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Reassembly of Active Caspase-3 Is Facilitated by the Propeptide*

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Abstract

Changes in ionic homeostasis are early events leading up to the commitment to apoptosis. Although the direct effects of cations on caspase-3 activity have been examined, comparable studies on procaspase-3 are lacking. In addition, the effects of salts on caspase structure have not been examined. We have studied the effects of cations on the activities and conformations of caspase-3 and an uncleavable mutant of procaspase-3 that is enzymatically active. The results show that caspase-3 is more sensitive to changes in pH and ion concentrations than is the zymogen. This is due to the loss of both an intact intersubunit linker and the prodomain. The results show that, although the caspase-3 subunits reassemble to the heterotetramer, the activity return is low after the protein is incubated at or below pH 4.5 and then returned to pH 7.5. The data further show that the irreversible step in assembly results from heterotetramer rather than heterodimer dissociation and demonstrate that the active site does not form properly following reassembly. However, active-site formation is fully reversible when reassembly occurs in the presence of the prodomain, and this effect is specific for the propeptide of caspase-3. The data show that the prodomain facilitates both dimerization and active-site formation in addition to stabilizing the native structure. Overall, the results show that the prodomain acts as an intramolecular chaperone during assembly of the (pro)caspase subunits and increases the efficiency of formation of the native conformation.

Apoptosis is a type of cell death that occurs in eumetazoans, is responsible for maintaining the balance between cell growth and death, and can occur via two major pathways (1). The extrinsic pathway occurs in response to death receptor ligation (2) and results in a cascade of limited proteolytic cleavages by upstream activator caspases that ultimately lead to the activation of the executioner caspase-3. The intrinsic pathway triggers the release of ATP and cytochrome *c* from the mitochondria in response to antineoplastic drugs (3), growth factor withdrawal (4), or ionizing radiation (5). The intrinsic pathway results in a proteolytic cascade that activates caspase-9 and, subsequently, caspase-3 (6). Both pathways give rise to the proteolysis of structural and protective components of the cell, which in turn leads to the coordinated disassembly of the cell (7).

A relatively well characterized event leading up to the commitment to apoptosis is the change in the ionic homeostasis of the cell. Apoptotic cells are generally characterized as having low $[K^+]_i$, normal to slightly increased $[Na^+]_i$, high $[H^+]_i$, and normal to moderately increased $[Ca^{2+}]_i$. The efflux of potassium (leading to the depletion of intracellular potassium) is a key early step in apoptosis (8). Under normal conditions, $[K^+]_i$ is ~140 mM,

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and levels decrease to <50 mM in apoptotic cells (9, 10). The decrease in $[K^+]_i$ and associated water movement are contributing factors to the change in cell volume observed during apoptosis. Normal potassium concentrations are inhibitory to apoptosis and may act by inhibiting an apoptotic nuclease, NUC18 (10). In addition, normal potassium levels inhibit the cytochrome *c*-dependent activation of procaspase-3, but do not inhibit the activity of caspase-3 (6, 11). Although free potassium is a major contributor to intracellular ionic strength, Hughes and Cidlowski (10) showed that substitution of potassium with other monovalent ions results in similar effects. This indicates that the response is not specific to potassium cations, but is instead controlled by the ionic strength.

Thus far, potassium is the only cation that has been described as having a role in the inhibition of apoptosis, although studies of the direct effects of cations on caspase-3 activation and apoptotic commitment are incomplete at best. Although the results are less clear, studies with other ions, such as magnesium, zinc, copper, and iron, show that these ions also may have a role in apoptosis in certain tissues (reviewed in Ref. 9). In most cases, the effects of changes in ionic homeostasis occur at a stage prior to the maturation of caspase-3 and, in some cases, may result from the prevention of the formation of the apoptosome. For example, fluctuations in ion levels may inhibit the expression of caspase-9 as well as prevent the recruitment of caspase-9 to the apoptosome (6). Putney and co-workers (12) showed that Ca^{2+} -ATPase inhibitors initiate apoptosis by decreasing $[Ca^{2+}]_i$, and Segal and Beem (6) demonstrated a 50% inhibition of caspase activity in cytosolic extracts with ~ 60 mM $CaCl_2$. However, Stenicke and Salvesen (13) found that calcium has no effect on caspase-3 activity at 100 mM and suggested that the effects of calcium on apoptosis are unlikely to be due to an effect on caspases. In contrast, the cleavage of poly(ADP-ribose) polymerase by caspase-3 has been shown to be inhibited by zinc at concentrations as low as 0.1 mM (14). This is due to a direct inactivation of caspase-3 by zinc (14).

Although studies of the direct effects of cations on caspase-3 activity remain incomplete, comparable studies on the activity of procaspase-3 are lacking. Until recently, procaspase-3 was thought to be enzymatically inactive; thus, studies of the effects of ions on procaspase activity were not warranted. Recently, however, it was shown by Nicholson and co-workers (15) that an uncleavable procaspase-3 mutant contains catalytic activity. In this mutant, the three processing sites (Asp⁹, Asp²⁸, and Asp¹⁷⁵) have been replaced with glutamate. Likewise, we showed previously that a procaspase-3 mutant with the processing sites replaced with alanine is active (see Fig. 1) (16). In the maturation of procaspase-3, the cleavage at Asp¹⁷⁵ separates the large and small subunits and results in a large increase in activity, whereas the cleavages at Asp⁹ and Asp²⁸ remove the prodomain (17). We further showed that the uncleavable procaspase is ~ 200 -fold less active than mature caspase-3 and that the lower activity is a result of a low catalytic efficiency rather than a difference in substrate binding (16). Here, we describe the effects of physiologically relevant salts on the activity of procaspase-3 and the accompanying changes in the active-site environment. In addition, it has been noted that the procaspase-3 homodimer is more stable than the caspase-3 heterotetramer between pH 4 and 7 (18, 19), although it is not clear whether this is due to the presence of the propeptide or to an intact intersubunit linker in the procaspase. Indeed, Denault and Salvesen (20) showed that the propeptide of caspase-7 plays a role in stabilizing the zymogen and alters the properties of procaspase-7 as a substrate for caspase-7. By analogy, the lower stability of caspase-3 may be due to the loss of the propeptide upon maturation. We investigated whether the prodomain or the intact intersubunit linker affects dimer stability, and we further investigated the roles that salts and pH play in the stability of the (pro)caspase-3 dimer. Based on the results, we propose a model for the salt- and propeptide-dependent assembly of the caspase subunits.

EXPERIMENTAL PROCEDURES

Materials

Caspase-3, -6, and -7 prodomains were synthesized by the Peptide Facility at the University of North Carolina (Chapel Hill, NC). Sodium chloride, ammonium chloride, Tris base, and β -mercaptoethanol were from Fisher. Zinc chloride, magnesium chloride, dithiothreitol (DTT),² sodium citrate, and monobasic and dibasic potassium phosphate were from Sigma. Potassium chloride and sucrose were from Mallinckrodt Chemical Works. Calcium chloride was from Acros Organics. CHAPS and Ac-DEVD-7-amino-4-trifluoromethylcoumarin were from Calbiochem, and EDTA was from EM Science.

Mutagenesis

A mutation was introduced into the background of pro-less caspase-3 using the template pHC329 (21) and the D175A forward and reverse primers (referred to as primers 1 and 2) as described previously (16). This generated plasmid pHC32902 and produces an uncleavable pro-less procaspase-3 with the D175A mutation. In this mutant, the N-terminal 28 amino acids were replaced previously with methionine (21). Construction of plasmid pHC33209, which produces procaspase-3(D9A,D28A,D175A), was described previously (16). The D9A,D28A double mutant was produced using the template pHC33209 and primer 1 (5'-GTGGCATTGAGACAGACAGTGGTGTGATGATG-3') and primer 2 (5'-CATCATCAACACCACTGTCTGTCTCAATGCCAC-3'). The resulting plasmid is called pHC33260. In this plasmid, the *NheI* site that was incorporated previously for screening plasmid pHC33209 was removed, and its absence was used for screening. All plasmids were sequenced (both DNA strands) to confirm the mutations. A schematic diagram of the proteins produced from these plasmids and used in the studies presented here is shown in Fig. 1.

Protein Purification

Caspase-3, uncleavable procaspase-3(D9A,D28A,D175A) (called procaspase-3(D₃A)) (16), pro-less procaspase-3(D175A), and procaspase-3(D9A,D28A) were purified following overexpression in *Escherichia coli* as described previously (16). Protein concentrations were determined using $\epsilon_{280} = 26,500 \text{ M}^{-1} \text{ cm}^{-1}$ (21).

Enzymatic Activity Versus Salt Concentration

Protein was incubated at 25 °C in buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM DTT for >1 h, and substrate (Ac-DEVD-7-amino-4-trifluoromethyl-coumarin) was then added. Hydrolysis of the substrate was monitored as described previously (16, 21). Salts were added to the buffer such that the final concentrations are those shown in the figures. Assays were performed in duplicate in the same buffer, and relative activity was determined *versus* salt concentration by comparing the initial velocity in the absence or presence of the salt. The concentration of substrate was 50 μM , and 10 nM caspase-3 or 50 nM procaspase-3 was used for each assay. Because DTT chelates Zn^{2+} , the DTT was replaced with 20 mM β -mercaptoethanol in the assay buffer as described previously (13). We showed previously that one can measure ~50-fold less enzymatic activity than the maximal activity observed for procaspase-3(D₃A) (or 10,000-fold less than that of mature caspase-3), establishing the sensitivity of the assay (16).

²The abbreviations used are: DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; procaspase-3(D₃A), procaspase-3(D9A,D28A,D175A); PIPES, 1,4-piperazinediethanesulfonic acid; IMCs, intramolecular chaperones.

Changes in Fluorescence Emission as a Function of pH and Salt

To determine the structural effects of different cations on caspase-3 and procaspase-3, the proteins (2 μM) were incubated in 50 mM citrate (pH 3.0–6.2) or 50 mM phosphate (pH 6.0–9.0) containing NaCl, KCl, NH_4Cl , MgCl_2 , or CaCl_2 at 50–1000 mM. Samples were excited at 280 nm, and fluorescence emission was acquired between 305 and 400 nm (PTI C-61 spectrofluorometer, Photon Technology International). The average emission wavelength was then calculated at each pH as described previously (16, 18, 22) and compared with the spectra of the wild-type protein.

Salt Titrations at Low pH

As described in Fig. 3, salts affected the average emission wavelength for caspase-3 but not for procaspase-3 at low pH. Because of this, we examined whether the effect was cooperative, reversible, or dependent on the protein concentration. To examine cooperativity, caspase-3 was incubated in 20 mM citrate (pH 3.0) and in the absence or presence of 2 M NaCl, KCl, NH_4Cl , MgCl_2 , or CaCl_2 . Using an Olis titrator coupled to the PTI C-61 spectrofluorometer, a solution of caspase-3 in buffer containing 2 M salt was titrated into a solution of caspase-3 without salts. The solutions were mixed and incubated for 20 min between each measurement to allow equilibration. The salt-dependent conformational changes at pH 3.0 were determined by calculating the change in the fluorescence average emission wavelength (22) at each concentration of salt. Reversibility was examined using 2 μM protein and a two-phase reverse titration. The first phase consisted of salt concentrations between 1000 and 250 mM, and the second phase consisted of salt concentrations between 250 and 50 mM. Two additional points were taken manually to obtain 25 and 12.5 mM salt concentrations. We observed that the data from forward or reverse titrations were superimposable, demonstrating that the transitions are reversible. The protein concentration dependence was examined by performing the titrations at several protein concentrations (2–12 μM).

Reversibility of Activity as a Function of Salt and pH

To examine the reversibility of enzymatic activity following incubation at low pH as well as at high salt concentrations, both caspase-3 and procaspase-3(D₃A) were dialyzed for 4 h at 25 °C in 50 mM citrate (pH 3.0) plus 1 M salt or in 50 mM Tris-HCl (pH 7.5) plus 1 M salt for monovalent cations or 0.5 M salt for divalent cations. All buffers contained 1 mM DTT. The proteins were then dialyzed for 9 h at 25 °C in 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. In all cases, the volume of the dialysis buffer was >500-fold that of the protein sample. The enzymatic activity was measured in 50 mM Tris-HCl (pH 7.5), 0.1% CHAPS, 1% sucrose, and 10 mM DTT (assay buffer) (16, 21) using the substrate and protein concentrations described above. The initial velocity was compared with that of a control protein that had been stored at –20 °C (*i.e.* not dialyzed for 13 h).

Activity as a Function of pH and Protein Concentration

The pH at which caspase-3 loses activity irreversibly was determined by dialyzing the protein (10 μM) for 4 h at 25 °C in 50 mM citrate (pH 3.0–6.0) or in 50 mM Tris-HCl (pH 6.5–7.5) containing 1 mM DTT every 0.5 pH units. Samples were then dialyzed for 9 h at 25 °C in 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. The enzymatic activity was measured in assay buffer and compared with that of a control sample of caspase-3 that had been incubated for 13 h at 25 °C in 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. In all cases, the volume of the dialysis buffer was >500-fold that of the protein sample.

To investigate the effect of protein concentration on solubility and return of activity in the pH-dependent folding of caspase-3, protein concentrations ranging from 2 to ~50 μM were

used. Samples of caspase-3 were incubated at 25 °C in 50 mM citrate (pH 4) and 1 mM DTT for 24 h to dissociate the heterotetramer (18, 19), and the protein was then refolded by dialyzing the samples against 50 mM Tris-HCl (pH 7.5) and 1 mM DTT at 25 °C for 24 h. The samples were centrifuged at 14,000 rpm at 25 °C for 5 min, and the supernatant was transferred to a new tube. The absorbance at 280 nm was compared with that at time 0. The enzymatic activity was measured in assay buffer. The final protein concentrations in the enzymatic assays were as follows: caspase-3, 10 nM; pro-less procaspase-3(D175A), 50 nM; procaspase-3(D9A,D28A), 10 nM; and procaspase-3(D3A), 50 nM.

To measure the loss of enzymatic activity over time, protein (1 μ M) was incubated in 50 mM Tris-HCl (pH 7.5) at 25 °C for the times indicated in Fig. 7. In separate experiments, DTT (1 or 10 mM), KCl (1 M), MgCl₂ (0.5 M), or caspase-3 propeptide (15 μ M) was added to the buffer. Aliquots were removed at various times, and the enzymatic activity was measured in assay buffer. The final protein concentrations were 10 nM in the assays.

To examine the effect of the prodomain concentration on the return of activity, the caspase-3, -6, or -7 prodomain was added to caspase-3 or pro-less procaspase-3(D175A) at 2–200-fold excess concentrations. The (pro)caspase concentrations were 10 μ M. In these experiments, the proteins were dialyzed initially in 50 mM citrate (pH 4.0) for ~24 h at 25 °C. Following the initial dialysis, the samples were then dialyzed against 50 mM Tris-HCl (pH 7.5) for ~24 h at 25 °C, and the enzymatic activity was measured as described above. All buffers contained 1 mM DTT. In these experiments, a dialysis membrane with an M_r 1000 cutoff was used. The final protein concentrations in the enzymatic assays were as follows: caspase-3, 10 nM; and pro-less procaspase-3(D175A), 50 nM.

RESULTS

Activity Versus Salt Concentration

We examined the activities of caspase-3 and procaspase-3(D₃A) in the presence of the monovalent cations Na⁺, K⁺, and NH₄⁺ at concentrations up to 500 mM, and the results are shown in Fig. 2. As was observed previously (13), the activity of caspase-3 increased slightly (~20%) in the presence of low concentrations of Na⁺ (~25 mM) (Fig. 2A). This was true also for NH₄⁺ (~50 mM). However, K⁺ had little to no effect on the activity of caspase-3. In contrast, there was no effect of Na⁺ or NH₄⁺ on the activity of procaspase-3(D₃A) (Fig. 2B). Unlike the activity of caspase-3, that of procaspase-3 was sensitive to the presence of K⁺ and decreased to ~50% in the presence of >25 mM K⁺.

In the presence of Mg²⁺, the activities of caspase-3 and procaspase-3 decreased to ~40%, and the midpoints of the transitions were similar, ~100 mM (Fig. 2, C and D). In addition, the activities of both proteins decreased in the presence of Ca²⁺ (Fig. 2, C and D). For caspase-3, the activity decreased to ~50% between 10 and 100 mM Ca²⁺ and then decreased further at higher Ca²⁺ concentrations. For procaspase-3, the activity decreased to ~25% in an apparent single transition, with a midpoint of ~10 mM Ca²⁺. This is in contrast to results reported by Stennicke and Salvesen (13), who observed no effect on the activity of caspase-3 at concentrations up to 100 mM. It is not clear why there is a discrepancy in the results, although the experimental conditions are similar but not identical. For these studies, our assays were carried out at 25 °C rather than at 37 °C, and our buffer contained 50 mM Tris-HCl (pH 7.5) rather than 20 mM PIPES (pH 7.2) and did not contain sucrose. In addition, the control with which the activities were compared was taken from a fresh stock of protein that had been stored at -20 °C until just prior to use. At present, it is not clear which of these parameters would result in the discrepancy. Regardless, it is interesting to note that [Ca²⁺]_i is ~100 nM and either increases slightly or does not change significantly,

depending on the cell type, during apoptosis (9). Thus, in the effects described here, the Ca^{2+} concentrations are at least 4 orders of magnitude higher than those found in apoptotic cells. Our conclusion from these studies is that, although Ca^{2+} does affect the activities of both caspase-3 and procaspase-3 *in vitro* and at high concentrations, it is unlikely that Ca^{2+} fluctuations in apoptotic cells directly affect the activity of either protein. However, this is not true of K^+ because the concentrations used in our experiments are comparable with those found *in vivo*. Therefore, although the activity of caspase-3 is unaffected by changes in the levels of K^+ , the activity of the procaspase would be expected to increase if the efflux of potassium during apoptosis resulted in concentrations below 25 mM.

Caspase-3 is known to be inactivated by micromolar concentrations of Zn^{2+} , and it has been suggested that Zn^{2+} coordinates one or both catalytic residues His¹²¹ and Cys¹⁶³ (14). As shown in Fig. 2E, caspase-3 was completely inactivated by ~10 mM Zn^{2+} , in agreement with previous studies (13, 14). Surprisingly, procaspase-3(D₃A) is much less sensitive to Zn^{2+} . We observed that the procaspase was inactivated by Zn^{2+} concentrations between 300 and 400 μM , >3 orders of magnitude that required to inactivate caspase-3 (compare the midpoints of the curves in Fig. 2E). This is in agreement with our assertion that the catalytic residue Cys¹⁶³ is less solvent-accessible in the procaspase than in the mature caspase (16).

It has been shown that ionic effects on caspase activity are due to cations rather than anions (6). We confirmed this by examining chloride, acetate, sulfate, and phosphate anions using the sodium or magnesium salts of each. We observed no effect on the enzymatic activity of either caspase-3 or procaspase-3(D₃A) beyond those described above for the cations (data not shown).

Structural Changes as a Function of pH and Salt

The active site of procaspase-3(D₃A) was shown to be distinctly different from that of mature caspase-3, as evidenced in part by the differences in pH-dependent activity profiles (16). Procaspase-3(D₃A) exhibits maximal activity between pH 8.0 and 8.5, in contrast to caspase-3, which has a maximal activity between pH 7.2 and 7.8 (13, 16). In addition, we examined changes in the fluorescence average emission wavelength ($\langle\lambda\rangle$) (Fig. 3), which report on conformational changes in the protein that result from changes in pH (16, 18). (Pro)caspase-3 contains two tryptophan residues (Trp²⁰⁶ and Trp²¹⁴), and both are located in the active site and are in close proximity. As a consequence, changes in fluorescence emission report on the active-site structure or environment. For example, as the pH was lowered from pH 9 to 2.5, the $\langle\lambda\rangle$ of procaspase-3(D₃A) decreased from 343 to ~341 nm, and two transitions were observed with $\text{p}K_a$ values of 4.7 and 3.7, respectively (Fig. 3) (16, 18). We further demonstrated that the major transition ($\text{p}K_a = 4.7$) corresponded to the dissociation of the procaspase homodimer, such that the monomer was populated almost exclusively at pH 4 (18, 19). In contrast, caspase-3 at higher pH exhibited a $\langle\lambda\rangle$ of 345 nm, which showed that the fluorescence emission is red-shifted in the mature caspase relative to that of the procaspase. These and other results led us to suggest that the active site of caspase-3 is in a more open, solvent-accessible conformation than that of procaspase-3 (16), which is consistent with structural data available for (pro)caspase-7 (23, 24) and with the results of enzymatic inactivation by Zn^{2+} described above (Fig. 2). As the pH is lowered, caspase-3 exhibits two transitions with $\text{p}K_a$ values of 5.7 and ~3. The first transition results in a large blue shift in the fluorescence emission maximum and correlates with the dissociation of the heterotetramer to yield the heterodimer, as shown by gel filtration chromatography (18).

We examined the effects of monovalent salts on the pH-dependent fluorescence emission, and the results are shown in Fig. 3. In these experiments, the pH-dependent changes in $\langle\lambda\rangle$

were examined in the presence of 1 M Na⁺, K⁺, or NH₄⁺. As shown in Fig. 3A, there was no effect of the cations on the transitions of procaspase-3(D₃A). This suggests that the cations do not affect the dissociation of the dimer because the p*K_a* of the major transition did not change. Moreover, although the addition of K⁺ resulted in a decreased activity of procaspase-3 (Fig. 2), there was no effect on the fluorescence emission properties; so the mechanism by which K⁺ affects procaspase activity remains unclear.

Similar studies with caspase-3 demonstrate that the cations affect $\langle\lambda\rangle$ at lower pH primarily (Fig. 3B). However, the addition of K⁺ to caspase-3 resulted in an additional transition at higher pH (p*K_a* = 6.4) that was not observed with either Na⁺ or NH₄⁺. Although the nature of this transition is unknown currently, the observed blue shift is similar to that observed for the K242A mutant of caspase-3 (18). The K242A mutation removes a salt bridge that stabilizes active-site loop L4, and a similar transition to that shown here for caspase-3 is observed for K242A in the absence of salts (18). Thus, although K⁺ had no effect on the activity of caspase-3, the cation does affect the fluorescence emission and hence the active-site environment. In contrast, the major transition (between pH 6 and 4) appears to be similar for all cations, suggesting that the cations do not affect the dissociation of the heterotetramer. At lower pH (below ~4), however, the cations had significant effects on the $\langle\lambda\rangle$ of caspase-3 (Fig. 3B). For example, at pH 3, $\langle\lambda\rangle$ was blue-shifted in the presence of 1 M Na⁺ or K⁺ and significantly red-shifted in the presence of 1 M NH₄⁺.

To further examine the effects of the cations at low pH, titrations were performed under conditions in which caspase-3 was preincubated at pH 3 to allow dissociation of the heterotetramer. We showed previously by gel filtration chromatography that the starting oligomeric state of the protein under these conditions is the heterodimer rather than the heterotetramer (18). The results of these titrations are summarized in Fig. 4A. Upon the addition of Na⁺, $\langle\lambda\rangle$ decreased in a single transition from 342 to ~339.5 nm between 0 and ~600 mM NaCl, consistent with the blue shift reported in Fig. 3B. The addition of K⁺ resulted in two transitions. At low concentrations of K⁺ (~150 mM), a red shift occurred in the fluorescence emission (to 343 nm), and this was followed by a blue shift in $\langle\lambda\rangle$ at higher K⁺ concentrations. In contrast, the addition of NH₄⁺ at 0–600 mM resulted in an increase in $\langle\lambda\rangle$ from 342 to 345 nm in an apparent single transition. Under these conditions, the fluorescence emission is similar to that of protein unfolded in 8 M urea at pH 7.5 (data not shown), indicating that the active-site tryptophans are exposed to solvent and that the subunits are likely unfolded. We also examined the effect of the divalent cations Mg²⁺ and Ca²⁺ on $\langle\lambda\rangle$. The results show that moderate concentrations of Mg²⁺ resulted in a blue shift in $\langle\lambda\rangle$, whereas higher concentrations of Mg²⁺ resulted in a red shift in $\langle\lambda\rangle$. Overall, there was little difference in $\langle\lambda\rangle$ at Mg²⁺ concentrations of 0 and 1 M. In contrast, Ca²⁺ had no effect on $\langle\lambda\rangle$ up to 1 M.

To examine whether the observed transitions were the result of dissociation of the heterodimer, titrations were performed at several protein concentrations. As shown in Fig. 4B, an increase in the protein concentration resulted in an increase in the midpoint of the transition in the presence of Na⁺, demonstrating that the heterodimer was favored at the higher protein concentrations. In the presence of K⁺ (Fig. 4C), both transitions were observed, but the lower protein concentrations had a pronounced red shift at low concentrations of K⁺, again indicating that the heterodimer was stabilized at the higher protein concentrations. This was readily observed also in the titrations with NH₄⁺ (Fig. 4D), in which the higher protein concentration demonstrated no change in $\langle\lambda\rangle$ until >600 mM NH₄Cl.

Our interpretation of the results shown in Figs. 3 and 4 utilizes the simple folding model shown in Fig. 5. In this model, P17 represents the large subunit (17 kDa), and P12 represents the small subunit (12 kDa). During assembly of the heterotetramer, the large and small subunits form a heterodimer (P17/P12), and two heterodimers associate to form the heterotetramer (P17/P12)₂. Although this folding scheme is unlikely to occur *in vivo* because the protein initially folds as the procaspase, where P17 and P12 are covalently connected, the subunits have been shown to associate *in vitro* to form the enzymatically active tetramer (25). Our previous studies using gel filtration chromatography have shown that the heterotetramer dissociates between pH 5 and 4; so at pH 3 and in the absence of salt, the protein is a heterodimer (P17/P12) (18). Fig. 4 shows that increasing the cation concentration promoted the dissociation of the heterodimer to give the two individual subunits (P17 and P12). In this regard, the cations appear to be equally effective. In contrast, an increase in the protein concentration promotes formation of the heterodimer (P17 + P12 → P17/P12). Following dissociation, the fluorescence emission varies depending on the cation present. This suggests that the cations shield negative charges in the subunits, which in turn affects the fluorescence emission. One would predict that this effect is limited primarily to the small subunit (P12) because both tryptophanyl residues and 7 of 10 tyrosines reside in the small subunit. Thus, the fluorescence emission observed under these conditions is primarily that of the small subunit. If it is true that the cations shield negative charges in P12, then the effect generally follows the lyotropic series

(Na⁺ > K⁺ > NH₄⁺ > Mg²⁺ > Ca²⁺). This would explain why there is no red shift in $\langle\lambda\rangle$ in the presence of Na⁺. As the subunits dissociate, the charges are shielded by the cation. In the case of K⁺, subunit dissociation is promoted at lower salt concentrations (red shift in $\langle\lambda\rangle$), and shielding occurs at higher concentrations (blue shift in $\langle\lambda\rangle$). In contrast, NH₄⁺ promotes dissociation but does not shield charges. Hence, only the red shift in $\langle\lambda\rangle$ is observed.

There are nine negatively charged amino acid side chains on P12. Of the nine amino acids, four are close to the active site on loops L3 and L4 and thus are close to the two tryptophans, and three are close to two tyrosines that reside at the C terminus of P12. The data suggest that one or more of the side chains are partially charged and that the partial charge affects fluorescence emission. By shielding the charge, either the tryptophanyl or tyrosinyl residues reside in a less polar environment, and the blue shift in $\langle\lambda\rangle$ results.

We showed previously that pH-dependent changes in $\langle\lambda\rangle$ are reversible in both the caspase and procaspase-3 (16, 18). To determine whether the salt-dependent structural changes observed at low pH were reversible as well, reverse titrations were performed as described under “Experimental Procedures.” The results demonstrate that the data from Figs. 3 and 4 were superimposable regardless of whether forward or reverse titrations were performed and regardless of the salt used in the titrations (data not shown). This shows that the subunits reassemble at low pH when the salt is removed.

The data in Fig. 4 (B and D) were fit to a simple dissociation model (P17/P12 \rightleftharpoons P17 + P12), whereas the data in Fig. 4C were fit to a model in which the heterodimer first dissociates, followed by quenching of the subunits (P17/P12 \rightleftharpoons P17 + P12 \rightleftharpoons P17* + P12*). The results of the fits are shown as the *solid lines*. By fitting the data to these simple models, we can estimate the free energy of heterodimer dissociation in the presence of salts. For the data shown in Fig. 4, the average free energy for heterodimer dissociation is 4.5 ± 0.65 kcal/mol. This is in good agreement with the conformational free energy for unfolding of the procaspase-3 monomer, which was determined by urea denaturation studies to be 4.0 kcal/mol at pH 4 (19). This correlation suggests that, following heterotetramer dissociation, the resulting heterodimer has a similar stability compared with the procaspase monomer. We used the parameters obtained from the fits to calculate the fraction of species (P17/P12 and

P17 + P12) as a function of salt, and representative data are shown in Fig. 4E for NaCl at two protein concentrations. The calculated fractions also show the heterodimer to be more stable at the higher protein concentration, as was shown experimentally in Fig. 4B.

Reversibility of Subunit Assembly

For procaspase-3, it was demonstrated that there were no conformational changes that could be detected by fluorescence emission when titrating salt up to 1 M (Fig. 3). To examine whether the salts affected the return of enzymatic activity upon reassociation of the homodimer, procaspase-3(D₃A) was incubated at pH 3.0 with and without salt (1 M). Following equilibration, the samples were returned to pH 7.5; the salt was removed; and the activity was measured. In control experiments, the protein was incubated at pH 7.5 with and without salt. The results are shown in Fig. 6A and demonstrate that ~100% of the activity was obtained regardless of the starting conditions. In these studies, the salt was removed in the final dialysis, so the loss of activity due to the presence of the salt described for Fig. 2 was not observed. Overall, the results show that procaspase subunit assembly and active-site formation are reversible processes and that salts had no effect on the reversibility. These results agree with our protein folding data showing that the procaspase subunits fold reversibly at pH 7.2 when unfolding is initiated by the addition of urea (19, 26).

Caspase-3 was treated under the same conditions as procaspase-3, and the results are shown in Fig. 6 (B–D). Initially, the protein was incubated at pH 7.5 with and without salts (1 M) (Fig. 6B); the salts were removed; and the activity was measured. The results show that the activity of caspase-3 decreased to ~40% of the starting activity under the conditions of this experiment, *i.e.* incubation at 25 °C for 13 h at pH 7.5 without salt. Although the activity loss was not affected by Na⁺ or NH₄⁺, the protein was stabilized by the presence of K⁺, Mg²⁺, and Ca²⁺. In fact, full activity was retained for 13 h when the divalent cations were included. We suggest that there are three possible reasons for the observed activity loss. First, it is thought that the loss of caspase-3 enzymatic activity over time results from oxidation of the catalytic residue Cys¹⁶³ (13). Although this probably occurred in our experiments as well, we note that the addition of 10 mM DTT to the assay buffer should reduce the sulfhydryl unless a covalent modification occurs to Cys¹⁶³. Second, caspase-3 may undergo autoproteolysis during the long incubation times of these experiments. As shown in Fig. 2, for example, both Ca²⁺ and Mg²⁺ inhibit caspase-3 activity at high concentrations, whereas neither Na⁺ nor NH₄⁺ affect activity. This suggests that the divalent cations may prevent autolytic cleavage by decreasing the enzymatic activity. However, analysis of caspase-3 by SDS-PAGE at the end of the incubation period (13 or 48 h) showed that no proteolysis had occurred (data not shown). Furthermore, the function of K⁺ in the protection against autolysis is less clear because K⁺ was shown to have no effect on activity (Fig. 2). Third, active caspase-3 may slowly inter-convert to an inactive conformation. If this occurs, then Ca²⁺, Mg²⁺, and Na⁺ may inhibit the conformational change. A partitioning between two forms of the protein, one of which is active, may explain the slow activity loss. Although the cause of the time-dependent loss of caspase-3 activity is unknown currently, further investigations are described below (see Fig. 7).

To examine subunit reassembly at low pH, caspase-3 was dialyzed at pH 3.0 with or without salt, and the pH was then returned to 7.5. The results shown in Fig. 6C demonstrate that ~96% of the activity was lost when the protein was returned to pH 7.5, and this effect was independent of the presence of salt. These data show that the return of caspase-3 activity is irreversible following subunit dissociation. To determine whether the irreversibility was due to pH, *i.e.* a change in [H⁺], the salt was removed following equilibration at pH 3, and the pH was then returned to 7.5 (Fig. 6C). In this experiment, the added salt promotes the dissociation of the heterodimer at pH 3 (Figs. 3 and 4); and as described above for the

fluorescence emission studies, removal of salt at pH 3 allows the subunits to reassociate. In a separate experiment, following equilibration at pH 3, the pH was first returned to 7.5, and the salt was then removed (Fig. 6D). As described above (Fig. 3), the presence of salts does not affect heterotetramer dissociation at pH 7.5, but the salts may prevent the subunits from reforming the heterodimer at higher pH, thus preventing reassembly of the heterotetramer. The results were the same regardless of the order of salt removal (Fig. 6, C and D). This suggests that the irreversible step occurs during dissociation of the heterotetramer rather than the heterodimer. This interpretation agrees with the reversibility of the titrations shown in Figs. 3 and 4, described above, indicating that the subunits (P17 and P12) were able to reassemble following the removal of salt at pH 3.

To examine the pH at which assembly becomes irreversible, caspase-3 was incubated at several pH values from 7.5 to 3.0; the pH was returned to 7.5; and the activity was measured (Fig. 6E). The results show that the return of activity became irreversible between pH 5 and 4.5, at which dissociation of the heterotetramer occurs (18). For protein that was initially incubated at pH 3 and then returned to pH 7.5, we examined the refolded protein by gel filtration chromatography to determine whether the protein reassembled to a heterotetramer or whether the heterodimer remained when the pH was returned to 7.5. The results demonstrate that the protein was indeed a heterotetramer, as the peak was superimposable with the control of caspase-3 at pH 7.5 (data not shown). Together, the results shown in Figs. 3, 4, and 6 demonstrate that the caspase-3 heterotetramer reassembles and that the fluorescence emission is the same as that of the native control, but that the enzyme is not active.

Time-dependent Loss of Enzymatic Activity

To further examine the time-dependent loss of caspase-3 enzymatic activity shown in Fig. 6B, we measured the activity over 72 h and in the presence of several factors (Fig. 7). The results shown in Fig. 7A demonstrate that changes in caspase-3 activity over time occurred in two phases. The first phase resulted in a slight increase in activity (~10%) and occurred with a half-time of ~1 h, whereas the second phase resulted in a large loss of activity, with a half-time of ~15 h. After 72 h of incubation, ~10% of the original activity remained. To determine whether the activity loss resulted from oxidation of the catalytic residue Cys¹⁶³, the experiment was repeated using 10 mM DTT. The results show that, after 72 h, ~55% of the original activity remained, although the half-time for activity loss was unchanged. This suggests that the activity loss is due, at least in part, to modification of Cys¹⁶³. The effects of salts were assessed by incubating caspase-3 in the presence of KCl (1 M) or MgCl₂ (0.5 M) for 72 h (Fig. 7B). The results show that both salts increased the half-time for the loss of activity such that essentially full activity remained after ~20 h. This is in agreement with the results shown in Fig. 6B. However, after 72 h, the activity of caspase-3 incubated in the presence of MgCl₂ was similar to that of the control, which retained only ~10% of the original activity. In contrast, the activity after 72 h was ~60% that of the original activity when incubated with KCl. Thus, both KCl (Fig. 7B) and increased concentrations of reducing agent (Fig. 7A) had similar effects on the final activity. KCl also increased the half-time of the transition to ~30 h, whereas increased concentrations of DTT had little effect on the half-time.

The largest effect on activity was observed when caspase-3 was incubated in the presence of excess propeptide (15-fold) (Fig. 7A). As with the caspase-3 control, two phases were observed in the data. In the presence of the propeptide, however, the half-time for the loss of activity was increased to ~30 h. In addition, ~70% of the original activity was retained after 72 h. Although the propeptide has been shown to have no effect on the activity of mature caspase-3 (21, 27), we showed previously that the propeptide binds to the protease domain with micromolar affinity when present in *trans* (21). The results shown here demonstrate

that the propeptide, when bound to the protease, decreases the time-dependent loss of enzymatic activity and thus acts in a stabilizing capacity.

To determine whether the propeptide affected the activity loss when in *cis* to the protease domain, we examined the time-dependent loss of activity of the D9A,D28A variant of procaspase-3. As shown in Fig. 1, the propeptide is retained on this protein because the two processing sites were removed; however, the intersubunit linker is cleaved at Asp¹⁷⁵, as for wild-type caspase-3. The results for this protein show that 100% of the activity was retained after 72 h of incubation, and the results were independent of whether 1 or 10 mM DTT was present in the buffer (Fig. 7C). In contrast, in the presence of MgCl₂ (0.5 M), virtually all activity was lost after 72 h, similar to the results observed for wild-type caspase-3 in the presence of MgCl₂. Similar results were obtained in the presence of NaCl and KCl (data not shown). This suggests that the propeptide binds to the protease domain via ionic interactions and that the salts disrupt the interactions. Thus, the results for procaspase-3(D9A,D28A) in the presence of salts are similar to those for caspase-3 in the presence of salts.

As described above, we suggest that the activity loss may be due to covalent modification of Cys¹⁶³, to autoproteolysis, or to a slow inter-conversion to a non-active conformation. Although we have shown that the activity loss is not due to autoproteolysis (described above), it is not yet clear whether the propeptide, reducing agent, and salts prevent the modification of Cys¹⁶³ or affect the rate of interconversion to a non-active conformation.

Role of the Prodomain in Subunit Reassembly

It is plausible that the irreversible loss of activity upon reassociation of the subunits following incubation at low pH (shown in Fig. 6) may be attributed to structural rearrangements in the active-site loops that are mediated by some factor other than cation concentration. In comparing procaspase-3 with caspase-3, the proteins differ in two regards. The procaspase contains the 28-residue propeptide, and it contains an intact subunit linker. So, in this and the subsequent section, we describe investigations of the roles that each plays in subunit reassembly and active-site formation.

Initially, we examined reassembly at several concentrations of caspase-3 because, as shown in the model in Fig. 5, there are two protein concentration-dependent steps in the reassembly of the heterotetramer. In these studies, the dependence of activity recovery following incubation at pH 4 was determined over the protein concentration range of 2 μ M to 25 μ M. In addition, the amount of precipitated protein was estimated based on the absorbance at 280 nm remaining in the clarified solution following equilibration. The results show that, as the protein concentration was increased, the amount of protein precipitation increased in a sigmoidal manner from near zero at the lower protein concentration to ~70% at the higher protein concentration (Fig. 8A). The midpoint of the transition was ~10 μ M caspase-3. The return of activity was also dependent on the protein concentration; we observed that a maximum of ~25% of the original activity was obtained at protein concentrations >10 μ M (Fig. 8B). Reassembly was then examined under conditions in which various concentrations of the prodomain were added in *trans* to the caspase-3 subunits. In the experiments shown in Fig. 8C, caspase-3 (10 μ M) was incubated at pH 4 in the presence of propeptide to allow subunit dissociation, and the pH was then adjusted to 7.5 as described under "Experimental Procedures." The results were compared with the original activity at time 0 for protein at pH 7.5. As shown in Fig. 8 (A and B), ~25% activity was recovered for 10 μ M caspase-3, and ~40% of the protein was precipitated. The results shown in Fig. 8C demonstrate an increase in caspase-3 activity at 2–10-fold excess propeptide, and a maximal activity of 85–90% was obtained at >10-fold excess propeptide over caspase-3. Thus, excess propeptide in *trans* not only stabilized caspase-3 at pH 7.5 from time-dependent loss of activity (Fig. 7A), it resulted in a large recovery of activity upon reassociation of the subunits (Fig. 8C). In addition, there

was a dramatic decrease in precipitation at the higher protein concentrations (Fig. 8C). For example, the amount of precipitated caspase-3 decreased from ~40 to <5% at a 2-fold (or greater) concentration of propeptide. The effects described here were not due to the prevention of subunit dissociation ($P17/P12 \rightarrow P17 + P12$) (Fig. 5), as it was shown previously that the prodomain does not bind to caspase-3 below pH ~5 (16). Rather, the results suggest that the prodomain facilitates assembly of the heterotetramer ($2(P17/P12) \rightarrow (P17/P12)_2$) (Fig. 5), formation of the proper active-site conformation after assembly, or both.

We examined whether the effect of the propeptide was specific for the prodomain of caspase-3 or whether other propeptide sequences would provide the same function. The increase in activity was not observed for the prodomain of either procaspase-6 or -7; there was little to no effect of these prodomains on the return of caspase-3 activity (Fig. 8C, *open symbols*). In addition, the prodomains had no effect on the amount of precipitation that occurred. In both cases, there was ~40% precipitation at 10 μ M caspase-3 regardless of the propeptide concentration (data not shown). Overall, these data show that the prodomain of caspase-3 affects the efficient formation of the active site in a specific manner because the propeptides of caspase-6 and -7 had no effect. Furthermore, when caspase-3 was reassembled in the presence of the procaspase-3 propeptide, the return of activity was nearly 100%, and the time-dependent decrease in activity was diminished. Thus, the caspase-3 prodomain stabilizes the protease activity, similar to that described above for Mg^{2+} , Ca^{2+} , and K^+ ions (Figs. 2, 6, and 7), and facilitates proper active-site formation when the heterotetramer is reassembled in the presence of the propeptide.

We suggest that there are two ways in general that the prodomain can affect active-site formation. First, the caspase-3 prodomain may act as a substrate and bind to the active site at high concentrations, and this binding event may facilitate efficient active-site formation. However, if this is the primary effect, then one would also expect the prodomains of caspase-6 and -7 to have similar effects, as they also should bind to the active site of caspase-3. In addition, measurements of caspase-3 activity following incubation in the presence of up to 100-fold excess caspase-3 propeptide showed no change in activity (data not shown). Second, the prodomain binds to the protease domain outside of the active site and facilitates proper folding of the active site. Although this suggestion remains under investigation, it has been shown that the prodomain binds to the caspase in an apparent extended conformation at a region outside of the active site (21). If this suggestion is true, then the prodomain may act as an intramolecular chaperone to facilitate the proper folding of the heterotetramer late in the folding process. Because of the specific nature of intramolecular chaperones, one would not expect the prodomains of caspase-6 and -7 to function with caspase-3, as was observed here.

Role of the Intersubunit Linker in Reassembly

To examine the role of the intersubunit linker in reassembly, we examined a pro-less variant of procaspase-3 containing the D175A mutation (Fig. 1). In this variant, the prodomain has been replaced with an N-terminal methionine (21), and the D175A mutation in the intersubunit linker prevents processing. As with caspase-3 (Fig. 8, A–C), the dependence of activity recovery following incubation at pH 4 was determined over the protein concentration range of 2–25 μ M, and the amount of precipitated protein was estimated. The results show that activity increased in a sigmoidal manner such that ~10% of the original activity was obtained at low protein concentrations, whereas full activity was obtained at higher protein concentrations (Fig. 8E). We note that the activity of this mutant was ~2-fold lower than that of procaspase-3(D3A) at pH 7.5, but the results shown in Fig. 8E are compared with controls of the pro-less variant at each protein concentration that had not been incubated at the low pH. At present, it is not clear why having an intact subunit linker

would result in a sigmoidal rather than a hyperbolic curve (Fig. 8, compare *B* and *E*), although the intact subunit linker may decrease the affinity of the monomers for dimerization. In addition, the intact subunit linker resulted in greater precipitation at lower protein concentrations. As shown in Fig. 8*D*, the percent of precipitated protein increased considerably at lower protein concentrations (2–10 μM): ~60% of the protein precipitated at 5 μM . This is in contrast to caspase-3 (Fig. 8*A*): ~10% of the protein precipitated at 5 μM . For the pro-less D175A variant, a second transition occurred at higher protein concentrations so that ~70% of the protein precipitated at 25 μM . We note that, at 25 μM , ~70% of the protein precipitated, yet full activity was recovered. This shows that the activity of the pro-less variant results from a small population of active protein (~30%). As described previously for procaspase-3(D₃A) (16), the activity of this variant is not due to alternate processing of the intersubunit linker because no cleavage was observed by SDS-PAGE or Western blotting (data not shown). Thus, at present, it is not clear why the majority of this protein is not active.

The prodomain was added in *trans* during reassembly of pro-less procaspase-3(D175A), and the effect on the return of activity as well as precipitation was determined. In these experiments, 10 μM pro-less variant was incubated with 2–200-fold excess propeptide, as described above for caspase-3, to allow the monomers to reassemble in the presence of the prodomain. The results are shown in Fig. 8*F* and are similar to those obtained for caspase-3 (Fig. 8*C*), *i.e.* the gain in activity plateaued (at 200% in this case) with ~10-fold excess prodomain. This should be compared with ~40% activity in the absence of the prodomain (Fig. 8*E*). When the pro-less variant was reassembled in the presence of the prodomain, there was an ~2-fold increase in activity compared with the control. Thus, when the monomers of the pro-less D175A variant were reassembled in the presence of excess prodomain, the final activity was similar to that of procaspase-3(D₃A), or ~2-fold higher than the starting activity of the pro-less variant. In addition, the presence of the propeptide decreased the amount of precipitation to <5%, even at the lower peptide concentrations (Fig. 8*F*, \circ).

The effects were examined further by studies with the D9A,D28A variant of procaspase-3 (Fig. 1). The results show that only ~25% of the protein precipitated at the higher protein concentrations (Fig. 8*G*), and, like caspase-3, the shape of the curve was sigmoidal. Thus, compared with caspase-3, the presence of the prodomain in *cis*, even with a cleaved subunit linker, resulted in much lower protein precipitation. In addition, ~95% of the original activity was obtained when the monomers were reassembled at the lower protein concentrations (Fig. 8*H*), and this increased to ~200% of the original activity at the higher protein concentrations. Thus, when the mutant heterotetramer was reassembled *in vitro* (Fig. 8*H*), the activity increased 2-fold over that of the control. We note that, in our hands, the activity of this mutant was ~25% higher than that of wild-type caspase-3 when isolated upon *E. coli* overexpression. This is in contrast to the results of Salvesen and co-workers (27), who showed that the activity of this mutant is similar to that of wild-type caspase-3. However, their data also showed that the mutant is slightly more efficient than wild-type caspase-3 in cleaving poly(ADP-ribose) polymerase, a substrate of caspase-3. This is consistent with the increased activity described here for this mutant. Nevertheless, the reassembled D9A,D28A variant resulted in ~10-fold higher activity compared with reassembled caspase-3 in the absence of the propeptide (Fig. 8, compare *B* and *H*).

The results for caspase-3 and the two variants were compared with those for uncleavable procaspase-3(D₃A), in which both the intersubunit linker and propeptide remain intact (Fig. 1). In this protein, there was very little precipitation at each protein concentration (Fig. 8*I*); we observed that a maximum of ~15% precipitated at the higher protein concentrations. In contrast to caspase-3 (Fig. 8*B*), the recovery of activity was efficient: 100% of the initial

activity was recovered at the low protein concentration, and this value decreased to ~90% at the higher protein concentration (Fig. 8J).

Overall, from the data shown in Fig. 8, we conclude that the prodomain both decreases aggregation and increases folding efficiency. The data show that the lowest amount of precipitation occurred when the prodomain was in *cis* to the protease domain (Fig. 8, compare *G* and *I* with *A* and *D*) or in excess of the protease domain.

In addition to decreasing aggregation, the presence of the prodomain, either in *cis* or in *trans*, improves the efficiency of active-site formation. The yield of activity recovery for caspase-3 increased from ~25 to ~85% at 10-fold excess propeptide. The recovery of activity for pro-less procaspase-3(D175A) increased 2-fold at the higher protein concentrations, but there was a dramatic effect at the lower protein concentrations: the activity increased from ~10 to ~150% that of the control at only 2-fold excess propeptide (Fig. 8, compare *E* and *F*). When the propeptide was present in *cis* to the protease domain, the recovery of activity increased from ~10 to ~100% that of the control at the low protein concentration (Fig. 8, compare *B* and *H*).

Fig. 8D shows that the intact subunit linker did not prevent aggregation of the procaspase-3 monomer. The intersubunit linker converts a second-order step in assembly to a first-order process because the individual subunits are covalently attached (Fig. 5). This should increase the efficiency of assembly at the low protein concentration. However, this was not observed for pro-less procaspase-3 (Fig. 8, compare *B* and *E*). This may be due to the orientation of the linker, which must extend over the body of the protein so that the terminal β -strand of the large subunit and the first β -strand of the small subunit are aligned in a parallel orientation (23–25, 28). This may result in the slow folding of the procaspase monomer or an inefficient dimerization process that is rescued by the propeptide.

DISCUSSION

Acidification and ion fluxes are two of the hallmarks of the initial stages of apoptosis (29). It is also relatively well established that osmolarity and anions do not play a direct role in the induction of apoptosis, whereas some cations have been implicated as key facilitators in the pathway (6). Most effects of ions on the maturation of caspase-3 *in vivo* are indirect, being mediated through the inhibition of cytochrome *c* release from the mitochondria or apoptosome formation. We have shown here that the presence of low concentrations of the monovalent cations Na^+ and NH_4^+ results in a modest increase in the activity of caspase-3 *in vitro*, whereas neither cation affects the activity of procaspase-3. In contrast, there was little effect of K^+ on the activity of caspase-3, whereas the activity of the zymogen decreased 2-fold at concentrations >10 mM. Although the total concentration of K^+ *in vivo* is ~140 mM, the concentration of free K^+ has been measured at ~13 mM (6). Thus, our results suggest that the efflux of K^+ that occurs during the onset of apoptosis may result in an ~2-fold increase in the activity of procaspase-3. However, the activity of the zymogen is significantly lower than that of the mature protease, so a 2-fold increase in activity is not likely to have a dramatic effect *in vivo*. Several studies have shown that cation effects on (pro)caspase-3 activity are stage-specific during apoptosis, and although inhibitory at certain stages, some cations may have little to no effect after commitment to apoptosis, *i.e.* caspase-3 maturation (6, 13, 30).

The activities of both caspase-3 and procaspase-3 decreased in the presence of the divalent cations Ca^{2+} and Mg^{2+} . However, the concentrations required for inhibition are several orders of magnitude greater than those found intracellularly, so fluctuations of these ions during apoptosis would not be predicted to affect the activity of either form of caspase-3 *in*

vivo. In contrast, Zn^{2+} inactivates both caspase-3 and procaspase-3, although the zymogen is much less sensitive to the cation. Because Zn^{2+} is thought to chelate either the catalytic cysteine or histidine residue or both (14), our data further implicate a more closed active-site conformation of the procaspase-3 zymogen with respect to mature caspase-3 and are consistent with our suggestion that the catalytic cysteine of procaspase-3(D₃A) is rotated away from the active site in the zymogen, making it less solvent-accessible (16). For this reason, the free Zn^{2+} ion concentration of 10 μ M observed in the cell (14, 31) would not be sufficient to contribute directly to procaspase-3 zymogen quiescence.

We showed previously that the procaspase-3 homodimer dissociates between pH 5 and 4 (18, 19), and we have shown here that dissociation is not sensitive to the presence of salts. In addition, the data demonstrate that subunit dissociation and active-site formation of the procaspase are reversible processes, as shown by fluorescence emission and activity assays. In contrast, the caspase-3 heterotetramer dissociates between pH 6 and ~4.5, as shown by gel filtration chromatography (18), indicating that the caspase heterotetramer is less stable than the procaspase homodimer. Like the procaspase, the dissociation of the heterotetramer was not affected by the presence of salts. Unlike the procaspase, however, dissociation of the heterodimer into the individual subunits was facilitated by salts. This was observed by protein concentration-dependent changes in the fluorescence emission at low pH in the presence of Na^+ , K^+ , or NH_4^+ .

Overall, the data presented here show that the structure of caspase-3 is more sensitive to changes in pH and ion concentrations than is the zymogen. This is due both to the cleavage of the intersubunit linker and to the absence of the prodomain. The results show that, although the caspase-3 subunits reassemble to the heterotetramer, the return of activity is not reversible after the protein is incubated at or below pH 4.5. Under these conditions, the caspase-3 heterotetramer is completely dissociated, but the individual subunits are not significantly populated (18), so the irreversible step occurs following heterotetramer rather than heterodimer dissociation. Upon return to higher pH, the protein reassembles to an inactive heterotetramer, as observed by gel filtration chromatography and activity studies. This suggests that active-site formation following heterotetramer assembly is irreversible in the caspase.

Salvesen and co-workers (27) showed that the prodomain has no inhibitory effect on caspase-3 activity if it is not removed during maturation. Recently, Denault and Salvesen (20) also showed that the propeptide of caspase-7 plays a role in stabilizing the zymogen and alters the properties of procaspase-7 as a substrate of mature caspase-7, although the mechanism is unknown currently. We have shown here that the prodomain indeed has a function in the pH-dependent folding of caspase-3. This was shown by the rescue of >90% of the caspase-3 activity when the propeptide was present in >10-fold excess. In addition, the propeptide is required to reach maximal catalytic activity in the procaspase. However, the most dramatic response may be in stabilizing the enzyme such that a time-dependent loss of enzymatic activity is not observed in 72 h. Together, the data show that the prodomain of caspase-3 plays an important role in the formation and stability of the active site.

The prodomains of many proteases function as intramolecular chaperones (IMCs) either to regulate proper folding or to affect cellular function (for review, see Ref. 32). In some cases, the propeptide directly catalyzes correct folding by stabilizing the rate-limiting folding transition state (termed class I propeptide), whereas in other cases, the propeptide is indirectly involved in folding, but rather affects other functions (class II propeptide). Examples of class I propeptides include α -lytic protease (33) and subtilisin (34), among many others. Interestingly, the prodomains of upstream caspases, such as caspase-8, are classified as class II propeptides because their interactions with receptors affect other

cellular pathways (35). We suggest that the propeptide of caspase-3 (and by analogy, those of caspase-6 and -7) functions as an IMC because it facilitates assembly of the (pro)caspase to ensure proper formation of the native conformation. As with other IMCs (32), our data show that the interactions between the caspase-3 protease domain and the propeptide are specific because the propeptides of caspase-6 and -7 do not convey the same effect. At present, it is not clear whether the caspase-3 propeptide is a class I or II IMC because it is not known whether the propeptide affects the kinetics of folding or, in this case, the kinetics of subunit assembly, which is a hallmark of the class I propeptides. Although the propeptide appears to function during folding, it clearly interacts with the native protein and stabilizes the native conformation. The caspase-3 subunits are able to assemble in the absence of the propeptide, albeit in a rather inefficient manner. Thus, the propeptide is not absolutely required to attain the active conformation; it is required, however, to fold efficiently into the active conformation. In addition to the results shown here, the propeptides of the executioner caspases have been shown to have other functions in the cell, as described below. Together, these features suggest that the propeptides are class II IMCs, although the precise mechanism by which the caspase-3 propeptide functions as an IMC is unknown currently.

Recent evidence from other investigators demonstrates that the propeptides of effector caspases are not simply evolutionary remnants from apical caspases, with no functional purpose. It has been shown, for example, that the propeptide of caspase-7 may sequester the protein in the cytosol to prevent activation by upstream activators (20). In addition, the propeptides of caspase-3 and -6 have been proposed to silence the zymogens *in vivo* (36, 37). This proposal is intriguing because the effect was not observed in bacterial expression systems or in yeast cells (36). This suggests that factors exist in mammalian cells that bind the prodomain and mediate caspase activation. Our data show that the prodomain stabilizes the enzymatic activity of the caspase *in vitro* and suggest that the zymogen should have a longer half-life *in vivo* relative to the processed enzyme. Thus, if factors do bind to the prodomain *in vivo* and facilitate maturation, then this ultimately results in a decrease in caspase stability and should result in a shorter half-life of the enzyme.

The data presented here suggest the assembly model shown in Fig. 9. As described previously (18) and as shown in Figs. 2–4, an increase in $[H^+]$ results in dissociation of the heterotetramer, whereas increases in $[Na^+]$, $[K^+]$, and $[NH_4^+]$ facilitate dissociation of the heterodimer. An intact intersubunit linker, as found in the zymogen, facilitates assembly of the subunits by converting the second-order association reaction into a first-order process. In addition, one or both subunits aggregate when not covalently connected to the other subunit. However, the largest aggregation occurs from the heterodimeric intermediate (P17/P12). The intact intersubunit linker increases aggregation in the absence of the propeptide, although it presumably increases the efficiency of subunit assembly. Formally, the intact linker also affects the formation of the active site in the procaspase because the active-site loop rearrangements that allow full activity, as observed in the mature enzyme, do not occur in the wild-type zymogen. However, this is not included in the model shown in Fig. 9 because dimer interface mutants of procaspase-3 have shown that loop rearrangements occur in the zymogen that result in activation of the mutant procaspase without the accompanying chain cleavage (38). It was suggested that the interface mutants affect active-site formation by perturbing the position of the flexible linker. In contrast to the linker, the propeptide decreases aggregation, facilitates active-site assembly, and stabilizes the native protein. The results shown here suggest that the procaspase monomer (or the caspase heterodimer) assembles into an inactive dimer (or heterotetramer in the case of caspase), which isomerizes to the native active conformation. The propeptide facilitates dimer (heterotetramer) assembly. If the procaspase monomer (or caspase heterodimer) partitions between aggregation and dimer (heterotetramer) formation, then facilitating dimerization should decrease aggregation, and this is observed experimentally. In addition, we observed

that, in the absence of the propeptide, the heterotetramer reassembled following the initial incubation at low pH, although the protein was not enzymatically active. This suggests that the propeptide can bind to the inactive dimer (heterotetramer) and affect assembly of the active site following formation of the heterotetramer. This aspect of the model is consistent with our protein folding data for procaspase-3 showing that the protein undergoes isomerization following assembly of the dimer (19, 26). The model shown in Fig. 9 suggests that the caspase-3 heterodimers assemble into an inactive heterotetramer, and in the presence of the propeptide, a further isomerization results in formation of active protease. This indicates that, in the absence of the propeptide, the caspase-3 heterotetramer may be trapped in an inactive conformation, explaining why the heterotetramer can reassemble following incubation at low pH, yet the enzyme is not active unless the propeptide is present. Together, the data suggest that an inactive heterotetramer initially forms upon heterodimer association, and the propeptide increases both the efficiency of heterotetramer assembly as well as proper active-site formation from this inactive structure.

The mechanism described here for the caspase-3 propeptide may be more general than for the executioner subclass of caspases (caspase-3, -6, and -7). Caspases that contain an N-terminal caspase recruitment domain, such as procaspase-1 for example, also contain a segment between the domain and the N terminus of the large subunit. If the mechanism of the caspase-3 propeptide is generalized for the other caspases, then these segments would also function in the folding and assembly of those caspases. Although the length and sequence of these segments is not conserved, we note that the peptides generally have a net negative charge, consistent with our data showing that the caspase-3 prodomain binds to the protease domain via ionic interactions.

References

1. Earnshaw WC, Martins LM, Kaufmann SH. *Annu Rev Biochem.* 1999; 68:383–424. [PubMed: 10872455]
2. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. *Cell.* 1995; 81:801–809. [PubMed: 7774019]
3. Fearnhead HO, McCurrach ME, O'Neill J, Zhang K, Lowe SW, Lazebnik YA. *Genes Dev.* 1997; 11:1266–1276. [PubMed: 9171371]
4. Deckwerth TL, Johnson EM Jr. *Ann N Y Acad Sci.* 1993; 679:121–131. [PubMed: 8512180]
5. Datta R, Banach D, Kojima H, Talanian RV, Alnemri ES, Wong WW, Kufe DW. *Blood.* 1996; 88:1936–1943. [PubMed: 8822910]
6. Segal MS, Beem E. *Am J Physiol.* 2001; 281:C1196–C1204.
7. Salvesen GS, Dixit VM. *Proc Natl Acad Sci U S A.* 1999; 96:10964–10967. [PubMed: 10500109]
8. Yu SP. *Prog Neurobiol (N Y).* 2003; 70:363–386.
9. Yu SP, Canzoniero LMT, Choi DW. *Curr Opin Cell Biol.* 2001; 13:405–411. [PubMed: 11454444]
10. Hughes FM Jr, Cidlowski JA. *Adv Enzyme Regul.* 1999; 39:157–171. [PubMed: 10470372]
11. Bilney AJ, Murray AW. *FEBS Lett.* 1998; 424:221–224. [PubMed: 9539155]
12. Bian X, Hughes FM Jr, Huang Y, Cidlowski JA, Putney JW Jr. *Am J Physiol.* 1997; 272:C1241–C1249. [PubMed: 9142849]
13. Stennicke HR, Salvesen GS. *J Biol Chem.* 1997; 272:25719–25723. [PubMed: 9325297]
14. Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG, Hannun YA. *J Biol Chem.* 1997; 272:18530–18533. [PubMed: 9228015]
15. Roy S, Bayly CI, Gareau Y, Houtzager VM, Kargman S, Keen SLC, Rowland K, Seiden IM, Thornberry NA, Nicholson DW. *Proc Natl Acad Sci U S A.* 2001; 98:6132–6137S. [PubMed: 11353841]
16. Bose K, Pop C, Feeney B, Clark AC. *Biochemistry.* 2003; 42:12298–12310. [PubMed: 14567691]
17. Denault JB, Salvesen GS. *Chem Rev.* 2002; 102:4489–4499. [PubMed: 12475198]

18. Feeney B, Pop C, Tripathy A, Clark AC. *Biochem J.* 2004; 384:515–525. [PubMed: 15312047]
19. Bose K, Clark AC. *Protein Sci.* 2005; 14:24–36. [PubMed: 15576551]
20. Denault JB, Salvesen GS. *J Biol Chem.* 2003; 278:34042–34050. [PubMed: 12824163]
21. Pop C, Chen YR, Smith B, Bose K, Bobay B, Tripathy A, Franzen S, Clark AC. *Biochemistry.* 2001; 40:14224–14235. [PubMed: 11714276]
22. Royer CA, Mann CJ, Matthews CR. *Protein Sci.* 1993; 2:1844–1852. [PubMed: 8268795]
23. Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. *Cell.* 2001; 107:399–407. [PubMed: 11701129]
24. Riedl SJ, Fuentes-Prior P, Renucci M, Kairies N, Krapp S, Huber R, Salvesen GS, Bode W. *Proc Natl Acad Sci U S A.* 2001; 98:14790–14795. [PubMed: 11752425]
25. Rotonda J, Nicholson DW, Fazil KM, Gallant M, Gareau Y, Labelle M, Peterson EP, Rasper DM, Ruel R, Vaillancourt JP, Thornberry NA, Becker JW. *Nat Struct Biol.* 1996; 3:619–625. [PubMed: 8673606]
26. Bose K, Clark AC. *Biochemistry.* 2001; 40:14236–14242. [PubMed: 11714277]
27. Stennicke HR, Jurgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, Zhou Q, Ellerby M, Ellerby LM, Bredesen D, Green DR, Reed JC, Froelich CJ, Salvesen GS. *J Biol Chem.* 1998; 273:27084–27090. [PubMed: 9765224]
28. Mittl PRE, DiMarco S, Krebs JF, Bai X, Karanewsky DS, Priestle JP, Tomaselli KJ, Grutter MG. *J Biol Chem.* 1997; 272:6539–6547. [PubMed: 9045680]
29. Bortner CD, Cidlowski JA. *Biochem Pharmacol.* 1998; 56:1549–1559. [PubMed: 9973175]
30. Suzuki H, Yanaka A, Shibahara T, Matsui H, Nakahara A, Tanaka N, Muto H, Momoi T, Uchiyama Y. *Am J Physiol.* 2002; 283:G986–G995.
31. Pattison SE, Cousins RJ. *Fed Proc.* 1986; 45:2805–2809. [PubMed: 3533646]
32. Shinde U, Inouye M. *Semin Cell Dev Biol.* 2000; 11:35–44. [PubMed: 10736262]
33. Silen JL, Agard DA. *Nature.* 1989; 341:462–464. [PubMed: 2507926]
34. Ikemura H, Takagi H, Inouye M. *J Biol Chem.* 1987; 262:7859–7864. [PubMed: 3108260]
35. Chaudhary PM, Eby MT, Jasmin A, Hood L. *J Biol Chem.* 1999; 274:19211–19219. [PubMed: 10383428]
36. Meergans T, Hildebrandt AK, Horak D, Haenisch C, Wendel A. *Biochem J.* 2000; 349:135–140. [PubMed: 10861221]
37. Cowling V, Downward J. *Cell Death Differ.* 2002; 9:1046–1056. [PubMed: 12232792]
38. Pop C, Feeney B, Tripathy A, Clark AC. *Biochemistry.* 2003; 42:12311–12320. [PubMed: 14567692]

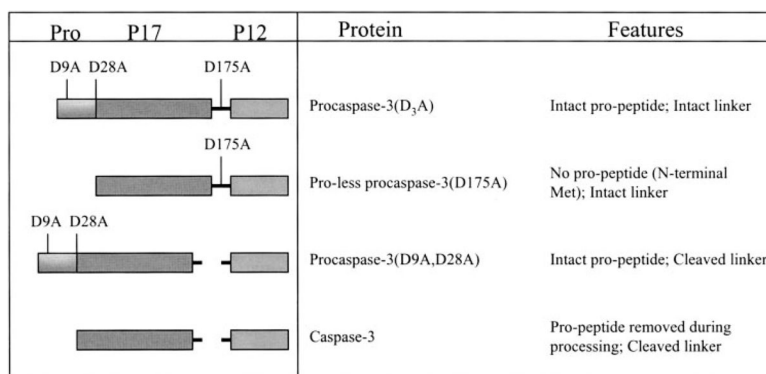


FIGURE 1. Schematic diagrams of caspase-3 proteins used in this study
Pro refers to the prodomain. P17 and P12 are the large (17 kDa) and small (12 kDa) subunits, respectively. Features of each protein are listed to the *right*.

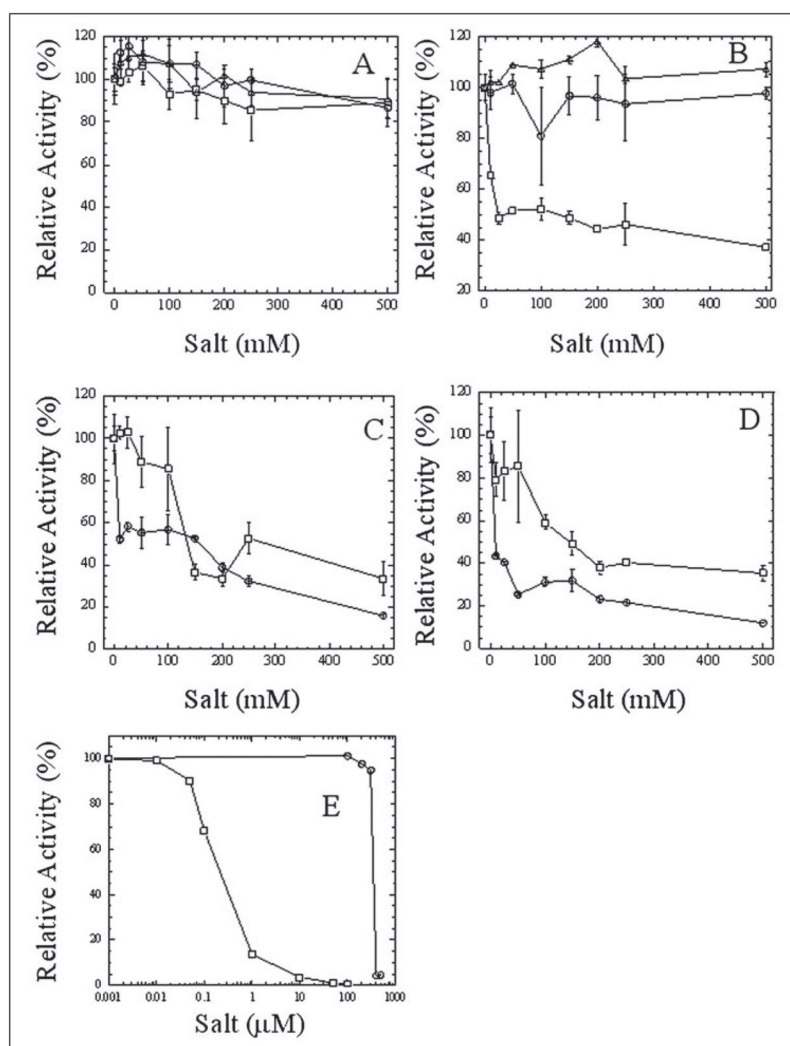


FIGURE 2. Relative activity versus salt concentration

A, activity of caspase-3 in the presence of Na⁺ (○), K⁺ (□), or NH₄⁺ (Δ); B, activity of procaspase-3(D3A) in the presence of Na⁺ (○), K⁺ (□), or NH₄⁺ (Δ); C, activity of caspase-3 in the presence of Mg²⁺ (□) or Ca²⁺ (○); D, activity of procaspase-3(D3A) in the presence of Mg²⁺ (□) or Ca²⁺ (○); E, activity of caspase-3 (□) or procaspase-3(D3A) (○) in the presence of Zn²⁺. For A–E, the activity was compared with that of a control as described under “Experimental Procedures.” Error bars show the S.E. from at least three independent experiments.

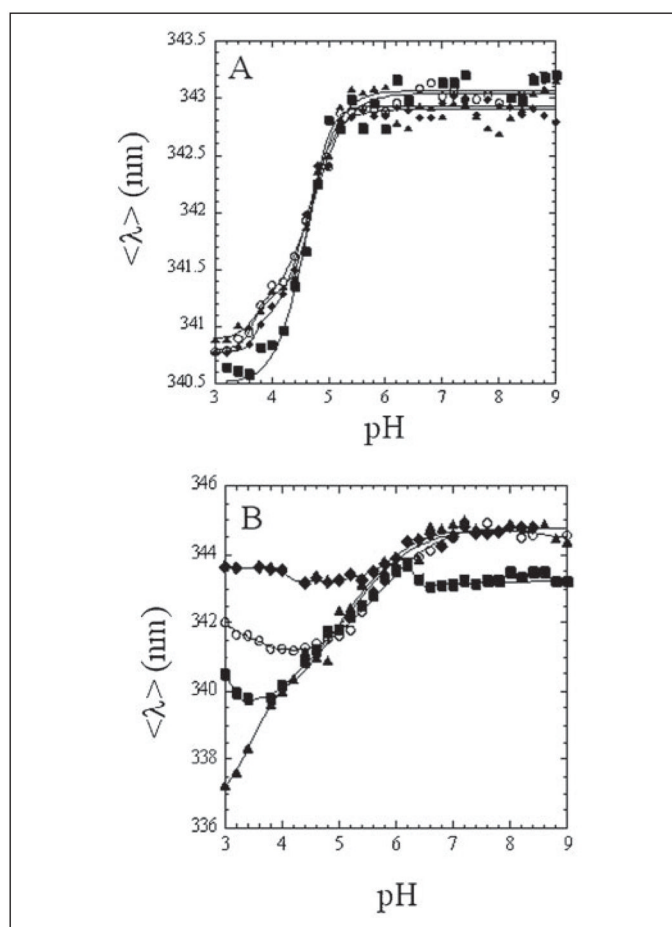


FIGURE 3. Fluorescence average emission wavelength ($\langle \lambda \rangle$) versus pH in the presence of salts A, procaspase-3(D₃A); B, caspase-3. For A and B, the following salts were examined: no salt (○), 1 M NaCl (▲), 1 M KCl (■), or 1 M NH₄Cl (◆). Solid lines represent fits of the data as described under “Experimental Procedures.”

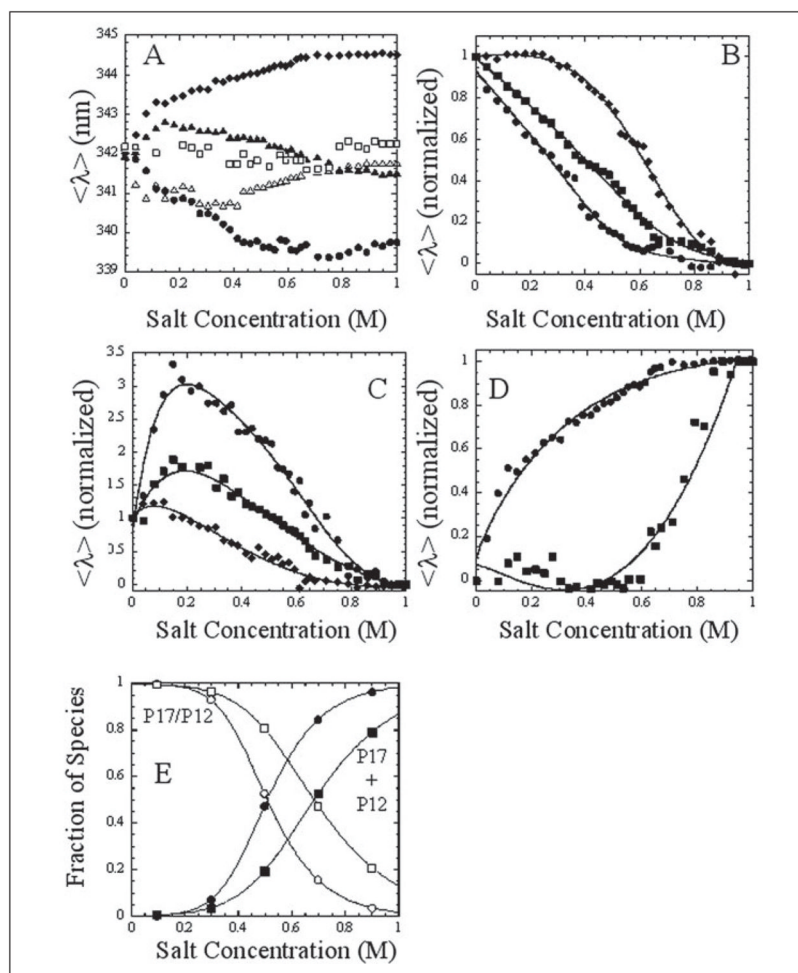


FIGURE 4. Changes in fluorescence average emission wavelength ($\langle\lambda\rangle$) versus salt concentration
 A, caspase-3 was incubated at pH 3 as described under “Experimental Procedures” to allow heterotetramer dissociation and then titrated with NaCl (●), KCl (▲), NH₄Cl (◆), MgCl₂ (△), or CaCl₂ (□). B, titrations were performed as described for A in the presence of NaCl at caspase-3 concentrations of 2 (●), 4 (■), and 12 (◆) μM. C, titrations were performed as described for A in the presence of KCl at caspase-3 concentrations of 2 (●), 4 (■), and 12 (◆) μM. D, titrations were performed as described for A in the presence of NH₄Cl at caspase-3 concentrations of 2 (●) and 10 (■) μM. In B and C, the data were normalized relative to the starting and ending values of $\langle\lambda\rangle$. In D, the data were normalized relative to the ending and starting values of $\langle\lambda\rangle$ so that the changes were consistent with those shown in A. Solid lines represent fits of the data as described in the text. E, fraction of heterodimer (open symbols) and subunits (closed symbols) calculated from fits of the data in B. Representative data are shown for 4 (circles) and 12 (squares) μM protein.

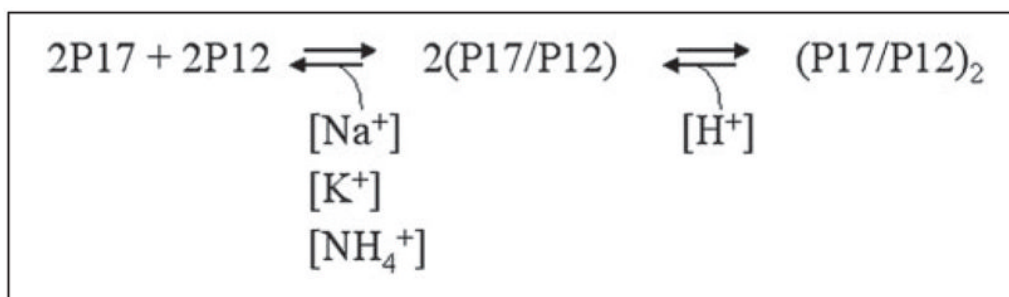


FIGURE 5. Proposed model for the effects of cations on caspase assembly

P17 is the large subunit (17 kDa); P12 is the small subunit (12 kDa); P17/P12 is the heterodimer; and $(P17/P12)_2$ is the heterotetramer. The model shows that an increase in $[H^+]$ (decrease in pH) facilitates dissociation of the heterotetramer, whereas an increase in $[Na^+]$, $[K^+]$, or $[NH_4^+]$ facilitates dissociation of the heterodimer.

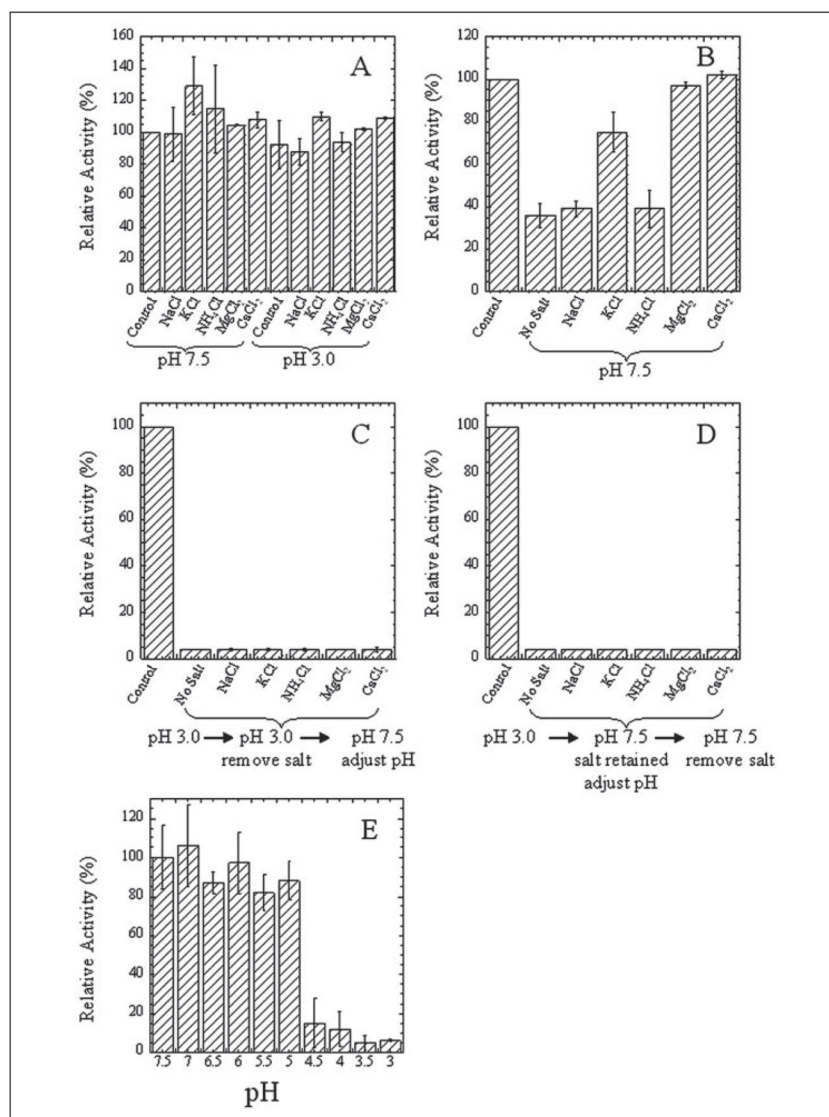


FIGURE 6. (Pro)caspase-3 activity recovery upon reassembly

A, shown is the relative activity of procaspase-3(D₃A) in the presence of salts. Protein was incubated with 1 M monovalent or 0.5 M divalent salts as described under “Experimental Procedures” at pH 7.5 or 3.0. Samples were subsequently dialyzed at pH 7.5 to remove the salts, and the activity was measured. B, shown is the relative activity of caspase-3 in the presence of salts at pH 7.5. Following the initial incubation, samples were dialyzed at pH 7.5 to remove the salts, and the activity was measured. C, caspase-3 was incubated at pH 3.0 in the presence of salts. Following the initial incubation, samples were dialyzed at pH 3.0 to remove the salts and then at pH 7.5 to adjust the pH, and the activity was measured. D, caspase-3 was incubated at pH 3.0 in the presence of salts. Following the initial incubation at pH 3.0, samples were dialyzed at pH 7.5 to adjust the pH, but the salts were retained. The samples were then dialyzed at pH 7.5 to remove the salts, and the activity was measured. E, caspase-3 was incubated at the indicated pH without salts as described under “Experimental Procedures”; the pH was then returned to 7.5; and the activity was measured. For A–E, activities were compared with those of controls as described under “Experimental Procedures.” Error bars show the S.E. from at least three independent experiments.

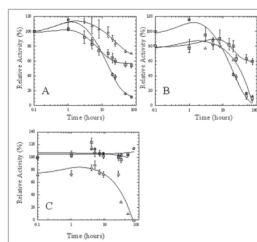


FIGURE 7. Time-dependent loss of activity

A, caspase-3 in the presence of 1 mM DTT (\circ), 10 mM DTT (\square), or 15 μ M propeptide (Δ); *B*, caspase-3 in the presence of 1 mM DTT (\circ), 1 M KCl (\square), or 0.5 M MgCl_2 (Δ); *C*, procaspase-3(D9A,D28A) in the presence of 1 mM DTT (\circ), 10 mM DTT (\square), or 0.5 M MgCl_2 (Δ). Note that the results for caspase-3 in the presence of 1 mM DTT are presented in *A* and *B* for comparison with the other conditions. In all cases, the caspase concentration was 1 μ M. *Error bars* show the S.E. from at least three independent experiments.

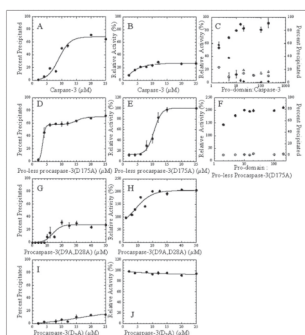


FIGURE 8. Efficiency of subunit reassembly

A, caspase-3 solubility as a function of protein concentration. Caspase-3 was initially incubated at pH 4, and the pH was then readjusted to 7.5. Solubility was measured as the absorbance at 280 nm remaining in a clarified solution as described under “Experimental Procedures.” *B*, relative activity of samples in *A*. In each case, the activity was compared with that of a control of equal concentration that had been incubated at pH 7.5. *C*, caspase-3 incubated initially at pH 4 in the presence of various concentrations of prodomains from caspase-3 (●), caspase-6 (○), and caspase-7 (Δ). The pH was readjusted to pH 7.5, and the activity was measured. Activities were compared with that of a control as described under “Experimental Procedures.” Changes in solubility at each propeptide concentration are shown (◆). *D*, pro-less procaspase-3(D175A) solubility as a function of protein concentration. *E*, relative activity of samples in *D*. *F*, return of activity for pro-less procaspase-3(D175A) incubated initially at pH 4 in the presence of various concentrations of caspase-3 prodomain (●), followed by return to pH 7.5. Changes in solubility at each propeptide concentration are shown (○). *G*, procaspase-3(D9A,D28A) solubility as a function of protein concentration. *H*, relative activity of samples in *G*. *I*, procaspase-3(D₃A) solubility as a function of protein concentration. *J*, relative activity of samples in *I*. For all panels, *error bars* indicate the S.E. from at least three independent experiments. *Solid lines* are meant as guides and do not represent fits of the data to specific models.

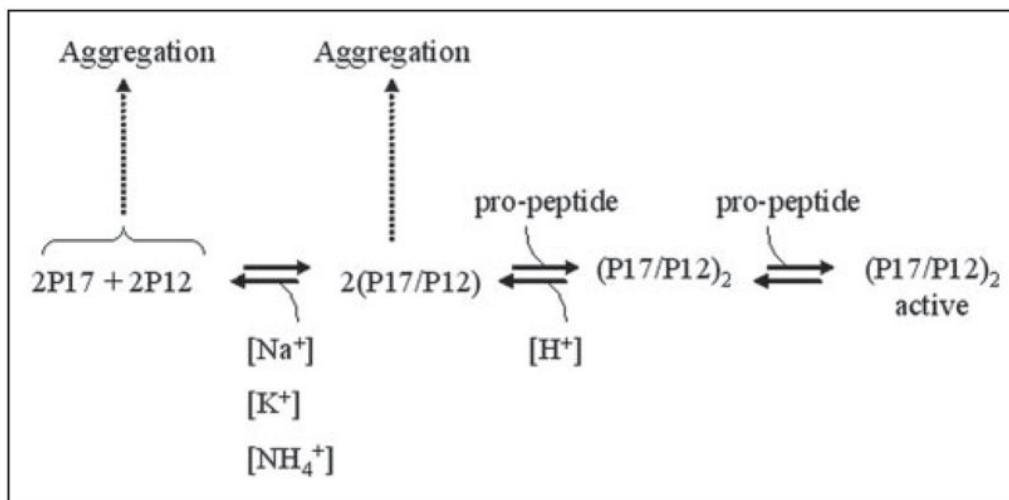


FIGURE 9. Proposed model for assembly of the caspase-3 subunits

Subunits P17 and P12 assemble into a heterodimer (P17/P12). The assembly step is dependent on the cation concentration, and either the subunits or the heterodimer or both aggregate at higher protein concentrations. Two heterodimers assemble into an inactive heterotetramer ((P17/P12)₂), which then isomerizes to the native active conformation. Assembly of the heterotetramer is sensitive to [H⁺]. The propeptide assists in formation of the inactive and/or active heterotetramer. The propeptide also stabilizes the native conformation.