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Rapid Folding and Unfolding of Apaf-1 CARD

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Abstract

Caspase recruitment domains (CARDs) are members of the death domain superfamily and contain six antiparallel helices in an α -helical Greek key topology. We have examined the equilibrium and kinetic folding of the CARD of Apaf-1 (apoptotic protease activating factor 1), which consists of ninety-seven amino acids, at pH 6 and pH 8. The results showed that an apparent two state equilibrium mechanism is not adequate to describe the folding of Apaf-1 CARD at either pH, suggesting the presence of intermediates in equilibrium unfolding. Interestingly, the results showed that the secondary structure is less stable than the tertiary structure, based on the transition midpoints for unfolding. Single mixing and sequential mixing stopped-flow studies showed that Apaf-1 CARD folds and unfolds rapidly and suggests a folding mechanism that contains parallel channels with two unfolded conformations folding to the native conformation. Kinetic simulations show that a slow folding phase is described by a third conformation in the unfolded ensemble that interconverts with one or both unfolded species. Overall, the native ensemble is formed rapidly upon refolding. This is in contrast to other CARDs in which folding appears to be dominated by formation of kinetic traps.

Keywords

caspase recruitment domain; protein folding; folding kinetics; parallel folding channels; sequential mixing stopped-flow

INTRODUCTION

Studies with several small helical bundle proteins ^{1; 2; 3} (see review by Eaton and coworkers ⁴) have shown that folding can be very fast, on the order of 300 s⁻¹ to >5000 s⁻¹, and in many cases folding rates can be predicted by the contact order, an algorithm based on the average sequence distance between all contacting residues in the native conformation ⁵. The correlations suggest a relationship between the rate of folding and the topology of the native state ⁵. For the more complex topology found in the caspase recruitment domain (CARD) of RICK (RIP-like interacting CLARP kinase) or of procaspase-1 (CP1-CARD), which consists of six antiparallel α -helices in the Greek key topology, folding is too slow to be accounted for by the contact order ^{6; 7}. For those CARDs, the folding pathways appear to contain kinetically trapped species that significantly slow the folding rate. Kinetic traps in folding have been observed for many other proteins ^{8; 9; 10; 11; 12}.

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While relatively little is known about the folding of α -Greek key proteins, much more is known about β -Greek key proteins, as the folding of proteins in this class has been studied for many years. For proteins such as $\beta\gamma$ -crystallin, superoxide dismutase, fibronectin type III domain and azurin^{12; 13; 14; 15}, several nucleation models have been proposed to explain the folding properties. The models focus on early formation of a β -hairpin¹⁶, hydrophobic zippers¹⁷, tyrosine corner motifs^{15; 18}, or hydrogen bonding¹⁹. For example, a highly conserved “tyrosine corner” motif is found in several β -Greek key proteins and is important for early structure formation or stability^{15; 18}. However, no such folding models have been proposed for the α -Greek key family, and the tyrosine corner motif is not found in this family.

At present, the nature of the intermediates found in the folding of the CARDs is not known, nor is it known why the folding process is so complex. In order to further examine this issue, we studied the folding of Apaf-1 CARD, a 97 amino acid domain found at the amino terminus of Apaf-1 (apoptotic protease-activating factor 1). Apaf-1 is a ~130 kDa multidomain protein that is involved in the intrinsic apoptotic pathway²⁰, which is activated when cytochrome c is released from the mitochondria into the cytoplasm^{20; 21}. The binding of cytochrome c and of ATP cause Apaf-1 to oligomerize into a complex called the apoptosome^{22; 23}, a ~1 MDa wheel-like particle with seven spokes that radiate from a central hub²². The Apaf-1 CARD binds the CARD of procaspase-9^{21; 22; 24} and results in dimerization of the inactive procaspase-9 to form the active protease, which in turn activates executioner caspases such as caspases-3 and -7²⁰. Apaf-1 CARD is a member of the death domain superfamily, which is subdivided into four families consisting of CARD (caspase recruitment domain), DD (death domain), DED (death effector domain) and the PYD (pyrin domain)^{25; 26}. To date, the α -Greek key proteins have been found only in this superfamily. The function of these domains is to mediate homo- and heterotypic protein-protein interactions in apoptotic pathways. Within the death domain superfamily there is a low sequence identity, but all members share a common “death fold” consisting of six antiparallel α -helices arranged in a Greek key topology²⁵.

We show here that the equilibrium folding of Apaf-1 CARD at pH 6 and at pH 8 is not well described by a two-state process, in contrast to RICK-CARD and CP1-CARD, demonstrating the presence of non-native folding intermediate(s) during equilibrium folding. Single mixing kinetic studies indicate that both folding and unfolding occur rapidly, while sequential mixing kinetic studies show that both reactions apparently occur without the presence of folding intermediates. Contrary to RICK-CARD, kinetically trapped or misfolded species are not present in the folding of Apaf-1 CARD, so the protein both folds and unfolds rapidly.

RESULTS

Structural studies

The structure of Apaf-1 CARD was determined previously by crystallography²⁷ and NMR²⁸. In addition, a structure of the complex of Apaf-1 CARD bound to caspase-9 (CP9-) CARD was determined^{28; 29}. Our clone of Apaf-1 CARD contains two extra residues at the amino terminus, a GS sequence that arises from the cloning vector³⁰. These residue positions are labeled -2 and -1, respectively (Figure 1). Since the side-chain of M1 forms part of the hydrophobic core²⁷, it was important to determine whether the two residues affected the structure in any significant way. We were able to grow crystals of the new Apaf-1 CARD construct using the published crystallization conditions²⁷, and they were isomorphous with those previously reported, with symmetry of the space group $P2_12_12_1$ and one molecule in the asymmetric unit (Table 1). Diffraction data were collected at the SER-CAT beam line at APS (Argonne National Laboratory, Argonne, IL) and the structure was refined to 2.0 Å resolution using the published model for initial phases (PDB code 1CY5). While the N-terminal G and S residues were both clearly visible in the electron density maps, they did not make hydrogen bonds or van der Waals contacts with other protein atoms. The side-chain of M1 contributed

to the hydrophobic core of the protein exactly as in the published structure where the GS segment is not present. The backbone root mean square deviation (RMSD) between our model and the previously published 1CY5 model was 0.36 Å, demonstrating that the GS sequence had no significant effect on the protein structure.

Equilibrium unfolding

We examined the secondary structure of Apaf-1 CARD by far-UV circular dichroism (CD) (data not shown), and the results demonstrated double minima at 206 nm and 218 nm, consistent with the α -helical CARD proteins^{6; 7}. In the presence of 6 M urea-containing buffer, the signal decreased to that of a random coil, and the spectrum was consistent with that of an unfolded protein.

The equilibrium unfolding of Apaf-1 CARD was examined by monitoring changes in CD at 222 nm and fluorescence emission at 305 nm following incubation of the protein in urea-containing buffers. The experiments were performed using three protein concentrations and two different buffers as described in Materials and Methods. In addition, the experiments were performed at pH 6, which is near the isoelectric point of the protein, and pH 8. The data collected at pH 8 allow for a comparison with that of RICK-CARD and CP1-CARD^{6; 7; 31}, while the data collected at pH 6 should provide the stability of the protein where the net charge is approximately zero³². Overall, the data demonstrate that Apaf-1 CARD does not form an oligomer under these conditions (25 °C, pH 6 or pH 8, <20 μ M protein) (data not shown). Based on the midpoints of the transitions, the results showed that the protein was more stable at pH 6 than at pH 8 in both CD and fluorescence emission measurements (Figure 2A and 2B). While the individual data sets could be fit to a two-state equilibrium model (Table 2), an overlay of the CD and fluorescence emission data showed that the curves are not coincident (Figure 2C and 2D). This demonstrated that a two-state folding model was not adequate to explain the equilibrium folding of Apaf-1 CARD. Interestingly, the tertiary structure, as measured by fluorescence emission, appeared to be more stable than the secondary structure. At both pH values, the midpoint of the transition as monitored by fluorescence emission was greater than that monitored by CD, and the difference between the two curves was greater at pH 8 than at pH 6 (Figure 2C and 2D). In addition, the free energy values, determined from fits of the data to a two-state equilibrium model, were higher for the fluorescence emission data than for the CD data (Table 2). We note that the data were not better fit to a three-state model that included an equilibrium intermediate ($U \rightleftharpoons I \rightleftharpoons N$), so at present the conformational free energy of Apaf-1 CARD is not known. The thermodynamic parameters obtained from fits of the equilibrium data are listed in Table 2 as a comparison to the kinetic data described below.

Single mixing refolding studies

The protein was unfolded initially in 6 M urea-containing buffer, and it was refolded by rapid dilution to conditions that favor the native conformation. As with the equilibrium folding studies described above, the experiments were performed at pH 6 and 8, and both fluorescence emission (>305 nm) and far-UV CD (222 nm) were used to monitor the time course for refolding. Unless otherwise noted, the results from fluorescence emission at pH 6 are presented in Figure 3, and those using CD at pH 6 as well as both probes at pH 8 are shown in the Supplemental Figures. As shown in Figure 3A (*circles*), refolding occurred within the mixing dead-time as the signal at 1 msec was similar to that of the native protein control. Following this burst phase, a small increase in signal occurred in the first 10 seconds that encompassed less than ten percent of the total signal change. This phase is described in more detail below. Stopped-flow CD studies at pH 6 showed similar results (Supplemental Figure 1A), and results of fluorescence emission and CD studies at pH 8 were similar to those at pH 6 (Supplemental Figures 2 and 3).

We monitored the amplitude of the refolding burst phase at several final urea concentrations, from 0.55 M to 6.9 M, and representative data are shown in Figure 3B for fluorescence emission at pH 6. Data collected by CD at pH 6 and by fluorescence emission and CD at pH 8 are presented in Supplemental Figures 1-3. The data show a cooperative decrease in signal so that the burst phase of refolding was no longer observed beyond 4 M urea (Figure 3B) at pH 6 or 3 M urea at pH 8 (Supplemental Figures), demonstrating that the protein folds cooperatively during the burst phase. The results were fit to a two-state equilibrium folding model³³, and the conformational free energies, ΔG^{H_2O} , and m -values are presented in Table 2. At both pH values, the conformational free energy determined by fluorescence emission was greater than that determined by CD by >1 kcal/mol. This is consistent with the equilibrium unfolding data (Figure 2, Table 2) that showed the secondary structure unfolded prior to the tertiary structure. Overall, the mid-points for the transitions were lower at pH 8 compared to pH 6, again consistent with the equilibrium unfolding data.

In addition to the burst phase amplitudes, the final signals at 10 seconds were determined at several final urea concentrations (Figure 3B, *squares*) (Supplemental Figures 1-3). The data showed that the transition mid-point was significantly higher than that observed for the burst phase amplitude. The data also were fit to a two-state folding model, and the parameters obtained from the fits are presented in Table 2. In contrast to the burst phase amplitudes, the ΔG^{H_2O} and m -values determined from fluorescence emission or CD were similar at pH 6, ~ 5 kcal mol⁻¹ and ~ 1.3 kcal mol⁻¹ M⁻¹, respectively, although we note that the m -values were similar for the burst phase refolding and the final signals, within experimental error of 1.2 kcal mol⁻¹ M⁻¹. The same was true for data collected at pH 8 (Table 2).

The mid-points for the transitions of the burst phase and final amplitudes (Figure 3B) are not coincident because of a kinetic phase that occurs at intermediate urea concentrations. For example, an increase in fluorescence emission was observed over 10 seconds when the protein was refolded in 3.6 M urea-containing buffer (Figure 3A, *diamonds*). Under these conditions, the amplitude of the burst phase was small (see also Figure 3B), although it still was present, and most of the signal change occurred from the observable kinetic phase. Similar results were obtained by CD at pH 6 (Supplemental Figure 1) and by both probes at pH 8 (Supplemental Figures 2 and 3). The data were best fit to a two exponential process (Figure 3A, lower panels), where the slower phase was the same as that described above for refolding at low urea concentrations. Therefore, the data showed that the protein refolded rapidly (burst phase) when it was refolded in low urea concentrations, and refolded through a somewhat slower kinetic phase (half-time of approximately 20 msec) at higher urea concentrations. Under the latter conditions, the amplitude of the burst phase was low. In both cases, a slow kinetic phase was observed that encompassed less than ten percent of the total signal change.

In order to further study refolding at higher urea concentrations, where the burst phase amplitude is low, the two apparent rate constants and their associated amplitudes were determined at several final urea concentrations. While the observed rate associated with the first phase is described in detail later (Figure 3F), the amplitude of this phase reached a maximum at 3.6 M urea (pH 6) or 2.6 M urea (pH 8) (Figure 3C) and decreased at lower and higher urea concentrations. Although the experiments in Figure 3C used fluorescence emission as a probe, the amplitude maxima determined from the data were similar to the mid-point of unfolding determined from equilibrium experiments when the secondary structure was monitored by CD (Figure 2 and Table 2). Overall, the data showed that during refolding under intermediate urea concentrations (~ 2 M to ~ 4 M urea), both the burst phase and the two kinetic phases were observed, and the amplitudes of the burst and fast phases depended on the final urea concentration. In contrast, only the burst and the slower kinetic phases were observed under conditions that strongly favor the native conformation (0.55 M urea). We note that neither

the rate associated with the slowest phase (0.6 s^{-1}) (data not shown) nor the amplitude of this phase (Figure 3C, *squares*) varied with final urea concentration or with pH.

Single mixing unfolding studies

We monitored the time course for unfolding Apaf-1 CARD by rapidly mixing native protein with urea-containing buffer. Representative data are shown in Figure 3A for protein unfolded in 6 M urea at pH 6 (*triangles*). Unfolding monitored by CD at pH 6 or by both probes at pH 8 is presented in Supplemental Figures 1-3. The data showed that unfolding occurred in a single phase that encompassed the entire signal between native and unfolded protein. There was no further change in signal between 0.1 and 200 seconds (data not shown), suggesting that the protein unfolded rapidly under these conditions, with a half-time of about 10 msec.

We determined both the amplitude and the rate of the unfolding transition from fits of the unfolding data to a single exponential equation, and this was done at several final urea concentrations. We also monitored the final signal at each final urea concentration. As shown in Figure 3D, the amplitude of the unfolding phase increased cooperatively as the urea concentration increased. The data were fit to a two-state equilibrium process, and the ΔG^{H_2O} and m-value were determined to be $5.1 \pm 0.8 \text{ kcal mol}^{-1}$ and $1.2 \pm 0.4 \text{ kcal mol}^{-1} \text{ M}^{-1}$, respectively, at pH 6 (Table 2). The parameters were similar at pH 8 (Supplemental Figure 3 and Table 2). The final signals in unfolding (Figure 3E and Supplemental Figures) also were fit to a two-state process, and the ΔG^{H_2O} and m-value determined from the fits were $\sim 5 \text{ kcal mol}^{-1}$ and $\sim 1.20 \text{ kcal mol}^{-1} \text{ M}^{-1}$, respectively, at pH 6 (Table 2). The conformational free energy was somewhat lower at pH 8 ($\sim 4.5 \text{ kcal mol}^{-1}$), but the m-value was similar to that at pH 6. Because the thermodynamic parameters were the same regardless of whether one monitored the amplitude of the unfolding phase or the final signal of unfolding versus urea, the data showed that the protein unfolded in a single transition that reached equilibrium within 100 msec.

The apparent rate constants, k_{obs} , of the fast phase of refolding and of unfolding, obtained from fits of the data as described above, generate a chevron plot (Figure 3F), demonstrating that these phases represent the folding and unfolding of the same species. Similar rates were obtained regardless of whether fluorescence emission or CD was used to monitor refolding or unfolding (Table 2). In addition, the minimum of the chevron was similar to that determined from equilibrium unfolding at pH 6 (4.3 M *versus* 4.4 M urea, respectively). At pH 8, however, this was true only for the fluorescence emission data (3.9 M *versus* 3.5 M urea, respectively). In contrast, the data collected by CD at pH 8 demonstrated a mid-point that was significantly higher than that determined by equilibrium unfolding (3.8 M *versus* 2.8 M, respectively).

The data were fit to a two-state kinetic mechanism^{34; 35} using equation 1

$$k_{\text{obs}} = k_{\text{UN}} \exp\left(-m_{\text{U-TS}} * [\text{urea}] / RT\right) + k_{\text{NU}} \exp\left(m_{\text{N-TS}} * [\text{urea}] / RT\right) \quad [1]$$

where k_{obs} is the observed rate constant of the refolding or unfolding phase, and k_{UN} and k_{NU} are the rate constants for refolding and for unfolding, respectively, in the absence of urea. The parameters $m_{\text{U-TS}}$ and $m_{\text{N-TS}}$ reflect the change in solvent exposed surface area between the unfolded or refolded states, respectively, and the transition state ensemble. At pH 6, values of $3537 \pm 640 \text{ s}^{-1}$ and $0.72 \pm 0.05 \text{ kcal mol}^{-1} \text{ M}^{-1}$ were obtained for k_{UN} and $m_{\text{U-TS}}$, respectively, while values of $0.4 \pm 0.3 \text{ s}^{-1}$ and $0.50 \pm 0.11 \text{ kcal mol}^{-1} \text{ M}^{-1}$ were obtained for k_{NU} and $m_{\text{N-TS}}$, respectively. Overall, the data show that the higher pH affects both the rate of refolding and of unfolding. At pH 8, values of $1427 \pm 174 \text{ s}^{-1}$ and $0.68 \pm 0.04 \text{ kcal mol}^{-1} \text{ M}^{-1}$ were obtained for k_{UN} and $m_{\text{U-TS}}$, respectively, while values of $0.21 \pm 0.3 \text{ s}^{-1}$ and $0.64 \pm 0.19 \text{ kcal mol}^{-1} \text{ M}^{-1}$ were obtained for k_{NU} and $m_{\text{N-TS}}$, respectively. Although there are large errors in these values due to the long extrapolations to zero urea, the conformational free energy and total m-

value were calculated from these parameters, where $\Delta G^{H_2O} = -RT \ln(k_{UN}/k_{NU}) = -5.3$ kcal mol⁻¹ at pH 6 and -5.1 kcal mol⁻¹ at pH 8. The m-values were calculated as $m_{total} = m_{U-TS} + m_{N-TS} = 1.22$ kcal mol⁻¹ M⁻¹ at pH 6 and 1.32 kcal mol⁻¹ M⁻¹ at pH 8. These results suggest that the fast phase represents rapid folding to the native conformation since the conformational free energy and m-value are similar to those obtained from equilibrium unfolding data (Table 2).

The compactness ($\beta^\ddagger$) of the transition state ensemble relative to the native ($\beta^\ddagger=1$) and unfolded ensembles ($\beta^\ddagger=0$) can be calculated from the ratio of m_{U-TS}/m_{total} ³⁶. For Apaf-1 CARD, $\beta^\ddagger=0.59$ at pH 6, and $\beta^\ddagger=0.52$ at pH 8, demonstrating that the transition state for folding is somewhat more like the native ensemble than the unfolded ensemble.

Proposed folding models

The single mixing kinetic studies show that three phases are observed during refolding, two of which depend on the final urea concentration: a burst phase, a fast phase with a time constant of about 10 msec, and a slow phase with a time constant of about 1 sec. A minimal model that might explain these results is presented in Scheme 1, which shows that the unfolded protein folds sequentially through two non-native intermediate structures prior to forming the native conformation. In this Scheme, the burst phase is represented by the transition of U to I₁, the fast phase is represented by the transition of I₁ to I₂, and the slow phase is represented by the transition of I₂ to N. There are features of the kinetic data, however, that are not compatible with this sequential folding scheme. Under conditions that strongly favor the native conformation, Apaf-1 CARD folded within the mixing dead-time to a conformation, presumably the native structure, that had a ΔG^{H_2O} and m-value similar to those determined from equilibrium unfolding data. Thus, a significant amount of hydrophobic surface area was buried in the burst phase. As shown in Scheme 1, if the hydrophobic surface is buried in the burst phase, then there should be little buried in the fast and slow phases. The data show, however, that a significant amount of hydrophobic surface area was buried during the fast folding phase as well. In fact, the conformational free energy and m-value derived from the observed rate constants of the fast phase also were similar to those determined from equilibrium folding studies, suggesting that the native conformation formed during this phase. Consequently, there is seemingly a paradox in which two separate refolding transitions result in burial of surface area that is approximately equivalent to that of the native protein. We note that this is not due to formation of transient aggregates because the kinetic data were independent of protein concentration between 5 and 20 μ M (data not shown).

A second model that might explain the single mixing kinetic data is the parallel folding model shown in Scheme 2. In this Scheme, the unfolded ensemble consists of two conformations, U₁ and U₂, each of which fold to native conformations, N₁ and N₂. This Scheme allows refolding in the burst phase or in the fast phase to be separated into parallel folding channels. For example, folding in the burst phase yielded a species with a conformational free energy of ~3-4 kcal mol⁻¹, depending on the probe used to monitor refolding, and an m-value of 1.2-1.4 kcal mol⁻¹ M⁻¹. Regardless of the probe, the conformational free energy was about 1 kcal mol⁻¹ lower than that obtained from fitting the equilibrium unfolding data to a two-state model, although the m-values were similar. Likewise, folding in the fast phase yielded a species with a conformational free energy of 5.3 kcal mol⁻¹ and an m-value of 1.22 kcal mol⁻¹ M⁻¹, also similar to those determined from equilibrium unfolding. If the transition of U₁→N₁ represents folding that occurs in the burst phase, then the transition of U₂→N₂ represents folding in the fast phase. Under conditions that strongly favor the native conformation, that is, low urea concentrations, the protein folds from U₁→N₁ in the burst phase. At intermediate urea concentrations, refolding occurs from both channels, and at higher urea concentrations (>4 M), the protein refolds from U₂→N₂ in the fast phase. This simple scheme explains the burial of

hydrophobic surface area from two major transitions and the urea dependence to each major folding phase. The slow phase, which is independent of urea concentration, could represent isomerizations in the unfolded ensemble or the native ensemble. We performed sequential mixing stopped-flow studies, described below, to further examine the proposed folding schemes.

Sequential mixing kinetic studies

Initially, we examined the transitions using double jump studies. In these experiments, the native protein was mixed rapidly with urea, and after a specified time of unfolding (delay time), the protein was returned to native conditions^{37; 38}. The fluorescence signal was monitored experimentally after the second mix. This experiment examined the formation of unfolded species that were able to fold to the native conformation upon dilution of the denaturant, and the refolding traces should resemble those shown in Figure 3A only when those species are formed. As described above for single mixing refolding studies, when the protein was refolded in buffer containing low urea concentrations, only the burst phase and slow refolding phases were observed. Thus, in the double jump experiment, one should monitor the formation of the unfolded conformation by monitoring these two phases as a function of unfolding delay time. A measure of the refolding burst phase signal *versus* unfolding delay time showed that the signal reached that of the native conformation within 100 msec of unfolding (Figure 4, *circles*). That is, when the protein was unfolded for 100 msec, it formed a species that could refold to the native conformation. This was true at pH 6 and pH 8 (Figure 4). The data are in agreement with the single mixing unfolding studies (Figure 3A), which showed that Apaf-1 CARD unfolded rapidly in a single transition. The double jump experiments showed that the signal of the refolding burst phase remained constant even after extended periods of unfolding (>200 seconds). In addition, the amplitude of the slow phase reached a maximum at one second and did not change with longer delay times (data not shown).

The double jump experiment was repeated to monitor refolding in 4 M urea. In this experiment, the protein was initially unfolded in 5 M urea-containing buffer for various delay times, and then the urea was diluted to 4 M. As shown in Figure 3B, the burst phase of refolding was not observed at urea concentrations of 4 M or greater, although the fast and slow phases were observed under these conditions (Figures 3A and 3C). The amplitude of the fast phase *versus* delay time is shown in Figure 4 (*triangles*) for data collected at pH 6 (Figure 4A) or pH 8 (Figure 4B). The results showed that the fast phase of refolding appeared at short unfolding delay times (100 msec), and the amplitude of the phase did not change with longer times of unfolding. Therefore, the unfolded species that refolds via this phase formed fully within 100 msec of unfolding. As described above, the amplitude of the slow phase reached a maximum at one second and did not vary with longer delay times (data not shown).

In addition to double jump experiments, we examined the refolding pathways in interrupted refolding sequential mixing studies^{39; 40}. In these experiments, the unfolded protein was rapidly mixed with buffer, conditions that favor the native conformation, and the protein was returned to denaturing conditions after a specified time of refolding (delay time). The fluorescence signal was monitored experimentally following the second mix. This experiment monitored formation of the native conformation as a function of refolding time, and the unfolding traces should resemble those shown in Figure 3A when the native species is formed. As shown in Figure 3A, unfolding occurred from a single transition with a half-time of ~10 msec, so in the interrupted refolding experiment one should observe the fast unfolding transition only when the native conformation is formed. The results from this experiment are summarized in Figure 4 (*squares*) for data collected at pH 6 (Figure 4A) or pH 8 (Figure 4B). The amplitude of the unfolding transition and the apparent rate constant, k_{obs} , were obtained by fitting the raw data to a single exponential process. We note that k_{obs} did not vary with

refolding delay time and was similar to that determined from the single mixing unfolding studies (Figure 3A). In addition, the maximum amplitude of the phase was obtained within 1 msec of refolding, and the amplitude did not change with longer refolding delay times (Figure 4). Attaining the maximum amplitude within 100 msec of refolding showed that the native conformation was fully populated within this time and further demonstrated the absence of slow folding species on the folding pathway.

Kinetic simulations

The sequential mixing kinetic studies described above ruled out the sequential folding model shown in Scheme 1 because the native ensemble formed rapidly upon refolding, either in low urea concentrations or in 4 M urea. As described for the single mixing studies, these refolding phases were observed as burst phase or as fast phase kinetics, respectively. Furthermore, the unfolded ensemble formed within 100 msec of unfolding. Together, the results showed that the native ensemble was directly accessible from the unfolded ensemble. Formally, we have presented evidence for three kinetic phases during refolding: a burst phase, in which the native ensemble formed at low concentrations of urea, a fast phase, in which the native ensemble also formed at higher urea concentrations, and a slow phase, which encompassed less than ten percent of the total signal change but was observed at all urea concentrations examined. In intermediate concentrations of urea, the native conformation formed simultaneously from the burst phase and the fast phase of refolding. While the data are consistent with parallel folding channels, there is no direct evidence for four species as presented in Scheme 2. We further examined this folding model, as well as variations of the model, using kinetic simulations

We used the following reasoning in the simulations. The rate of refolding in the burst phase was assumed to be 5000 s^{-1} , which is sufficiently fast to allow for a burst phase in the simulations. In simulating refolding in 0.55 M urea, the final conditions in low urea concentrations, the fast phase was calculated to be 1800 s^{-1} (Figure 3F). The rate of refolding in 3.6 M urea was calculated to be 41 s^{-1} (Figure 3F). Those values were used to simulate refolding in low urea or in higher urea concentrations, respectively. In addition, simulations with a single unfolded species demonstrated that refolding occurred primarily in the burst phase since the rate was significantly faster than that of the fast phase in 3.6 M urea (5000 s^{-1} versus 41 s^{-1}). Thus, in order to observe refolding in the burst phase and the fast phase simultaneously, which is detected at intermediate urea concentrations, two unfolded species were populated.

We used a single native conformation in the simulations. This assumption was supported by single mixing unfolding experiments that showed that the protein unfolded in a single transition (Figure 3A). Furthermore, unfolding occurred from the native species that formed during the fast phase of refolding (Figure 3F). Therefore, regardless of whether the protein was refolded in low urea concentrations or in 4 M urea, unfolding occurred from a single species. If two native species were present at equilibrium, as suggested in Scheme 2, one might expect two transitions during unfolding. Otherwise, one must assume either that the two native species share the same rate-limiting step in unfolding or that one native species rapidly relaxes to the second native species prior to unfolding. Without further evidence to support these assumptions, we elected to use a single native conformation in the simulations. We note that sequential mixing unfolding experiments (that is, interrupted refolding studies) (Figure 4) demonstrated that the burst phase species was not positioned on-pathway between the unfolded and native ensembles. Indeed, those results showed that the native conformation formed within 100 msec of refolding. Likewise, sequential mixing refolding studies (that is, double jump experiments) (Figure 4) demonstrated that the unfolded ensemble formed rapidly from the native species. This was true also when the experiments were carried out in 4 M urea. In

addition, structural studies of Apaf-1 CARD using NMR²⁸ did not support the presence of two native conformations.

The slow phase in refolding was somewhat more problematic to simulate. For example, the slow phase was observed under conditions where the burst phase was not observed (Figure 3B). The slow phase also was observed when refolding occurred under conditions that favored the burst phase (Figures 3A and 3C). Neither the amplitude nor the rate of the slow phase was affected by urea concentration (Figure 3C). The simplest explanation for the slow refolding phase is the presence of slowly interconverting species in the unfolded ensemble. The simulations suggested, however, that the slow phase was not due to interconversion of the two unfolded species that yield the native conformation. Because the refolding rates were significantly faster than that of the slow phase, all of the unfolded species rapidly converted to the native conformation upon simulated refolding, leaving no species in the unfolded conformation to interconvert. In order to simulate the slow phase, we assumed that the unfolded ensemble contained a third unfolded conformation, U_3 , that slowly interconverted with U_1 and U_2 .

Based on those rationales, we propose the model shown in Scheme 3. In this model, the native conformation is accessible either from U_1 or U_2 . Refolding from U_1 to N occurs in the burst phase, and refolding from U_2 to N occurs in the fast phase. The slow phase is represented by the transition of U_3 either to U_1 or to U_2 . Furthermore, in this model, N unfolds to U_2 regardless of whether the protein was refolded in 0.55 M urea ($U_1 \rightleftharpoons N$) or in 3.6 M urea ($U_2 \rightleftharpoons N$). Thus, under the conditions used here to unfold the protein (6 M urea-containing buffer at pH 6), U_2 is the preferred conformation of the unfolded ensemble, with ~10% found as U_3 . In the simulations, we assumed that the protein unfolded through a single channel ($N \rightleftharpoons U_2$), so we have no information on the rate constant k_{-1} , that is, unfolding from N to U_1 . Upon refolding in 3.6 M urea, approximately 80% of the protein refolds from U_2 , and about 10% refolds from U_1 . Thus, by changing the distribution of U_1 and U_2 at intermediate urea concentrations, the burst phase and the fast phase are observed simultaneously during refolding. At low urea concentrations, the protein refolds primarily from U_1 (~80%). We note that the rates of interconversion between U_1 and U_2 are not known at present (k_2 and k_{-2} in Table 3), although the formation of U_1 from U_2 upon dilution from urea must be rapid in order to observe the burst phase. Under all conditions, about 10% of the protein refolds from U_3 . The rate constants determined from simulations of the data at pH 6 and at pH 8 are presented in Table 3 and are compared to those calculated from the experimental data. In addition, simulations of the refolding and unfolding data are shown in Figure 5 (panels A and B) for data at pH 6 and 8, respectively, and are compared to the experimental data. Overall, the results show good agreement between the simulated and experimental data at both pH values.

DISCUSSION

Since our Apaf-1 CARD construct contained two extra residues at the amino terminus, we crystallized the protein and determined the structure to 2 Å resolution in order to examine the effect of the residues on the structure. The results showed that the GS sequence does not hydrogen bond, nor does it form van der Waals contacts, with other atoms of the protein and thus does not significantly affect the structure of the protein.

We showed that refolding and unfolding of Apaf-1 CARD occurred rapidly. For example, at low urea concentrations, refolding occurred within the dead-time for mixing. An examination of the burst phase signals *versus* urea concentration showed that the mid-point of the transition was significantly lower than that of equilibrium unfolding regardless of whether fluorescence emission or CD kinetic studies were performed. At higher urea concentrations, the protein folded to the native conformation simultaneously through two distinct phases. Together the

kinetic data strongly suggest that the protein folds through parallel pathways, and the flux through each pathway depends on the final urea concentration. Simulations of the data suggested the model shown in Scheme 3 in which the unfolded ensemble consists of three conformations. The native conformation is accessible from U_1 and/or U_2 , depending on the final urea concentration, and isomerizations from U_3 account for the slow phase observed in the refolding experiments. At high urea concentrations (>5 M), the majority of the protein favored one unfolded conformation, which we designated U_2 .

We also have shown that equilibrium unfolding of Apaf-1 CARD is not consistent with a two-state process since unfolding of the secondary and tertiary structures were not coincident. Interestingly, the tertiary structure appeared to be more stable than the secondary structure since the mid-point of the unfolding transition ($urea_{1/2}$) was higher when fluorescence emission was monitored compared to far-UV CD. These results are contrary to those for RICK-CARD⁶ and CP1-CARD⁷, where there was no evidence for partially folded intermediates at equilibrium.

The equilibrium and kinetic studies of Apaf-1 CARD were carried out at pH 6, in order to examine folding near the isoelectric point, and at pH 8, in order to compare to the folding of other CARD proteins. The equilibrium data suggested that Apaf-1 CARD is less stable at pH 8 than at pH 6 since the mid-points of the unfolding transitions were lower compared to those at pH 6. In addition, there were larger differences between the two structural probes, CD and fluorescence emission, at pH 8 compared to pH 6. Because of the differences over this pH range, we suggest that charged histidine residues favor stability at pH 6. An examination of the structure shows that one histidine resides on each of helices 1, 2, 5, and 6 (Figure 1C). The tyrosines reside on helix 2 (Y24), helix 5 (Y68 and Y72), and turn 5 (Y80), which is located between helices 5 and 6. Based on the positioning of the tyrosines on one half of the protein, as shown in Figure 1, our equilibrium unfolding data suggest that helices 2 and 5, and possibly helix 6, are more stable than helices 1, 3 and 4 since the fluorescence emission unfolding profiles demonstrated a higher $urea_{1/2}$ than did the CD unfolding profiles, which monitor changes in secondary structure. The intermediates observed at equilibrium (Figure 2) likely represent the heterogeneity observed in the unfolded ensemble (Scheme 3). Changes in the final urea concentration affect the relative populations of U_1 and of U_2 , and we speculate that this also affects the rates at which helices 1, 3, and 4 fold and dock against the core. This interpretation is consistent with structural studies of the pyrin domain of Nalp1, which showed that helix 3 is disordered⁴². For Apaf-1 CARD at pH 8, where the histidines are uncharged, the secondary structure appeared much less stable than the tertiary structure. This may be due to loss of interactions between histidine 88, on helix 6, and tyrosine 72, on helix 5, which are 4.6 Å apart (Figure 1C). Although this requires further study, the loss of these putative interactions may destabilize helices 5 and 6.

When compared to previous results for RICK-CARD⁶ or CP1-CARD⁷, the results described here show that the folding of Apaf-1 CARD is complex, as it is for the other CARDS. Although the rates differed, the folding pathways were similar at pH 6 and pH 8 for Apaf-1 CARD, and the data collected at pH 8 allow a direct comparison of the three proteins. As noted above, there is no evidence to date for equilibrium intermediates in the folding of RICK or CP1 CARDS, and both proteins are adequately described by an apparent two-state equilibrium folding model. In addition, the apparent m -value for Apaf-1 CARD, as shown in Table 2, is similar to that of RICK-CARD (~ 1.3 kcal mol⁻¹ M⁻¹) and is much larger than that of CP1-CARD (0.65 kcal mol⁻¹ M⁻¹). As noted previously⁶, the m -values of RICK-CARD and of Apaf-1 CARD are consistent with proteins of 97 amino acids, based on the work of Scholtz and coworkers⁴³. It was suggested, however, that CP1-CARD contains non-native structure in the native ensemble⁷, explaining the low m -value. The folding pathways of CP1-CARD and of RICK-CARD are complex and contain misfolded or kinetically trapped species. While CP1-CARD appeared to

fold rapidly in single mixing kinetic studies, sequential mixing studies showed that the native conformation formed fully only after about 100 seconds of refolding⁷. This was true also for RICK-CARD, except that several phases were observed during folding⁶. In the case of RICK-CARD, an analysis of several proline-to-alanine replacement mutants demonstrated that the kinetic complexity was not due to the three prolines present in that protein³¹. That is, the kinetically trapped species was present in a triple mutant that lacked prolines. As noted, the data for RICK-CARD are difficult to reconcile with a sequential folding pathway, and it was suggested that the protein folds via parallel channels³¹. Like RICK-CARD, Apaf-1 CARD also appears to fold via parallel channels. Apaf-1 CARD contains two prolines, P47, in turn three, and P92 near the C-terminus of the protein (Figure 1), although at present it is not clear whether either proline is responsible for the slow isomerization observed in the refolding of Apaf-1 CARD. P47 is conserved between Apaf-1 CARD and RICK-CARD but is not conserved in other CARDS. While removal of P47 destabilized RICK-CARD, the folding kinetics were similar to those of the wild-type protein.

The results described here show that folding of Apaf-1 CARD is complex, as observed with other CARDS, although the complexity does not result from formation of kinetically trapped conformations. In contrast to Apaf-1 CARD, we showed previously that the fast phase in refolding of RICK-CARD has an apparent rate of $\sim 32 \text{ s}^{-1}$, and this transition represents the primary folding phase in terms of burial of hydrophobic surface area⁶. Interestingly, the transition state placements of Apaf-1 CARD ($\beta^\ddagger = m_{\text{U-TS}}/m_{\text{U-N}} = 0.52$ at pH 8) and of RICK-CARD ($\beta^\ddagger = 0.71$)⁶ correlate with their refolding rates. As described previously⁵, this suggests that the more poorly packed transition state for Apaf-1 CARD results in a lower loss in chain entropy while overcoming the rate limiting step to folding. In contrast, the more highly organized folding transition state for RICK-CARD may result in higher chain entropy losses and hence a decrease in the folding rate. To date, this is the best correlation that explains the differences in folding rates between the CARDS, although it does not explain the formation of kinetically trapped species in the folding of RICK-CARD or CPI-CARD.

METHODS

Materials

Ultrapure urea was purchased from ICN. Dithiothreitol (DTT) was from Acros. 2-(4-morpholino)-ethane sulfonic acid (MES), sodium chloride (NaCl), 2-mercaptoethanol, trizma base (Tris), and reduced glutathione were from Fisher. Acetic acid was from VWR. DEAE sepharose, monobasic and dibasic potassium phosphate (KH_2PO_4 , K_2HPO_4), ampicillin, and molecular weight markers were from Sigma. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was from Anatrace. All buffers were filtered through either 0.45 or 0.22 μm filter membranes. The urea-containing buffers were prepared as described previously⁴⁴. Apaf-1 CARD was expressed and purified from *E. coli* BL21(DE3) pLysS cells harboring plasmid pApaf-1CARD +GS as described previously³⁰. An extinction coefficient (ϵ_{280}) of $5,120 \text{ M}^{-1} \text{ cm}^{-1}$ was used to determine protein concentration. Caspase-3 was purified as described previously^{45; 46}.

Crystallography

Apaf-1 CARD was dialyzed at a concentration of 5 mg/ml in a buffer of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl. The best crystals were formed using the hanging drop method by mixing 2 μL of protein with 2 μL of reservoir buffer containing 200 mM Tris-HCl, pH 6.1, 200 mM zinc acetate, 17% PEG 6000. Crystals were grown for approximately one week at 18 °C and flash frozen using glycerol (10%) as a cryoprotectant. Data sets were collected at 100 K at the SER-CAT synchrotron beamline (Advance Photon Source, Argonne National Laboratory, Argonne, IL) and processed with HKL2000⁴⁷. Ten percent of the unique reflections were randomly selected and reserved for the calculation of R_{free} ⁴⁸. The crystal structure was solved by the

molecular replacement method using the CNS software package⁴⁹ with the previously published coordinates (PDB code 1CY5²⁷) as the search model. The model was refined with alternating rounds of manual rebuilding in O⁵⁰ with visualization of $F_o - F_c$ and $2F_o - F_c$ electron density maps and crystallographic refinement in CNS⁴⁹. The final structure was checked with the online resource MolProbity⁵¹ and deposited in the Protein Data Bank.

Equilibrium unfolding

Equilibrium unfolding experiments were performed as described previously^{6; 7} by mixing native protein with urea-containing MTA (10 mM MES, 20 mM Tris, 10 mM acetate, pH 6.0 or pH 8.0, 1 mM DTT) or potassium phosphate (50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6.0, 1 mM DTT) buffers. The final protein concentrations were 5, 10 or 15 μM , and a quartz cuvette with a 1 cm path length was used for data collection. The CD signal at 222 nm was measured for 20 seconds, and the data were averaged. In addition, fluorescence emission was measured at 305 nm following excitation at 280 nm. The data *versus* urea concentration were fit globally to a two-state equilibrium process³³ using the program IgorPro (Wavemetrics Inc.). All samples were maintained at a constant temperature of 25 °C using a circulating water bath. In control experiments, stock protein was initially unfolded in 6 M urea-containing MTA or phosphate buffer, the samples were diluted to several final urea concentrations that encompass the folding transition, and the samples were incubated overnight at 25 °C. The signals were identical to those shown in Figure 2, in which the protein stock was not initially unfolded (data not shown).

Single-mixing stopped-flow fluorescence

Single-mixing unfolding and refolding fluorescence (SX18, Applied Photophysics, UK) and circular dichroism (PiStar, Applied Photophysics, UK) experiments were performed as described previously⁶ with the following modifications. For refolding experiments, protein stock solutions (55, 110 or 220 μM) that had been prepared in MTA buffer containing 6 M urea were rapidly diluted into MTA buffer containing urea between 0 and 7 M. For unfolding experiments, protein stock solutions (55, 110 or 220 μM) in MTA buffer were mixed with MTA buffer containing urea between 0 and 7 M. For both refolding and unfolding, a mixing ratio of 1:10 was used, the final protein concentrations were 5, 10, or 20 μM , and the final urea concentrations are shown in the Figures.

Sequential mixing stopped-flow experiments

Double jump stopped-flow experiments were performed as described previously⁶ with the following modifications. For experiments at pH 6 or at pH 8, native protein (120 μM) in MTA buffer was mixed 1:1 with 10 M urea-containing MTA buffer for the first jump. After various delay times, as described in the text, the protein solution was mixed 1:5 with MTA buffer, and the signal trace was monitored for 10 seconds. The final protein concentration was 10 μM , and the final urea concentration was 0.83 M. For the native protein signal, a protein stock solution (120 μM) in 0.83 M urea-containing MTA buffer was mixed with 0.83 M urea-containing MTA buffer. For the unfolded protein signal, a protein stock solution (120 μM) in 5 M urea-containing buffer was mixed with 5 M urea-containing MTA buffer.

For sequential mixing double jump studies in 4 M urea (pH 6) or 3.18 M urea (pH 8) (final concentrations), native protein (120 μM) in MTA buffer (pH 6 or pH 8) was mixed 1:1 with 10 M urea-containing buffer for the first jump. After various delay times, as described in the text, the protein solution was mixed 1:5 with 3.8 M (pH 6.0) or 2.82 M (pH 8.0) urea-containing MTA buffer, and the signal trace was monitored for 10 seconds. The final protein concentration was 10 μM , and the final urea concentration was 4 M (pH 6) or 3.18 M (pH 8). For the native protein signal at pH 6, a protein stock solution (120 μM) in 4 M urea-containing MTA buffer was mixed with 4 M urea-containing MTA buffer.). For the native protein signal at pH 8, a

protein stock solution (120 μ M) in 3.18 M urea-containing MTA buffer was mixed with 3.18 M urea-containing MTA buffer. For the unfolded protein signal at pH 6 and pH 8, a protein stock solution (120 μ M) in 5 M urea-containing buffer was mixed with 5 M urea-containing MTA buffer.

Interrupted refolding stopped-flow experiments were performed as described previously⁶ with the following modifications. Unfolded protein (360 μ M) in 5 M urea-containing MTA buffer was mixed 1:5 with MTA buffer (pH 6 or pH 8) for the first jump. After various delay times, as described in the text, the protein solution was mixed 1:5 with 5.9 M urea-containing MTA buffer (pH 6 or pH 8). The final protein concentration was 10 μ M, and the final urea concentration was 5.1 M. For the native protein signal, a protein stock solution (120 μ M) in 0.83 M urea-containing MTA buffer (pH 6 or pH 8) was mixed with 0.83 M urea-containing MTA buffer. For the unfolded protein signal, a protein stock solution (120 μ M) in 5 M urea-containing buffer was mixed with 5 M urea-containing MTA buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of Apaf-1 CARD with GS sequence at the amino terminus. Side view (A) and top view (B) of Apaf-1 CARD. Helices 1 through 6 are labeled H1-H6. The four tyrosine residues are colored blue, and the two proline residues are colored yellow and are labeled. The first three residues (G-2, S-1, M1) are colored cpk and are labeled. (C) The four histidine residues of Apaf-1 CARD (H12, H28, H77, H88) are highlighted relative to their position in helices-1, -2, -5, and -6, respectively. The distances between H28 and H77 and between H88 and Y72 are shown. This figure was generated with PyMol (Delano Scientific, San Carlos, CA).

Figure 2.

Equilibrium studies of Apaf-1 CARD. (A) Equilibrium unfolding of Apaf-1 CARD was measured by CD at 222 nm in urea-containing MTA buffer at pH 6 (○) or pH 8 (●). Error bars show the standard deviation from three independent experiments. (B) Equilibrium unfolding of Apaf-1 CARD was measured by fluorescence emission at 305 nm with excitation at 280 nm in MTA buffer at pH 6 (○) or pH 8 (●). Error bars show the standard deviation from three independent experiments. (C) and (D) Normalized equilibrium unfolding of Apaf-1 CARD was measured by CD at 222 nm (●) or by fluorescence emission at 305 nm with excitation at 280 nm (○) in urea-containing MTA buffer at pH 6 (C) or pH 8 (D). Error bars show the standard deviation from three independent experiments. For A-D, solid lines represent fits of the data as described in the text.

Figure 3.

Single-mixing stopped-flow fluorescence emission experiments. (A) Refolding of Apaf-1 CARD in 0.55 M (○) or 3.6 M (◇) urea-containing MTA buffer at pH 6. Unfolding of Apaf-1 CARD in 6 M urea-containing MTA buffer (□, △). A control of Apaf-1 CARD in 6 M urea is shown (□). Solid lines represent fits to single (unfolding) or double (refolding) exponential equations. Parameters obtained from the fits are described in the text. The upper and lower panels are residuals of one or two exponential fits of 3.6 M refolding data (◇), respectively. (B) Burst phase (○) or final (□) fluorescence emission signal *versus* urea concentration obtained from single-mixing stopped-flow refolding experiments. The final signal was measured at 10 seconds. (C) Amplitude of the fast (pH 6 (○) or pH 8 (●)) and slow (pH 6 (□) or pH 8 (■)) phases *versus* urea concentration from single-mixing stopped-flow refolding experiments. Refolding data such as those in Panel A were fit to a single or double exponential equation, as described in the text, and the amplitudes obtained from the fits are plotted *versus* urea

concentration. (D) Single-mixing stopped-flow unfolding experiments were performed at the urea concentrations shown in the figure, and the data were fit to a single exponential equation. The amplitude associated with the unfolding transition at each urea concentration is shown. (E) Final fluorescence emission signal of stopped-flow unfolding experiments. The final signals for the unfolding experiments shown in Panel D were obtained after 10 seconds of unfolding. In Panels B, D, and E, the solid lines represent fits of the data to a two-state equilibrium folding model. Parameters obtained from the fits are described in the text and are shown in Table 2. (F) Apparent rates of refolding (pH 6 (○) and pH 8 (□)) and of unfolding (pH 6 (●) and pH 8 (■)) *versus* urea concentration. The apparent rates are associated with the refolding and unfolding data described in Panel C and D, respectively. The solid lines represent fits to a two-state kinetic folding model. Parameters obtained from the fits are described in the text.

A

B

Figure 4.

Sequential-mixing stopped-flow fluorescence studies. (A) Double-jump experiments were performed at final urea concentrations of 0.83 M (○) or 4 M (△) in MTA buffer at pH 6. The fluorescence emission signal of the burst phase (○) and the amplitude of the fast refolding phase (△) were determined *versus* unfolding delay time. Interrupted refolding experiments (□) were performed at a final urea concentration of 5 M in MTA buffer at pH 6. The amplitude *versus* refolding delay time was determined from fits of the unfolding transition to a single exponential equation. The signals of the native protein in 0.83 M or in 4 M urea are 0.05 and 0.028, respectively. (B) Double-jump experiments were performed at final urea concentrations of 0.83 M (○) or 3.18 M (△) in MTA buffer at pH 8. The fluorescence emission signal of the burst phase (○) and the amplitude of the fast refolding phase (△) were determined *versus* unfolding delay time. Interrupted refolding experiments (□) were performed at a final urea concentration of 5 M in MTA buffer at pH 8. The amplitude *versus* refolding delay time was determined

from fits of the unfolding transition to a single exponential equation. The signals of the native protein in 0.83 M or in 3.18 M urea are -0.17 and -0.185 , respectively.

Figure 5.

Simulations of single-mixing stopped-flow fluorescence emission experiments. (A) Refolding and unfolding of Apaf-1 CARD are as shown in Figure 3A (pH 6). (B) Refolding of Apaf-1 CARD in 0.45 M ($\circ$) or 3.2 M ($\diamond$) urea-containing MTA buffer at pH 8. Unfolding of Apaf-1 CARD in 5 M urea-containing MTA buffer ($\triangle$). A control of Apaf-1 CARD in 5 M urea is shown ($\square$). For A and B, the data were simulated as described in the text using the model shown in Scheme 3. The rate constants used in the simulations are shown in Table 3, and the results are represented by the solid lines.

Scheme 1.

Sequential folding model in which the unfolded protein, U, folds through two non-native intermediates, I_1 and I_2 , prior to forming the native conformation, N.

Scheme 2.

Parallel folding model in which the unfolded ensemble contains at least two conformations, U_1 and U_2 . Both unfolded conformations fold to native conformations, N_1 and N_2 , respectively. In this scheme, both the unfolded and the native conformations interconvert.

Scheme 3.

Parallel folding model in which the unfolded ensemble contains at least three conformations, U_1 , U_2 , and U_3 . Unfolded conformations U_1 and U_2 fold to a single native conformation, N . Conversion of U_3 either to U_1 or U_2 , or both, represent slow isomerizations observed during refolding. Rate constants determined from kinetic experiments as well as those estimated from simulations of the data are shown in Table 3.

Table 1
Summary of statistics for data collection and refinement for Apaf-1 CARD

Temperature (K)	100
Resolution (Å)	35-2.0 (2.00-2.07)
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å, deg.)	
a (Å)	33.47
b (Å)	47.52
c (Å)	54.75
α,β,γ (deg.)	90
No. reflections	5981 (458)
Completeness (%)	96 (75)
I/σ	24.4 (4.8)
R _{merge} (%) ^a	10.6 (23.1)
Redundancy	5.6 (1.4)
R _{work} (%) ^b	23.9 (31.7)
R _{free} (%) ^c	29.7 (32.0)
Stereochemistry (r.m.s. deviation)	
Bond lengths (Å)	0.006
Bond angles (deg.)	1.14
Dihedral angles (deg.)	17.4
Improper (Å)	0.6
Average B factor (Å ²)	42.4
No. protein atoms	753
No. Zn ²⁺	6
No. water molecules	55

^a $R_{\text{merge}} = \sum_h \sum_i |I(h,i) - I(h)| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ are symmetry-related intensities and $I(h)$ is the mean intensity of the reflection with unique index h .

^b $R_{\text{work}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors.

^c $R_{\text{free}} = \sum T ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum T |F_{\text{obs}}|$, where T is a test data set of 10% of the total reflections randomly chosen and set aside prior to refinement.

Table 2

Summary of thermodynamic parameters for Apaf-1 CARD

Experiment	ΔG^{H_2O} (kcal mol ⁻¹) FL	ΔG^{H_2O} (kcal mol ⁻¹) CD	m (kcal mol ⁻¹) FL	m (kcal mol ⁻¹) CD	Urea _{1/2} (M) ^d FL	Urea _{1/2} (M) ^d CD
pH 6						
Equilibrium unfolding ^b	5.2±0.9	4.2±0.6	1.17±0.20	1.12±0.17	4.4	3.8
Refolding burst phase ^c	4.2±0.6	2.9±0.8	1.40±0.43	1.23±0.30	3.0	2.4
Refolding final signal ^c	5.1±1.4	4.9±0.4	1.10±0.28	1.18±0.11	4.6	4.2
Unfolding fast phase amplitude ^d	5.1±0.8		1.20±0.40		4.3	
Unfolding final signal ^e	4.9±0.9	5.1±0.2	1.20±0.21	1.31±0.54	4.1	3.9
Fast phase rates ^f	5.3		1.22		4.3	
pH 8						
Equilibrium unfolding ^b	4.8±0.8	3.1±0.5	1.38±0.23	1.11±0.16	3.5	2.8
Refolding burst phase ^c	3.6±0.3	2.0±0.5	1.40±0.12	1.00±0.30	2.3	2.0
Refolding final signal ^c	5.0±0.5	4.4±0.6	1.60±0.18	1.32±0.16	3.1	3.3
Unfolding fast phase amplitude ^d	4.7±0.01		1.35±0.02		3.5	
Unfolding final signal ^e	4.4±1.8	4.6±0.3	1.26±0.54	1.34±0.41	3.5	3.4
Fast phase rates ^f	5.1	5.1	1.32	1.33	3.9	3.8

^a Calculated as Urea_{1/2} = ΔG^{H_2O} / m

^b From fits of data in Figure 2

^c From fits of data in Figure 3B or Supplemental Figures 1-3

^d From fits of data in Figure 3D or Supplemental Figure 3C

^e From fits of data in Figure 3E or Supplemental Figures 1-3

^f From fits of data in Figure 3F

Table 3

Summary of calculated and simulated rates using Scheme 3

	k_1^a (sec^{-1})	k_3 (sec^{-1})	k_{-3} (sec^{-1})	k_4 (sec^{-1})	k_{-4} (sec^{-1})	k_5 (sec^{-1})	k_{-5} (sec^{-1})
Refolding pH 6							
(3.6 M) calculated rate ^b	burst	0.6		0.6		41	8.4
(3.6 M) simulated rate	5000	0.6		0.6		37	13
(0.55 M) calculated rate	burst	0.6	0.01	0.6	0.01	1800	0.63
(0.55 M) simulated rate	5000	0.6	0.01	0.6	0.01	1800	0.6
Refolding pH 8							
(3.2 M) calculated rate	burst	0.6		0.6		37	6.6
(3.2 M) simulated rate	5000	0.6		0.6		35	8
(0.45 M) calculated rate	burst	0.6	0.01	0.6	0.01	850	0.34
(0.45 M) simulated rate	5000	0.6	0.01	0.6	0.01	850	0.3
Unfolding pH 6							
(6 M) calculated rate						61	2.3
(6 M) simulated rate						70	
Unfolding pH 8							
(5 M) calculated rate						48	4.6
(5 M) simulated rate						55	

^a Rates correspond to those shown in Scheme 3. k_{-1} , k_2 , and k_{-2} are not shown since no information was obtained in the simulations for these rates.

^b Final urea concentration is indicated in parentheses for each experiment.