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Thermodynamic, Enzymatic and Structural Effects of Removing a Salt Bridge at the Base of Loop 4 in (Pro)caspase-3

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Abstract

Interactions between loops 2, 2' and 4, known as the loop bundle, stabilize the active site of caspase-3. Loop 4 (L4) is of particular interest due to its location between the active site and the dimer interface. We have disrupted a salt bridge between K242 and E246 at the base of L4 to determine its role in overall conformational stability and in maintaining the active site environment. Stability measurements show that only the K242A single mutant decreases stability of the dimer, whereas both single mutants and the double mutant demonstrate much lower activity compared to wild-type caspase-3. Structural studies of the caspase-3 variants show the involvement of K242 in hydrophobic interactions that stabilize helix 5, near the dimer interface, and the role of E246 appears to be to neutralize the positive charge of K242 within the hydrophobic cluster. Overall, the results suggest E246 and K242 are important in procaspase-3 for their interaction with neighboring residues, not with one another. Conversely, formation of the K242–E246 salt bridge in caspase-3 is needed for an accurate, stable conformation of loop L4 and proper active site formation in the mature enzyme.

Keywords

Apoptosis; caspase activation; enzyme activity; protein folding and assembly

1. INTRODUCTION

Apoptosis relies on the activation of effector caspases, primarily the executioner caspase-3. Procaspase-3 is a stable dimer that has very little enzymatic activity until cleavage of the intersubunit linker (IL) at D175 by upstream initiator caspases, where IL cleavage results in rearrangement of several active site loops, formation of the substrate binding pocket and proper orientation of the catalytic dyad, C163 and H121 [1,2]. In contrast, initiator procaspases are stable monomers that are activated via dimerization on death scaffolds [3–6]. Importantly, cleavage of the IL is not required for activation of initiator procaspases since dimerization is sufficient for proper active site formation. Thus, procaspase dimerization is a vitally important control mechanism for the initiation of apoptosis. Presently, it is not clear why procaspase-3 is a stable dimer that exhibits little enzyme activity, nor is it clear why initiator procaspases are monomers until activated.

We showed previously that procaspase-3 unfolds with a unique four-state mechanism where the native dimer (N_2) isomerizes to an inactive dimeric intermediate (I_2), which dissociates to a monomeric intermediate (I) prior to monomer unfolding (U) [7]. The dimer is most stable at pH ~ 7 (~ 26 kcal/mol), where dimerization and a subsequent isomerization contributes significantly to protein stability. In addition, the dimer is destabilized relative to the monomer at pH below ~ 5 [8]. Although dimerization of procaspase-3 is very slow, with a second order rate of $\sim 70 \text{ M}^{-1} \text{ sec}^{-1}$ [9], the rate of dimerization of the initiator procaspase-8 is essentially zero in the absence of natural or artificial dimerization agents [10].

While the mechanisms that govern dimerization and stability remain elusive, the role of dimerization in active site formation is more clear due to structural studies of several caspases [11–15]. Even though the overall structure is very similar among the three caspase subfamilies, with several α -helices outside a 12-stranded β -sheet core, the dimer interfaces are very different. Caspase-3 contains a cluster of hydrophobic amino acids at the center of the interface, whereas initiator caspases contain more polar interfaces. Upon cleavage, the IL of caspase-3 is released from binding in the interface cavity and subsequently forms two active site loops, 2 and 2', which in turn form H-bonds, ionic interactions and van der Waals contacts in the newly formed active site as well as across the interface. Interactions in active site loops 2, 2', and 4 (called L2, L2', and L4) are known as the loop bundle and have been shown to stabilize the active site (Figure 1) [16]. In contrast, initiator procaspases contain longer ILs that may release conformational restraints observed in procaspase-3.

Due to the location of L4, situated between the substrate binding pocket and the interface, the interactions contributed by amino acids in L4 confer enzyme specificity, via interactions with P4 of the substrate, and dimer stability, since contacts are made across the interface. In particular, we showed that a salt bridge between K242 and E246, at the junction between L4 and helix 5 (Figure 1A) is important for stabilizing the active site [17]. Replacement of either amino with alanine resulted in differential effects on enzyme activity, where the activity decreased between ~ 15 and ~ 60 -fold compared to wild-type caspase-3. While neither mutation affected oligomerization of the procaspase, biochemical studies showed that the mutations affected loop bundle formation in the mature caspase.

Located at the C-terminus of helix 5 (Figure 1A), K242 not only interacts with E246, but it also makes several hydrophobic contacts with neighboring amino acids. In contrast, E246, at the base of L4, is mostly exposed to solvent (Figure 1B). Furthermore, several interactions are made across the dimer interface by residues upstream of K242. Of note, H234 and E231 form several ionic contacts across the interface, and we suggested that removal of the K242-E246 salt bridge may also affect dimer stability due to destabilizing helix 5 [17]. While our previous studies highlight the importance of the K242-E246 interaction in context of activity and active site formation, a number of questions remained regarding the importance of the interaction for maintaining procaspase stability in addition to structural changes in the mature caspase. We report here an examination of the double mutant, K242A,E246A, as well as structural and thermodynamic characterizations of the single and double mutants. X-ray crystallographic analyses of the three mutants show that removal of the salt bridge affects formation of the loop bundle as well as interface contacts. Importantly, our data also suggest that the salt bridge may not be intact in the procaspase but rather forms only upon maturation and formation of the loop bundle. Both K242 and E246 appear to be involved in other interactions in the procaspase, where stability measurements show that contacts provided by K242 are important for maintaining the native procaspase dimer, so the two residues function differently in the context of the zymogen or the mature caspase.

2. MATERIALS AND METHODS

2.1 Materials

The single caspase-3 mutants, K242A and E246A, were made in the background of C163S or D₃A as described previously [17]. The double mutant, K242A,E246A, was made in a similar manner using site-directed mutagenesis and the primers for the single mutants. All proteins were purified as described [18].

2.2 Enzyme activity assays

Enzyme activity was assayed in a buffer containing 20 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10% sucrose, and 10 mM DTT (enzyme assay buffer) at 25 °C, as described previously [17,19]. The total reaction volume was 200 μ L and the final enzyme concentration was 10 nM. Following addition of the substrate (Ac-DEVD-AFC), the samples were excited at 400 nm, and fluorescence emission was monitored at 505 nm for 60 seconds. All fluorescence emission measurements were acquired using a PTI C-61 spectrofluorometer (Photon Technology International, Birmingham, New Jersey). The steady-state parameters, K_M and k_{cat} were determined from plots of initial velocity versus substrate concentration, and the data shown are an average of four experiments.

2.3 Equilibrium unfolding

Equilibrium unfolding experiments were performed as described previously [7,20]. Briefly, protein stock solutions were prepared in phosphate buffer, and the unfolding samples were prepared by adding the stock protein to varying concentrations of urea-containing phosphate buffer as shown in the figures. The refolding samples were prepared similarly with the exception of the starting material being unfolded protein. The final protein concentrations varied between 0.5 and 2 μ M, as described in the figures. The samples were incubated at 25 °C for 24 hours prior to data collection, as this incubation time was determined to be adequate for allowing the reactions to equilibrate.

Fluorescence emission scans were measured using a PTI C-61 spectrofluorometer. Time-based scans were acquired at excitation wavelengths of 280 nm or 295 nm with fluorescence emission measured at 335 nm. Circular dichroism (CD) was measured using a PiStar spectropolarimeter (Applied Photophysics, Surrey, UK) at 228 nm. The data were averaged for 20 seconds. Both instruments were equipped with water jackets to maintain a constant temperature. Data were fit globally as described [7,20] to determine the ΔG° and m-values for each step in unfolding.

2.4 Crystallization and data collection

Proteins were dialyzed in a buffer of 10 mM Tris-HCl (pH 8.5) and 1 mM DTT. The protein was concentrated to 10 mg/mL using Amicon ultrafree centrifugal filter devices, and inhibitor, Ac-DEVD-CMK, reconstituted in DMSO, was then added at a 5:1 inhibitor:protein ratio (w/w). The protein was diluted to a concentration of 8 mg/mL by adding 10 mM Tris-HCl (pH 8.5), concentrated DTT, and concentrated NaN₃ so that the final protein buffer consisted of 10 mM Tris-HCl (pH 8.5), 10 mM DTT, and 3 mM NaN₃. Crystals were obtained at 18 °C by the hanging drop vapor diffusion method using 4 μ L drops that contained equal volumes of protein solution and reservoir buffer over a 0.5 mL reservoir. The reservoir solutions for optimal crystal growth consisted of 100 mM sodium citrate (pH 5), 3 mM NaN₃, 10 mM DTT, and 10% – 16% PEG 6000 (w/v). Crystals appeared within two weeks. Before flash-freezing for data collection, the crystals were briefly immersed in cryogenic solution containing 10% MPD and 90% reservoir solution. Data sets were collected at 100 K at the SER-CAT synchrotron beamline (Advance Photon Source, Argonne National Laboratory, Argonne, IL). The X-rays had a wavelength of 1 Å,

and one hundred and eighty frames of diffraction data were collected for each protein at 1° intervals. All three mutants crystallized with the symmetry of the orthorhombic space group I222 and were phased with a previously published caspase-3 structure (PDB entry 2J30), as described [21]. A summary of the data collection and refinement statistics is shown in Table 3. The atomic coordinates and structure factors for caspase-3(K242A), caspase-3(E246A), and caspase-3(K242A,E246A) have been deposited in the PDB under accession codes 3PD1, 3PD0, and 3PCX, respectively.

3. RESULTS

3.1 (Pro)caspase mutants

The single mutants, K242A and E246A, and the double mutant, K242A,E246A, were produced in three backgrounds of (pro)caspase-3. First, the mutations were introduced into wild-type procaspase-3, allowing one to examine the effects of the mutations on mature caspase-3 because the active procaspase autoprocesses during expression in *E. coli*. Second, the mutations were introduced into the uncleavable procaspase-3 variant, D9A,D28A,D175A (called D₃A), in order to examine enzyme activity of the procaspase. As described previously[21,22], removal of the processing sites provides a catalytically active yet uncleavable procaspase. Third, the mutations were introduced into the background of the catalytically inactive mutant C163S in order to examine the effects on procaspase stability compared to our previous data for procaspase-3(C163S) [7,8]. We have shown that there is little difference in the folding characteristics of the D₃A and C163S procaspases [9].

3.2 Enzyme activity

We previously determined the steady-state parameters, K_M and k_{cat} , of the two single mutants K242A and E246A, where our data showed that both mutations abrogated activity in the procaspase and that K242A had a larger effect on the activity of the mature enzyme [17] (see also Table 1). The specificity constant, k_{cat}/K_M , was about 60-fold lower for K242A compared to that of wild-type largely due to a decrease in k_{cat} . Although it was clear from the data for the single mutants that the salt bridge between K242 and E246 was important for maintaining the active site, it was unclear whether the effects would be additive or cooperative or why the loss of K242 was more detrimental than that of E246, which necessitated an examination of the double mutant.

The procaspase-3(D₃A,K242A,E246A) variant showed no activity against the tetrapeptide substrate (data not shown). As described previously, the lower limit for detection of enzyme activity in the fluorescence emission assay is approximately 50-fold less than that of procaspase-3(D₃A) [17]. Because the activities of the single mutants were below this detection limit, it is not surprising that the activity of the double mutant also was undetectable.

In contrast, the activity of caspase-3(K242A,E246A) was detectable, and the data showed that the double mutant affected both K_M and k_{cat} (Table 1). For this mutant, K_M was about 15-fold higher and k_{cat} was about 100-fold lower compared to wild-type caspase-3, providing a specificity constant about 1000-fold lower than that of wild-type. When viewed as a double-mutant cycle (Figure 1D), the data for the single and double mutants show a free energy change of 2.4 kcal mol⁻¹ upon replacement of K242 and 1.6 kcal mol⁻¹ upon replacement of E246, where the change in free energy is calculated as shown in equation 1.

$$\Delta\Delta G^\circ = -RT \ln \left(\frac{k_{cat}/K_{M(\text{mutant})}}{k_{cat}/K_{M(\text{wild-type})}} \right) \quad (1)$$

In the case of the double mutant, the free energy change is 4.3 kcal mol⁻¹, which is equal to the sum of the two single mutants in the double-mutant cycle. We note that the difference of 0.3 kcal mol⁻¹ for each parallel arm of the cycle is likely within the error for the measurements. Although K242 and E246 are important for active site stability in both the procaspase as well as the mature caspase, the data show that the effects of removal of each amino acid are additive rather than cooperative. This is likely due to the fact that neither residue is directly involved in catalysis but rather the interactions stabilize L4 and indirectly affect substrate binding and catalysis.

3.3 Equilibrium folding of procaspase-3 variants

As described previously [7,8,20], four protein concentrations and three spectroscopic probes are used to examine procaspase unfolding: fluorescence emission with excitation at 280 nm or 295 nm and far-UV circular dichroism. This method generally provides eleven data sets that are fit globally to the four-state equilibrium unfolding model (described below). Representative unfolding data are shown in Figure 2, and results of the global fits are shown in Table 2. All three proteins were unfolded reversibly, as shown by the solid symbols in the figure.

For the procaspase-3 variants, individual denaturation curves appear to be described by a three-state mechanism, with a plateau between ~3 and ~5 M urea and a mid-point for the first cooperative transition of ~2.5 M urea. When examined at various protein concentrations, however, one observes that the mid-point of the second transition increased from ~5.5 M to ~7 M urea as the protein concentration was increased. In addition, the relative signal of the plateau increased at higher protein concentrations. Together, the data are similar to those described previously for procaspase-3 in which a four-state equilibrium unfolding model (equation 2) adequately describes the data [7].

In the four-state model, the native dimer (N₂) isomerizes to a dimeric intermediate (I₂) that dissociates to a monomeric intermediate (I) prior to unfolding (U). The individual unfolding curves in Figure 2 contain two transitions, indicating three species, but when examined at several protein concentrations and spectroscopic probes, the data show that the four-state model is required. In this model, the two intermediates (I₂ and I) are present over a similar range of urea concentrations (~3–6 M), and the dissociation of the dimer is accompanied by a change in signal. Thus, the plateau at ~3–5 M urea decreases at lower protein concentration because the signal is lower for the monomer relative to the dimer. As a result, the first transition appears to be dependent on the protein concentration, however the urea^{1/2} for the transition is ~2.5 M for all data sets. In contrast, the mid-point for the second transition is dependent on protein concentration and reflects dimer dissociation. The free energy values listed in Table 2 ($\Delta G_1^\circ - \Delta G_3^\circ$) correspond to the equilibrium constants (K₁–K₃) shown in equation 2.

The data show that the single mutant E246A had no effect on the stability of procaspase-3, where the total free energy and m-value were within error of those of wild-type. In contrast, the K242A single mutant was significantly less stable than wild-type procaspase-3, by 7.5 kcal mol⁻¹. An examination of the values in Table 2 show that the lower free energy is due to a destabilization of the dimer, where ΔG_1° is about half that of wild-type and ΔG_2° is decreased by ~3 kcal mol⁻¹ so that the dimer is ~7 kcal mol⁻¹ less stable in the K242A mutant. The stability of the monomer is not affected by the mutation, where the free energy is within error of that of wild-type (~7 kcal mol⁻¹). In addition, the m-values for the first two steps in unfolding (m_1 and m_2), which correspond to the $N_2 \leftrightarrow I_2$ and $I_2 \leftrightarrow 2I$ transitions, respectively, are significantly lower for K242A. Because the m-value is correlated to a change in solvent accessible surface area (ΔASA) for the transition [23], the data suggest that less surface area is exposed during the isomerization and dissociation reactions for the mutant compared to wild-type. Although the reasons for this are not yet clear, the data are consistent with a partially unfolded native dimer of K242A.

Surprisingly, the stability of the double mutant was the same as that of wild-type procaspase-3, where one observes that the free energy and m-values are within error of wild-type. Overall, the data demonstrate that although the K242A mutation was destabilizing in the single mutant, it was not destabilizing when both K242 and E246 were replaced. The structures discussed below lend insight into why the double mutant appears to have regained stability when compared to the single K242A mutant.

3.4 X-ray crystal structures of the L4 mutants

During maturation, cleavage of the intersubunit linker at D175 releases two active site loops from binding in the dimer interface. Active site loop 2 (called L2) is the C-terminus of the large subunit, and active site loop 2' (L2') is the N-terminus of the small subunit (see Figures 1A and 1C). L2' rotates 180° degrees away from the interface to interact with active site loops 2 and 4 (L2 and L4), from the other monomer, forming the so-called loop bundle (L2-L2'-L4). Many of the newly formed contacts in the loop bundle stabilize the active site [16] and are not available to the procaspase due to the covalent connection between L2 and L2'. Pertinent to the discussion below, we note that in crystals with the symmetry of the I222 space group, L4 makes several contacts with L1 of a symmetry related molecule. As a result, L4 is ordered in the crystal even though our previous biochemical data show that the K242A and E246A mutations affect L4 in solution [17]. Consequently, we note here the structural changes that correlate to the findings of our biochemical assays. The structural changes described below are summarized in Table 4.

In wild-type caspase-3 (pdb code 2J30), no electron density is observed for residues 176'-179' (L2'), but L2' is ordered starting with D180', where the prime (') indicates residues in the second monomer (Figure 1C). In the loop bundle, the side chain of K186', from L2', is engaged in ionic interactions with the backbone carbonyls of A258 (from L4) and C170 (from L2). In addition, H185' (from L2') interacts with the backbone carbonyl of T245 (helix 5) as well as two water molecules that also H-bond to the backbone carbonyl of D181' (Figures 1C and 3A). Furthermore, there are several H-bonds between the backbone atoms of H185' and I187' (from L2'), I172 and T174 (both from L2) (Figure 3A). Overall, interactions in the loop bundle are important in stabilizing L2' as well as L2 and the base of L4 [16, 24]. L2' also extends beyond L4 to contact the substrate binding loop (L3), and it makes indirect contact with the substrate, where one observes that D180' H-bonds with D211 (in L3), which in turn interacts with the substrate at the P4 site via a network of water molecules (Figure 1C).

In contrast to wild-type caspase-3, the single mutant, E246A, demonstrates good electron density beginning with K186', while residues 173'–185' are mostly disordered (Figure 3B). Caspase-3(K242A) shows weak electron density for the backbone atoms of H185 but no electron density for the side chain in the $2F_o - F_c$ map contoured at the 1σ level (Figure 3C). In addition, the double mutant demonstrated reasonable electron density for A180' to D183', but there was no electron density for C184' and only main-chain electron density for H185' (Figure 3D). The positions of residues D180'-H185' in the double mutant should be viewed with caution as this region is stabilized by crystal contacts and probably would be disordered in the absence of the symmetry related molecule. In all cases, the contacts between K186', C170 and A258 are maintained, as are the H-bonds between backbone atoms of K186' and I172, but the single mutants have lost the H-bonds contributed by T174 (Figures 3B–D). The absence of electron density for H185' in the mutants suggests that the contacts with T245 (helix 5) and D181' (via water molecules) are lost. Diminishing the hydrogen bonding interactions between L2' and L4 in this region of the protein likely destabilizes both L3 and L4, providing one explanation for the observed decrease in activity.

The crystal structure of wild-type caspase-3 suggests that removing K242 would have negative effects not only due to the lost interaction with E246 but also due to the interactions of K242 with neighboring residues. While the positive charge is exposed to solvent, the aliphatic region of the K242 side chain is involved in a hydrophobic cluster with other amino acids in helix 5 as well as residues on the neighboring helix 4 (see Figure 1B). The data show that removing K242 resulted in small changes at the C-terminus of helix 5, where the side chain of E246 moved by ~ 1 Å, but there were few changes in helix 4 (Figure 4A). A water molecule fills the position of the lysine side chain and bridges a new H-bond between the side chains of E246 and S218. The S218 side chain also H-bonds to the backbone carbonyl of W214 (on L3). In contrast, the hydrophobic contacts contributed by K242 are lost, where removal of K242 leaves a groove between helices 4 and 5 (compare Figure 4B to Figure 1B), possibly explaining previous data that showed an increase in accessibility of active site tryptophans to quencher in caspase-3(K242A) [17].

In the E246A single mutant, an alternate rotamer is observed for K242 (Figure 4C). The shift is a rotation about the χ^2 torsion angle and results in the displacement of the K242 side chain such that it is only partially engaged in the hydrophobic interactions observed in the wild-type protein. The rotation moves the ζ -nitrogen ~ 3 Å away from its position in WT caspase-3 so that it partially occupies the site of the missing E246 side chain. In the double mutant, two water molecules occupy the sites for the missing K242 and E246 side chains (Figure 4D). For the K242A single mutant and for the double mutant there are no major structural changes, aside from localized changes at the carboxyl-terminus of helix 5. Overall, the structural data suggest that the salt bridge between K242 and E246 stabilizes the position of K242 within the hydrophobic cluster by neutralizing the positive charge.

As noted above, the discovery that the double mutant retained conformational stability comparable to that of procaspase-3 was surprising because the data for the K242A single mutant showed that its native dimer was less stable than that of procaspase-3 (Figure 2 and Table 2). While conformational stability was recovered in the double mutant, the active site remained impaired, as shown by a 100-fold decrease in k_{cat} and ~ 15 -fold increase in K_M (Figure 1D and Table 1). In WT caspase-3, R241, in helix 5, forms two H-bonds across the dimer interface: a strong H-bond with the backbone carbonyl of T270' (2.8 Å) and a weak H-bond with the backbone carbonyl of D34' (4.5 Å) (Figure 5A). In the double mutant, the side chains of R241 and D34' utilize different rotamers that move the charges to within ~ 3 Å of each other, resulting in a new salt-bridge across the dimer interface (Figure 5B). Using a structure-based thermodynamic approach, Rothlisberger and co-workers [25] suggested that 90% of caspase dimer stability is contributed by three key regions: interactions involving

amino acids residing on the anti-parallel β -sheet at the protein core, interactions in the loop bundle, and interactions involving residues on helix 5. Furthermore, we showed that ~70% of the conformational free energy of procaspase-3 is contributed by dimer formation [7]. Because R241 is located in the dimer interface in one of the “hot spots,” we suggest that the new salt bridge of D34'-R241 observed in the double mutant increases conformational stability compared to the single K242A variant, although at present it is not clear if the new interactions fully account for the increase in stability compared to caspase-3(K242A).

We recently generated homology models of inactive and active procaspase-3 using procaspase-7 as a template [21]. The models show that the salt bridge between K242 and E246 is *not* present in the inactive zymogen. This result was surprising because we previously had shown that both single mutations resulted in loss of activity for the procaspase [17], suggesting that the K242-E246 salt bridge was intact in procaspase-3. However, the model shows the side chain of E246 oriented toward the active site and H-bonded with the ϵ -nitrogen of the indole ring of W214 (Figure 5C). Upon activation, L4 rotates ~60° and is stabilized by newly formed contacts in the loop bundle, as described above. As a result of the rotation of L4, the C-terminus of helix 5 also rotates, although to a lesser degree, such that E246 moves ~10 Å away from W214. The movements are likely important for activation since the ϵ -nitrogen of the indole ring of W214 H-bonds to the P4 aspartate of the substrate. In addition, K242 is observed to interact with S218 from helix 4 in the inactive procaspase-3 (Figure 5C). In contrast, the active procaspase-3 conformer more closely resembles the mature caspase-3 due to the rotation of L4 into its active conformation (Figure 5C). As a result, the salt bridge between E246 and K242 is observed only in the active conformer, which, as described previously, is not favored in solution [9,21]. Overall, we suggest that the rotation of L4 upon activation allows E246 to rotate away from the active site and form a salt bridge with K242, disrupting interactions with S218. The rotations subsequently allow S218 to H-bond with W214, stabilizing the tryptophan in the active conformer (see Supplemental Movie).

4. DISCUSSION

The structural, biochemical and biophysical assays described here demonstrate the role of the K242-E246 salt bridge in (pro)caspase-3 stability and activity. In context of both procaspase-3 and caspase-3, the residues are important in maintaining the active site environment, as shown by increased K_M and decreased k_{cat} values in each mutant [17] (Figure 1, Table 1). Enzymatic studies show that the activity changes are not cooperative, possibly due to the fact that the residues indirectly stabilize the active site. Structural studies indicate that the mechanisms of inactivation are likely due to more than the loss of the salt bridge and may depend on the context of the mutation (procaspase or caspase).

We observed strong correlations in the model for inactive procaspase-3 with our biochemical and biophysical data. The interaction observed between E246 and W214 in procaspase-3 implies that E246 is involved in stabilizing the substrate binding pocket (L3) in its inactive conformation. Furthermore, there was no change in conformational stability (Table 2) implying that E246 is important for maintaining the active site environment but not the overall stability of the dimer. When the active conformer of the procaspase forms, however, the loss of E246 prevents active site stabilization due to the loss of the salt-bridge with K242, much like that for the mature caspase-3. In agreement, we observe no activity in this procaspase mutant, and the active site tryptophans become more accessible to quenching when E246 is removed [17]. Thus, E246 appears to have two roles depending on the conformer present. Conversely, procaspase-3(K242A) exhibits a decrease in conformational stability of ~7.5 kcal mol⁻¹. The structural data show that removing K242 disrupts the hydrophobic network between helices 4 and 5, likely destabilizing the two helices, which is

consistent with the lower conformational stability as a number of critical contacts across the interface are made in this region. Unexpectedly, the conformational stability of the double mutant is comparable to that of procaspase-3 (Table 2). The structural data show new contacts form across the interface between D34' and R241 and may explain the recovery of stability in the dimer.

Recent studies have shown that the dynamic nature of (pro)caspases is important for their allosteric regulation [26–28]. In the case of procaspase-3, the ensemble of native states consists of at least two major conformations, one of which is enzymatically active. While the active procaspase-3 conformer undergoes self-cleavage in the presence of a small molecule activator to yield mature caspase-3 [28], we have shown that the active procaspase-3 supports apoptosis in cell culture [21]. The conformational transition in procaspase-3 is not well-understood, nor is it clear why the inactive form is the preferred state. Studies that define interactions in the dynamic ensemble should help our understanding of the allosteric control. All together, the data presented here suggest that E246 is critical in maintaining the active site environment in both procaspase-3 and caspase-3. In procaspase-3 it forms a H-bond with W214 in the inactive conformer, whereas in caspase-3 it neutralizes the positive charge of K242 within a hydrophobic cluster of amino acids between helices 4 and 5. The data suggest that the interactions of K242 within the cluster are necessary both for active site stability and for dimer stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

K242-E246 salt bridge in caspase-3. **(A)** Structure of caspase-3 dimer highlighting K242 and E246 relative to the active site. **(B)** Cluster of hydrophobic amino acids in helices 4 and 5 involving K242. **(C)** Hydrogen bonding interactions between the N-terminus of active site loops 2' (cyan), 3 (blue), and helix 5. **(D)** Summary of enzymatic activity studies of wild-type and mutant caspase-3. The k_{cat}/K_M values in parentheses are presented in Table 1.

Figure 2.

Representative equilibrium unfolding of procaspase-3 variants E246A (**A**), K242A (**B**) and K242A,E246A (**C**). Unfolding was monitored by fluorescence emission at 335 nm following excitation either at 280 nm (shown here) or 295 nm (not shown) and CD at 228 nm (not shown), as described in section 2.3. Protein concentrations of 0.25 μM ($\circ$), 0.5 μM ($\square$), 1 μM ($\diamond$), and 2 μM (Δ) were examined, and reversibility of the folding transitions is shown in closed squares ($\blacksquare$). The data for each protein were fit globally (solid lines) to the four-state equilibrium unfolding model (equation 2, also shown at the top of panel A) as described in section 2.3. Results from the fits are shown in Table 2.

Figure 3.

Crystal structure of WT and mutant caspase-3 highlighting interactions in the loop bundle between H185' and K186' and active site loops 2' (L2'), L2, L4, and helix 5. (A) WT caspase-3, (B) caspase-3(E246A), (C) caspase-3(K242A), and (D) caspase-3(K242A,E246A). The prime (') indicates residues from the second monomer, and dashed lines represent H-bonds.

Figure 4. Structural features of helices 4 and 5. (A and B) caspase-3(K242A), (C) caspase-3(E246A), and (D) caspase-3(K242A,E246A). In panels A, C, and D, structures of the mutants are overlaid with that of WT (semi-transparent). Red spheres represent new water molecules not observed in WT. Dashed lines represent H-bonds. In panel B, the hydrophobic cluster shown in Figure 1B for wild-type is shown for the K242A variant.

Figure 5.

Interactions across the dimer interface. **(A)** In WT caspase-3, R241 (helix 5) H-bonds with backbone carbonyls of D34' and T270'. **(B)** In caspase-3(K242A,E246A), the side chains of R241 and D34' rotate and form a new salt bridge across the dimer interface. WT caspase-3 is shown as semi-transparent. **(C)** Comparison of K242 and E246 in inactive and active conformers of procaspase-3. The active conformer is shown as semi-transparent, and the arrows represent rotation or movement upon formation of the active conformer. Dashed lines indicate H-bonds. The direct interaction between E246 and W214 is predicted to stabilize the inactive conformer, and the salt bridge between E246 and K242 forms in the active conformer.

Table 1

Catalytic parameters of the caspase-3 loop 4 mutants

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1}\text{s}^{-1}$)
Cp-3 Wild-type ^a	2.2 ± 0.5	0.4 ± 0.05	1.8×10^5
Cp-3(K242A) ^b	13 ± 2	0.04 ± 0.01	3.1×10^3
Cp-3(E246A) ^b	23 ± 2	0.3 ± 0.02	1.3×10^4
Cp-3(K242A,E246A)	31 ± 3	$0.004 \pm 2.4 \times 10^{-4}$	1.3×10^2
Procaspase-3(D ₃ A) ^a	3.5 ± 0.8	$0.003 \pm 1.4 \times 10^{-4}$	8.6×10^2

^aFrom [22]^bFrom [17]

Table 2

Thermodynamic parameters of the procaspase-3 loop 4 mutants from equilibrium unfolding

	Wild-type ^a	E246A	K242A	K242A,E246A
ΔG_1° ^b	8.3±1.3	7.4±3.7	4.2±1.4	7.6±1.8
m ₁ ^c	2.8±0.5	3.1±1.4	1.2±0.2	2.7±0.6
ΔG_2°	10.5±1.0	10.9±2.1	7.4±1.5	11.4±1.4
m ₂	0.5±0.1	1.1±0.4	0.2±0.06	0.5±0.17
ΔG_3°	7.0±0.5	7.7±1.8	6.7±1.4	8.4±2.1
m ₃	1.2±0.1	1.4±0.3	1.1±0.2	1.4±0.3
ΔG_{Total}	25.8	26.0	18.3	27.1
m _{Total}	4.5	5.6	2.5	4.6

^aProcaspase-3(C163S). Data from [7]^b ΔG° values are in kcal mol⁻¹^cm-values are in kcal mol⁻¹ M⁻¹

Table 3

Summary of data collection and refinement statistics

		Wild-type ^a	K242A	E246A	K242A,E246A
Space Group		I222	I222	I222	I222
Unit Cell	a	68.73 Å	68.67 Å	67.61 Å	68.94 Å
	b	84.40 Å	84.53 Å	83.78 Å	84.69 Å
	c	96.35 Å	96.17 Å	96.23 Å	96.21 Å
	α	90°	90°	90°	90°
	β	90°	90°	90°	90°
	γ	90°	90°	90°	90°
Temperature		100 K	100 K	100 K	100 K
Resolution		1.40 Å	1.62 Å	2.00 Å	1.50 Å
Number of Reflections		54,279	34,489	18,646	48,708
Completeness (%)		97.9	99.0	99.4	95.1
Redundancy		5.8	5.1	6.8	5.1
Average I/ σ		34.48	2.35	3.71	2.77
R _{work} (%)		19.6	19.4	17.6	18.10
R _{free} (%)		20.7	21.1	20.3	19.9
R _{merge} (%) ^b		5.9	11.3	10.7	8.2
rmsd for bond lengths (Å)		0.005	0.004	0.005	0.005
rmsd for bond angles (deg)		1.3	1.26	1.25	1.27
No. of protein atoms		1974	1917	1919	1956
No. of water molecules		290	268	214	308

^aPDB code 2J30

^b $R_{\text{merge}} = \sum_h \sum_i |I(h,i) - I(h)| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ values are symmetry-related intensities and $I(h)$ is the mean intensity of the reflection with unique index h . $R_{\text{work}} = \sum_h ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_h |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors, respectively.

Table 4

Summary of amino acid interactions for (pro)caspase-3 loop 4 mutants described in the text

Wild-type	E246A	K242A	K242A,E246A
Electron density for N-terminus of L2' is observed to begin at (disordered residues in parentheses):			
D180' (S176'-D179')	K186' (S176'-H185')	K186' (S176'-H185')	D180'-A183'
Contacts in caspase-3 described in the text			
H185'(L2')-T245(helix 5)-Wat-D181'(L2')	Lost	Lost	Lost
K186'(L2')-I172(L2)-E173(L2)	Retained	Retained	Retained
K186'(L2')-C170(L2)-A258(L4)	Retained	Retained	Retained
T174(L2)-H185'(L2')	Lost	Lost	Retained
K242(helix5)-F247(L4)-W214(L3)-A221(helix4)-M222(helix4)-V239(helix5) {hydrophobic cluster}	Partial Loss	New cavity from loss of K242	New cavity from loss of K242 R241(helix 5) interactions
Comparison of contacts in procaspase-3 (inactive) and caspase-3			
Caspase-3	Procaspase-3		
K242(helix5)-E246(L4)	E246(L4)-W214(L3)		
W214(L3)-active site P4			
S218(helix4)-W214(L3)	S218(helix4)-K242(helix5)		

The prime (') indicates amino acids from the second monomer. L1, L2, L2', L3, and L4 refer to the five active site loops.