits. (e) GdnHCl dependence of the apparent rates for the major (upper chevron) and the minor phase (lower chevron) at pH 6. In each chevron, the data formine the left (darker symbols) and right (lighter symbols) arms
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indicative of refolding. The initial phase which is faster than the slow phase by at least two orders of magnitude and during which the fluorescence decay is associated with an unfolding event (Figure 7c). The GdnHCl distributions of the apparent rate constants ($k_{obs}$) for both kinetic phases of refolding and unfolding at pH 6 are shown in Figure 7e. Except for the unfolding by the slow kinetic phase, the rates clearly roll over as strongly native-like or strongly unfolding conditions are approached.

Classically, multiple chevrons indicate existence of interconverting ensembles of unfolded conformations with ensemble-specific refolding rates producing parallel folding routes, and chevron rollover is thought to arise from accumulation of kinetic intermediates [48]. For Apaf-1 CARD, the two distinct chevrons with limb rollovers then suggest the occurrence of two ensembles of unfolded conformations, one folds faster than the other. The folding routes for both fast-folding ($U_F$) and slow-folding ($U_S$) ensembles involve kinetic intermediates; but how many intermediates are involved in the folding of $U_F$ and $U_S$ cannot be determined with the data at hand.

Available results allow the description of Apaf-1 CARD folding by the following basic model

where $I_{Fi}$ and $I_{Si}$ represent intermediates. But for the kinetic intermediates invoked here, this model is consistent with the one proposed earlier based on the kinetic study of Apaf-1 CARD folding [47]. That work employed urea as the denaturant unlike
GdnHCl used here, and observed only one chevron with no rate rollover in the limbs. Part of the discrepancy in the results may arise from the use of two different chemical denaturants. The present study provides a direct evidence for distinct unfolded state ensembles based on the finding of two distinct chevrons.

Figure 7f shows the rate-denaturant distribution for CARD folding-unfolding at pH 3.1. At all concentrations of GdnHCl, the observable fast phase in the stopped-flow kinetics is due to unfolding. So is the case with the slow phase when the GdnHCl concentration is higher than ~0.3 M. Importantly, the GdnHCl dependences of both rates are associated with considerable positive slope or kinetic $m$-value (given by $m_u = 2.3RT \frac{\partial \log k_u}{\partial [\text{GdnHCl}]}$, where $k_u$ is the apparent rate constant of unfolding), indicating that CARD at pH 3.1 is still substantially structured with a defined core. Also, as it happens at pH 6, the unfolding rates at pH 3.1 roll over under strongly unfolding conditions, suggesting the occurrence of two ensembles of unfolded conformations and an unknown number of kinetic intermediates as depicted in folding model given above. It thus appears that the structure and topology of CARD at pH 3.1 resemble that of the pre-molten globule state characterized by fluctuating structural elements [45].

### 5.4.4 Kinetics of protofibril formation for Apaf-1 CARD

The indication provided by ANS fluorescence results (Figure 5c) that acid-denatured and molten globule-like CARD can form soluble oligomers or PA at pH near 2 led us to examine whether the precursors have the propensity to grow into protofibrils. Since the dye ThT is specifically used to probe amyloid fibrils [49, 50], we incubated a 15 μM CARD solution containing 50 μM ThT at 60°C held at pH 2.1 in Gly-HCl buffer, and periodically measured the time dependence of the dye
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fluorescence up to 100 hours. As Figure 8 shows, following a lag time of ~9 hours, the fluorescence increases in a single-exponential phase with an apparent rate constant of 0.04 per hour, suggesting the formation of amyloid fibrils [51]. Generally, the presence of the lag phase and the fibrillation rate both depend on the protein concentration as well as the incubation temperature and buffer conditions used. This should hold for the formation of CARD fibrils also, although we have not explored conditions that would reduce the lag time or increase the rate of fibril formation.

Figure 8. Fibrillation kinetics monitored by ThT fluorescence. The sample contained 15 µM CARD and 50 µM ThT was held at pH 2.1 and 60°C. The arrow indicates the end of the lag phase (~9 hours). The fit of the data after the lag phase yields \( k = 0.04 \text{ hr}^{-1} \).

5.4.5 Images and dimensions of Apaf-1 CARD fibrils

The formation of fibrils was confirmed by direct images of CARD samples held at pH 2.1 and incubated at 60°C for varying length of time (Figure 9). By using ~10 µM protein, we see signs of elementary combination of PA within ~15 minutes (Figure 9a, panel 1). The growth into protofibrils requires several hours shown here at the end of 8 hours (panel 2), and longer fibrillar structures begin to appear by ~14 hours of incubation (panel 3). At ~100 µM protein, the fibrillation kinetics was very rapid (Figure 9b). The rich lattice of amyloid fibrils observed after 1 hour of incubation (panel 1) becomes denser after ~3 hours (panel 2), but appears diffused.
Figure 9. AFM images of Apaf-1 CARD aggregates. (a) A 10 μM protein solution incubated at 60°C, pH 2.1, shows first signs of combination of the PA within ~15 min (panel 1). The growth and elongation of protofibrils are shown in panels 2 and 3 imaged at the end of 8 and 14 hours, respectively, of incubation. Yellow, blue, green, and white arrows indicate PA, nascent protofibrils, and elongated protofibrils, respectively. (b) At ~100 μM protein concentration, fast and rich growth of fibrils is observed. Images shown in panels 1, 2, 3, and 4 were recorded with samples incubated for 1
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To achieve a dimensional distinction of PA, protofibrils, and large fibrils, we determined the Gaussian distribution of diameter $d$ for each population. The diameters respectively, were arranged into groups of 0.25 nm increments, and the % population falling in each diameter group was determined (Figure 10). The solid lines through the data are 3-parameter Gaussian fits according to

$$P(d) = P(d_o) \exp \left[ -0.5 \left( \frac{d-d_o}{b} \right)^2 \right]$$

where, $P(d_o)$ is the amplitude corresponding to the mean diameter $d_o$, and $b$ is the Full Width at Half Maximum (FWHM). The value of $d_o$ for PA is 2.1 nm as against 2.7 and 2.63 nm for protofibril and elongated protofibrils, respectively. This dimensional difference between the initial PA and the fibrils should arise from differences in the content of presumably β-sheet. The FWHM values (1.5, 1.6, and 1.25 nm for the distributions corresponding to PA, protofibrils, and elongated protofibrils, respectively) indicate the population inhomogeneity, being largest for protofibrils and smallest for large fibrils.

**Figure 10.** Distribution of population diameters. The solid lines are three-parameter Gaussian fits to the measured data according to equation 1. (a) PA with a mean diameter, $d_o =$ 2.1 nm. (b) The protofibrils and elongated protofibrils $d_o$ values of 2.7 and 2.63 nm, respectively.
The differences in the inhomogeneity may partly arise from the fact that structurally PA and fully grown fibrils are characterized by $\alpha$-helical and $\beta$-sheet content, whereas the protofibrils at the initial formation stages contain both in proportion different from one set of population to another (large FWHM).

5.5 Discussion

Examinations of the pH-induced conformational transitions and folding stability of recombinant human Apaf-1 CARD have shown that the acid-denatured protein self-associates to form soluble precursor aggregates which can combine and undergo structural transitions to form amyloid protofibrils.

5.5.1 Soluble oligomers and protofibrils of Apaf-1 CARD.

Many pro- and anti-survival proteins of the apoptotic machinery are known to homodimerize, heterodimerize, and even homo-oligomerize to exert their survival and death effects [52-60]. Some are constitutively oligomeric due to ready accessibility of the interacting surfaces, and others are prevented from oligomerization by sequestration of the interacting surfaces until functional activation occurs. The former class is exemplified by the pro-survival protein Bcl-xL, for which a large fraction of the cellular population homodimerizes by homophilic interaction of the C-terminal hydrophobic tails [44, 60-62], and the latter is represented by quiescent Apaf-1 known to exist in the monomeric form [36, 63]. It appears that the structural surface of CARD of Apaf-1 required for homophilic interaction with the CARD of procaspase-9 [38] is partially buried in the inactive state of Apaf-1 due to intramolecular interactions, an observation based on the crystal structure of the WD40-deleted Apaf-1 [64]. When activated by binding with cytochrome $c$ and dATP [63], Apaf-1 forms a wheel-shaped homoheptamer complex or apoptosome having a seven-fold symmetry.
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[65, 66]. Although atomic details and packing interactions in the apoptosome are not known, the CARD can now interact with the CARD of procaspase-9. Thus, homo-heptameization of Apaf-1 seems to be crucial for sensitizing the CARD for binding interactions.

But, Apaf-1 CARD alone in neutral-pH solutions exists in the monomeric state (Figures 5-7), consistent with the earlier report that no dimerization occurs even at concentrations as high as 1 mM [38]. On the other hand, isolated Apaf-1 CARD forms a complex with isolated caspase-9 CARD in which the former deploys sets of acidic and hydrophobic residues in a manner that creates a contiguous binding surface, suggesting that intermolecular associations of Apaf-1 CARD require both electrostatic and hydrophobic interactions [38]. Should Apaf-1 CARD alone undergo homooligomerization, the operative forces of interaction must be hydrophobic in nature, because the surface charge complementarity is not available. The existence of Apaf-1 CARD in the monomeric form in the neutral-pH region then suggests that the hydrophobic interactions afforded by the native protein surface are insufficient for dimerization to occur. The situation is quite interesting under acidic conditions (~pH 3-4) where CARD undergoes a MG-like transition. Relative to the native state, the molecular surface in the MG state is more hydrophobic as evidenced by binding of small nonpolar molecules like ANS (Figure 5b, c). The increased surface hydrophobicity should favor CARD-CARD self association, but electrostatic repulsion due to excess positive charge and large-scale fluctuations of the structural elements in the MG-like state [45] are the principal opposing forces. A dramatic increase in surface hydrophobicity can occur under strongly acidic conditions (<pH 2.5) where the already weakened structural elements of the CARD MG break down causing exposure of buried nonpolar residues. We believe, the preponderance of hydrophobic surfaces in the acid-denatured proteins is now so overwhelmingly favorable toward CARD oligomerization that electrostatic repulsions are subdued, and
a monomer ⇔ oligomer equilibrium is readily established. The oligomerization event can be thought of as a transition to an alternative non-native global free-energy minimum [67]. We should also note that protein oligomerization by hydrophobic interactions at low pH very likely buries some positive charges in the protein-protein apolar interface, and because charge burial in the low dielectric apolar environment is energetically expensive, the CARD oligomers are unlikely to be very highly stable.

These soluble oligomers are precursor aggregates (PA) which grow in size to form elongated protofibrils (PA → protofibril). We have not investigated the mechanism and the events associated with the PA → protofibril condensation in this work, but the CARD fibrillation pathway may be depicted as: MG ⇔ PA → protofibril → elongated protofibril. The inference that the MG-like conformation facilitates PA formation is consistent with the survey-based observation that the amyloidogenic conformation shares many structural and dynamical properties with the pre-molten globule state [2].

5.5.2 Relevance to in vivo fibrillation of Apaf-1

Although we obtained amyloid fibrils from acid-denatured CARD at an elevated temperature, the question is: what relevance this has for in vivo situations, given that none of the pro- and anti-survival proteins is known to undergo fibrillation? This study rests on a limited empirical search for an in vitro condition that facilitate fibrillation. The low pH and high temperature encourage, respectively, higher surface hydrophobicity for the monomers and stronger hydrophobic interactions between them. Such conditions are certainly not physiological, but one cannot exclude yet unknown intrinsic cellular factors, accidental biochemical insults, or pathological conditions that could promote formation of soluble oligomers of Apaf-1 required for protofibril growth. As mentioned earlier, fibril growth may require some combination
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of the initial molecular structure, packing density, bonding, intramolecular dynamics, and surface dielectric. Since the underlying physico-chemical principles for fibril growth are not fully understood, the conditions that might promote fibrillation *in vivo* need to be empirically found. Facing the two counteracting scenarios—the absence of specific evidence for amyloidosis of apoptotic proteins on one hand, and the finding that CARD could undergo fibrillation, albeit under nonphysiological conditions, on the other— one could at the most say that there is a very low or a restricted likelihood of Apaf-1 fibrillation *in vivo*. Even this likelihood could narrow down for a protein the size of Apaf-1 given that amyloidogenic proteins and peptides are generally smaller in size. The CARD is a small domain of Apaf-1, and it independently might not form fibrillar aggregates under physiological conditions. Nonetheless, a discounted possibility of Apaf-1 fibrillation still exists.

It is often hard to foresee the cellular or physiological relevance of a phenomenon tested under non-physiological conditions. For example, the MG states are best stabilized under extreme pH conditions in the presence of counter ions [45], and indeed the significance of the MG state was elusive in the seminal days, except for the recognition that it represented the third thermodynamic state of proteins [45]. It is now clear that a class of intrinsically disordered proteins that resemble MG and lack tertiary folds [68] are involved in cell signaling and regulatory function by protein–DNA and protein–protein interactions [69, 70].

In the same spirit, fibrillation of apoptotic proteins might find relevance to cell survival. In the absence of specific evidence at the moment, we simply say that there is a finite likelihood of Apaf-1 fibrillation *in vivo*.

### 5.5.3 Amyloid fibrils and cell survival

Although cytotoxicity of amyloid fibrils and progression of degenerative disorders such as Alzheimer’s and Creutzfeldt-Jacob diseases, due to insoluble fibrils
of amyloid-β protein (Aβ) and prion protein (PrP), respectively [31-35, 71, 72], is well established, the molecular mechanism by which the amyloid fibrils and their precursors kill cells is less understood. It is however recognized that activation of an apoptotic pathway is one of the major causes of amyloid fibril-induced cell death [27-30]. Credible evidences for this recognition are provided by observation of morphological and biochemical characteristics of apoptosis in healthy neurons and cultured cells treated with fibrils of Aβ [27, 29], and with fibrils produced differentially from PrP [30-35]. But whether it is the extent of fibrillation or the fibril dimension or the inherent property of the precursor amyloid protein that forces cells to undergo apoptosis is unsettled. For example, insulin fibrils are non-toxic to neurons [30], but precursor oligomers of hen lysozyme fibrils decrease the viability of neuroblastoma SH-SY5Y cells by presumably apoptotic activation of caspases [73].

But the issue of cytotoxicity of prefibrillar aggregates of pro- and anti-survival apoptotic proteins, assuming that one or more of them could possibly undergo in vivo fibrillation, is redundant. Cytotoxicity is irrelevant here because prefibrillar aggregates of any of these proteins as such would be very detrimental to cell survival. Since Apaf-1 is the key anti-survival protein of the mitochondrial pathway of apoptosis in neuronal and non-neuronal cells alike, amyloid fibrillation of this protein will arrest normal development of organs in adults and lack of embryonic differentiation leading to death, just the way Apaf-1-null mice die due to a pronounced enlargement of the periventricular proliferative zone during late embryonic development [74]. To find out if this is one of the manners by which cell survival could be challenged, the in vivo fibrillation tendency of apoptotic proteins needs to be determined.
5.6 References


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