Role of Proteolysis in Caspase-8 Activation and Stabilization†

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ABSTRACT: Caspase-8 is an apoptotic protease that is activated at the cytosolic face of the cell membrane. Activation relies on adaptor-induced dimerization of monomeric caspase-8 and is followed by specific limited autoproteolysis of the linker which separates the two subunits of the catalytic domain. However, the role of this autoproteolysis, which directly activates executioner caspases-3 and -7, is unknown for the apical caspase-8. We have generated linker mutants of caspase-8 that can be proteolyzed in a controlled manner by thrombin or tobacco etch mosaic virus protease, and we use these to define the role of proteolysis in the activation and stability of the enzyme. We show that proteolysis is insufficient for generating enzymatic activity in recombinant caspase-8. Kinetic activation studies using Hoffmeister salts demonstrate that activation is the result of caspase dimerization. However, linker proteolysis significantly enhances the equilibrium for caspase-8 dimerization, thereby increasing the stability of the dimer. Kinetic and fluorescence measurements demonstrate that caspase-8 activation by Hoffmeister salts is at least a two-step event, with the required step being dimerization, followed by an intramolecular event that further stabilizes the catalytic conformation. Autoproteolysis of caspase-8 may be a mechanism for increasing the lifetime of the dimeric enzyme following dissociation from its activating complex at the cell membrane.

Caspases are intracellular proteases that cleave specific proteins thought to drive forward the activation of pro-inflammatory cytokines, apoptosis, or keratinocyte differentiation (reviewed in ref *1*). Most of the human caspases (caspases-2, -3, -6, -7, -8, -9, and -10) are members of the apoptotic group and become activated just a few minutes before the cell commits suicide by the process of apoptosis. Other caspases, such as caspases-1, -4, and -5, are implicated in executing the innate immune response by activating specific pro-inflammatory cytokines in response to sensitizing pathogen-derived signals (*2*). No matter the group to which it belongs, a caspase is never constitutively active unless an upstream signal triggers the conversion of the latent conformation to the active one. In the case of the inflammatory caspases and the initiators of apoptosis (caspases-8, -9, and -10), the trigger is a caspase-specific multiprotein complex that recruits the protease by interacting with its prodomain (*3*). Complexes assembled in response to a death stimulus, like the death-inducing signaling complex (DISC)[1] for initiator caspases-8 and -10 and the apoptosome for caspase-9, activate the caspases by favoring their dimerization via an induced proximity mechanism (*4*, *5*). In this case, activation-induced dimerization is necessary, sufficient, and reversible (*6*, *7*). By contrast, the activation trigger for the downstream executioner apoptotic caspases-3, -6, and -7, which are inactive dimeric proteins, is the proteolytic cleavage of the intersubunit linker as an indispensable and irreversible event (reviewed in ref *8*). Caspases are organized into an N-terminal domain (long for the initiators, short for the executioners) followed by a catalytic domain usually composed of a large subunit and a small subunit (see Figure 1). In the active conformation, caspases are obligatory dimers of catalytic domains, arranged in antiparallel symmetry with the small subunits at the dimer interface; thus, each molecule of active caspase contains two catalytic sites (reviewed in refs *1* and *8*).

Although caspase-8 is most of the time associated with apoptosis as an initiator activated at the DISC (*9*), it is also reported to participate in T-lymphocyte proliferation and activation without apparently evoking cell death (*10*, *11*). An interesting difference between these two apparently opposing roles is that caspase-8 is almost always found to be processed in the lysates prepared from apoptotic cells but present as an intact active full-length protein in lysates of the activated T-lymphocytes (*11*, *12*). By using a caspase activity-based probe, Tu et al. (*13*) have shown that during death receptor ligation, the active form of caspase-8 recruited to the DISC via its death effector domains (DEDs) is indeed the full-length unprocessed form; however, as a result of subsequent activation, caspase-8 undergoes autocleavage

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[1] Abbreviations: TEV, tobacco etch mosaic virus; Casp8-WT, two-chain caspase-8; Casp8-THR, caspase-8 that can be cleaved by thrombin; Casp8-TEV, caspase-8 that can be cleaved by TEV protease; Casp8-D_2A, uncleavable single-chain caspase-8; B-VAD-FMK, biotin-Val-Ala-Asp-fluoromethyl ketone; DISC, death-inducing signaling complex; DED, death effector domain; Ac-IETD-AFC, *N*-acetyl-Ile-Glu-Thr-Asp-(7-amino-4-trifluoromethylcoumarin); DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside.

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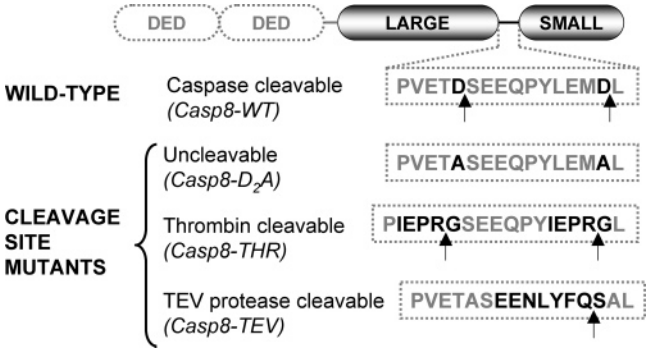

FIGURE 1: Caspase-8 mutants used in this study. Wild-type caspase-8 lacking the death effector domains (DEDs), Casp8-WT or "two-chain", is constitutively autocleaved after the two aspartate residues during expression in *E. coli*. Uncleavable single-chain mutant Casp8-D_2A replaces the aspartates with alanine. Mutants Casp8-TXR and Casp8-TEV have been mutated in the linker so that they can be cleaved by thrombin and TEV protease, respectively. The arrows indicate cleavage sites.

(*14*). Therefore, cell death is related to robust caspase-8 activation at the DISC followed by caspase cleavage, while activation of the NF-κB complex may involve only weak activation and preservation of the single-chain form (*11*). In vitro, recombinant caspase-8 prepared without the DEDs (see Figure 1) is a mixture of the two-chain inactive monomer and the active dimer (*6*), with a K_d in the low micromolar range (*7*). A single-chain mutant that cannot be processed is a stable inactive monomer. Addition of artificial dimerizing domains changes the equilibrium to an active dimer without the need for cleavage (*4*, *7*, *14*), reflecting the likely mechanism of activation at the DISC.

With regard to the physiological role of caspase-8 cleavage, several questions remain to be answered. One of them concerns the role of intersubunit linker proteolysis subsequent to dimerization, thought to be necessary for stabilizing the resulting heterotetramer once detached from the DISC (*6*, *7*). There is confusion in the literature relating to the use of the activity-based probe biotin-Val-Ala-Asp-fluoromethylketone (B-VAD-FMK), which reacts with the active form of caspases, to dissect the mechanism of caspase-8 activation in cell-based experiments. For example, using B-VAD-FMK, it has been concluded that proteolysis in the intersubunit linker of caspase-8 either does or does not activate it (*13*, *15*, *16*). Contradictory data from these papers may reflect the use of different cell lines, apoptotic paradigms, and treatment regimens using B-VAD-FMK. Consequently, we have taken an approach to determining the role of intersubunit proteolysis directly by generating site-specific mutants of caspase-8 and, importantly, mutants that can be specifically cleaved under controlled conditions by non-caspase proteases (see Figure 1). We delineate the activation mechanism before and after cleavage by determining the kinetics of activation in high concentrations of sodium citrate, an artificial activator, and also by conformational analysis through emission fluorescence properties upon activation.

EXPERIMENTAL PROCEDURES

Materials. CHAPS, dimethyl pimelimidate, EDTA, Hepes, imidazole, IPTG, sodium citrate, $NaClO_4$, $NaNO_3$, K_2HPO_4/KH_2PO_4, Pipes, thrombin, and Tris were from Sigma. $CaCl_2$, NaCl, and $MgCl_2$ were from Mallinckrodt. DTT and IPTG were from BioVectra. Caspase substrates and inhibitors were from MP Biomedicals, LLC.

Protein Expression and Purification. The catalytic domain of caspase-8 (residues 217−479) and the mutants thereof (see Figure 1) were expressed as His-tagged proteins and purified as previously described (*6*), except that the expression was conducted at 25 °C for 18 h. Caspase-3 and caspase-7 were obtained as described previously (*17*). Following purification on Ni beads (Amersham), the proteins were chromatographed on Superdex 200 or Mono Q for further purification, or to separate the monomeric and dimeric forms. The purification buffer contained 50 mM Hepes and 100 mM NaCl (pH 7.5). The extinction coefficients used for two-chain and single-chain caspase-8 were 19 770 and 21 050 M^{-1} cm^{-1}, respectively (*7*).

A plasmid encoding the TEV protease (pRK508) was the kind gift of A. Osterman from The Burnham Institute for Medical Research. The protease was expressed as a chimeric protein containing N-terminal maltose-binding protein, followed by a TEV protease cleavage site, followed by TEV protease containing a C-terminal six-His tag. Expression was carried out at 30 °C with 0.2 mM IPTG for 4 h. During expression, maltose-binding protein was removed by proteolysis autocatalytically. Following purification on Ni beads, the protein was dialyzed in 50 mM Tris-HCl (pH 8) and 50 mM NaCl.

Gel filtration experiments were performed with an AKTA LC system (Pharmacia) using Superdex 200. The running buffer contained 20 mM Tris (pH 8) and 1 mM EDTA. Fractions (0.4 mL) were tested for activity against Ac-IETD-AFC following a 10-fold dilution in low-salt or high-salt assay buffer.

Activity Measurements. Casp8-THR (4.4 μM) was cleaved with thrombin (400 nM) in PBS for 3 h at 37 °C. Casp8-TEV (4.4 μM) was cleaved with TEV protease (final concentration of 500 nM) in 50 mM Tris (pH 8), 0.5 mM EDTA, and 1 mM DTT for 3 h at 37 °C. The reaction was terminated by the addition of electrophoresis running buffer, and the samples were analyzed by 8 to 18% SDS−PAGE (*18*). For determination of the enzymatic activity, the uncleaved or cleaved caspase-8 mutants and wild-type caspase-8 purified from *Escherichia coli* were diluted in low-salt assay buffer [20 mM Pipes (pH 7.5), 100 mM NaCl, 0.05% CHAPS, 10 mM DTT, and 5% sucrose] or high-salt assay buffer (same as above but containing 1 M sodium citrate) at final concentrations of 50 nM. The samples were incubated at 37 °C for 30 min, and caspase-8 activity was measured by adding the substrate Ac-IETD-AFC (100 μM). The control consisted of measuring the activity of thrombin and TEV protease against Ac-IETD-AFC.

The catalytic parameters of caspase-8 mutants and active site titrations with the irreversible Z-VAD-FMK inhibitor were determined as described previously (*17*). The enzymes (500 nM) were diluted in high-salt assay buffer and incubated at 37 °C for 1 h to allow complete activation. Catalytic parameters were determined at final enzyme concentrations of ~10−50 nM.

Kinetics of Caspase-8 Activation. Monomeric caspase-8 or caspase-8(D_2A) was diluted in 0.7, 0.8, or 1.0 M sodium citrate {prepared in buffer A [50 mM sodium phosphate (pH 7.5), 10 mM DTT, and 0.01% CHAPS]} at various final concentrations ranging between 7.5 and 300 nM and

incubated at 23 °C. At indicated times, a 100 μL sample was withdrawn and the substrate Ac-IETD-AFC (100 μM) was added. Activity was determined immediately in a 96-well plate reader (Molecular Devices) at 23 °C for the first 60 s of the reaction to diminish the long-time effect of the substrate on caspase dimerization (*19*).

The kinetics of the transition of the inactive enzyme E to the active species E′ is described by eq 1:

$$\frac{d[E']}{dt} = k_{obs}[E'] \text{ or } [E'] = [E_t] - [E_t]e^{-k_{obs}t} \quad (1)$$

where d[E′]/dt represents the variation of concentration of active enzyme E′ at time t, [E_t] is the total concentration of the enzyme, and k_{obs} is the pseudo-first-order rate constant.

E′ production was monitored by cleavage of the substrate S with generation of the fluorescent product P. The initial velocity of substrate cleavage was plotted versus time, and the data were fit to eq 2 obtained by substituting eq 1 into the Michaelis−Menten equation:

$$v_{obs} = \frac{k_{cat}[E'][S]}{K_M + [S]} = \frac{v_{max}[S](1 - e^{-k_{obs}t})}{K_M + [S]} \quad (2)$$

where k_{cat} is the catalytic constant, K_M is the Michaelis−Menten constant (∼4 μM), and [S] is the substrate concentration.

For caspase-8 (two-chain), the second-order rate constant, k_{on}, was calculated by plotting the k_{obs} values versus the enzyme concentration. The data were fit with the linear eq 3, which assumes that at the end of the equilibration time in sodium citrate all the E_t is present as E′:

$$k_{obs}[E_t] = k_{on}[E_t]^2 \text{ or } k_{obs} = k_{on}[E_t] \quad (3)$$

For a correction of eq 3 that takes into account an additional unimolecular process, k_{obs} becomes

$$k_{obs} = k_{on}[E_t] + k^* \quad (3^*)$$

where k* is the first-order rate constant that characterizes the transformation of active E′ into a more active species E*.

For the caspase-8(D_2A) mutant (single-chain), the dependence of k_{obs} on the enzyme concentration did not exhibit a linear dependence, and eq 3 was modified so that it takes into account the fact that the active enzyme dimer, E′, is at equilibrium with its inactive monomer, E (*20*):

$$k_{obs} = \frac{k_{off}}{8[E_t]}(\sqrt{K_d + 8[E_t]} - \sqrt{K_d})^2 \quad (4)$$

where k_{off} is the maximum rate constant at high concentrations of enzyme, when all the enzyme is present as a dimer, and K_d is the dissociation constant. The second-order rate constant k_{on} was calculated using eq 5:

$$k_{on} = \frac{k_{off}}{K_d} \quad (5)$$

Kinetics of Caspase-8 Inactivation. For the experiments involving the decay of caspase-8 activity, a mixture of caspase-8 monomer and dimer was diluted into low-salt assay buffer at a final concentration of 10−200 nM and incubated at 37 °C for 3.5 h. Aliquots were withdrawn in time, and the activity was tested in low-salt buffer as described above. For an equilibration time of 3.5 h, the activity was plotted versus the enzyme concentration. Assuming that only the dimeric species is active, the data were fit using eq 6:

$$v_{obs} = v_{max}\frac{4[E_t] + K_d - \sqrt{K_d(8[E_t] + K_d)}}{4} \quad (6)$$

where v_{obs} is the activity measured at a certain total concentration of enzyme, [E_t], v_{max} is the maximum velocity when all the enzyme is present as a dimer, and K_d is the dissociation constant between the monomer and dimer (*21*).

For the experiments involving the kinetics of inactivation by dilution from high-salt buffer into low-salt buffer, caspase-8 in the range of 75 nM to 2 μM was dissolved in buffer A containing 1 M sodium citrate (pH 7.5), and complete activation was allowed to proceed at 23 °C, on the basis of the results for the kinetics of activation. Samples were then diluted 20-fold in buffer A and incubated at 23 °C, and at the specified times, a 100 μL sample was withdrawn and the activity determined as described above. As controls, samples were diluted 20-fold in buffer A containing 1 M sodium citrate and treated as described above. The activity of these controls was considered the "time zero" activity prior to dilution.

Chemical Cross-Linking. Caspase-8(D_2A) was dialyzed against 50 mM sodium phosphate (pH 7.5) containing 1 mM DTT and then activated in the same buffer containing sodium citrate in the concentration range of 0.2−1 M for 1 h at room temperature at final protein concentration of 7 μM. Dymethyl pymelimidate was added to at final concentration of 1 mM, and the samples were incubated for 30 min at room temperature (*22*). The protein was precipitated with 10% TCA, washed, and then analyzed via 12−18% SDS−PAGE.

Fluorescence Emission Studies. Caspase-8 mutants were diluted at a final concentration of 0.5 μM in 50 mM sodium phosphate (pH 7.5) with 1 mM DTT, or in the same buffer containing 1 M sodium citrate. Proteins were incubated for 30 min at room temperature. Samples were excited at 295 nm, and the fluorescence emission was read between 310 and 400 nm in a Perkin-Elmer LS50B fluorescence spectrometer coupled with the LF Win Lab software using a 1 cm pathway quartz cuvette (Helma). The average emission wavelength was calculated as described previously (*23*) using eq 7:

$$\langle\lambda\rangle = \frac{\sum_{i=1}^{N}\lambda_i I_i}{\sum_{i=1}^{N} I_i} \quad (7)$$

where $\langle\lambda\rangle$ is the average emission wavelength and I_i is the fluorescence emission for wavelength λ_i. The emission fluorescence was plotted against wavelength, and the background emission (buffers only) was subtracted from the protein spectra.

RESULTS

Cleavage Does Not Activate Recombinant Caspase-8. Following cellular activation by recruitment to an oligomeric

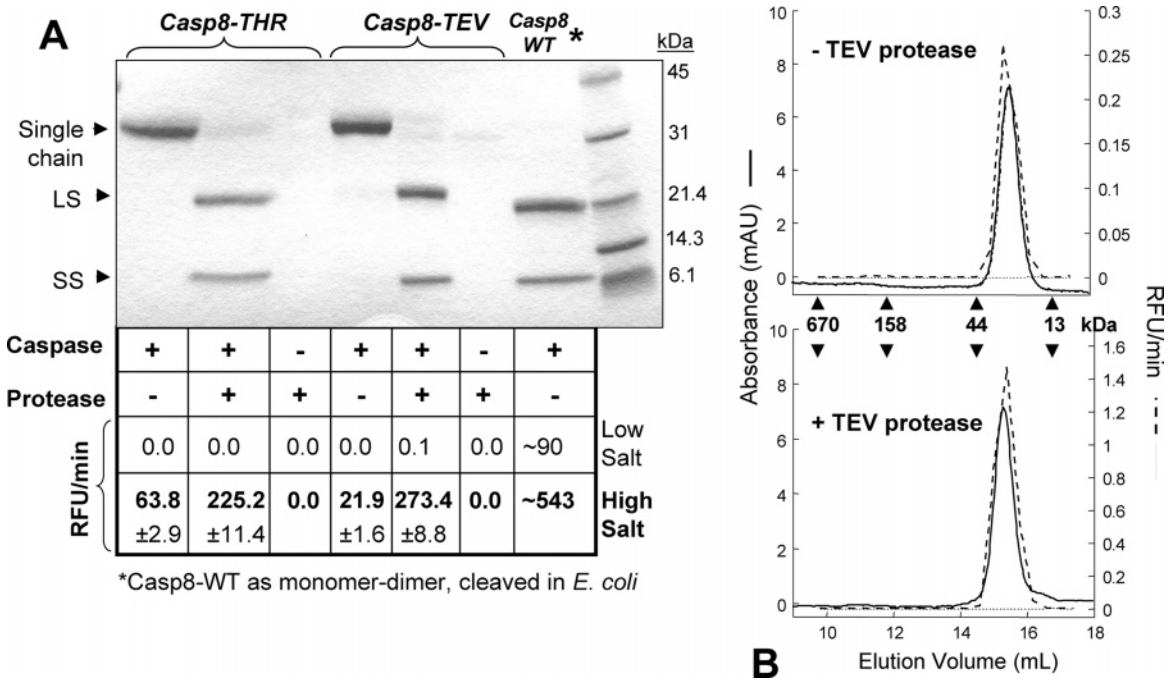

FIGURE 2: Cleavage does not activate caspase-8 or change its dimeric properties. (A) Recombinant caspase-8 mutants Casp8-THR and Casp8-TEV (4.4 μM) were cleaved by thrombin and TEV protease, respectively, at a substrate:protease ratio of 10:1. The cleavage was analyzed by SDS−PAGE. Caspase large and small subunits are labeled LS and SS, respectively. The values beneath the gel list the activity of the mutants after cleavage. Activity was determined vs Ac-IETD-AFC in low-salt assay buffer or high-salt assay buffer at a protein concentration of 50 nM. (B) Gel filtration analysis of the uncleaved Casp8-TEV or cleaved Casp8-TEV (200 μL of a 4.4 μM solution) monitored by absorbance at 280 nm (—). The activities of fractions assayed in low-salt (•••) and high-salt (- - -) buffer are shown.

Table 1: Catalytic Parameters of Caspase-8 Mutants in 1 M Sodium Citrate

		high salt			low salt
form	mutant	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
two-chain	Casp8-WT	4.5 ± 0.4	1.58 ± 0.04	3.5×10^5	1.2×10^4
	Casp8-THX	3.8 ± 0.7	0.60 ± 0.02	1.5×10^5	$<10^2$
	Casp8-TEV	9.3 ± 1.4	0.78 ± 0.03	8.3×10^4	$<10^2$
single-chain	Casp8-D_2A	16.0 ± 2.4	0.64 ± 0.04	4.0×10^4	$<10^2$
	Casp8-THX	3.6 ± 0.3	0.37 ± 0.009	1.0×10^5	$<10^2$
	Casp8-TEV	67.6 ± 16	0.67 ± 0.05	1.0×10^4	$<10^2$

activation complex known as the DISC, caspase-8 becomes cleaved into a large subunit and a small subunit. Moreover, *E. coli* expression of the single-chain catalytic domain results in rapid and complete cleavage of caspase-8 in the intersubunit linker. Since the analogous cleavage is the activation event for caspases-3 and -7, we first considered the possibility that this represents the same role in caspase-8. To investigate this, we designed caspase-8 mutants that can be cleaved in the intersubunit linker in a controllable manner by thrombin or TEV-protease (Figure 1). Recombinant caspase-8 purified from *E. coli* as a two-chain protein (Casp8-WT) and the single-chain mutant (D_2A) harboring the D297A and D314A mutations served as controls (Figure 1). Because recombinant full-length caspase-8 is insoluble in our hands, we used caspase-8 constructs from which the N-terminal DEDs were deleted.

Figure 2A shows that single-chain caspase-8 linker mutants could be cleaved by thrombin and TEV protease into small and large subunits, respectively. Casp8-WT also shows two-chain cleavage products due to autoproteolysis during expression. The faster migration of the large subunit of Casp8-WT is due to cleavage at both autolytic sites (Figure 1).

Next, we tested whether simple cleavage of caspase-8 mutants can generate enzymatic activity. The cleavage products were diluted in the assay buffer in the nanomolar range and tested for activity versus the preferred substrate Ac-IETD-AFC. Figure 2A shows that there is no substantial induction of enzymatic activity generated upon cleavage, if the assay is performed in a buffer with a low salt concentration (at most, 0.03% of the activity measured in sodium citrate). Monomeric Casp8-WT separated by gel filtration exhibits no activity in the same range of concentrations, consistent with the fact that monomeric species is inactive (*6*). This suggests that proteolysis of the intersubunit linker is insufficient for organizing an active site. The activity in low-salt buffer of Casp8-WT shown in Figure 2A is due to the dimeric component of the monomer/dimer mixture produced by *E. coli*. However, if the activity is measured in buffer containing 1 M sodium citrate known to induce activation without cleavage (*6*), the linker mutants all exhibit robust activity (Figure 2A). For comparison, the catalytic parameters of the cleavage mutants, measured before and after cleavage in 1 M sodium citrate, are similar to the values of the single-chain and two-chain caspase-8 measured under the same conditions (Table 1). The k_{cat} values are almost

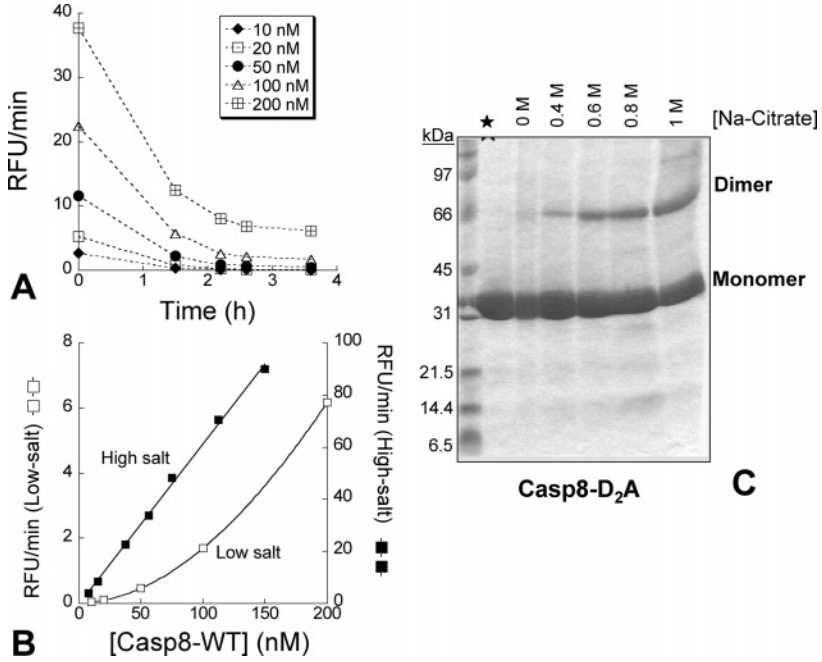

FIGURE 3: Sodium citrate dimerizes caspase-8. (A) Activity decay over time of a mixture of monomeric and dimeric caspase-8 measured in low-salt assay buffer. Different concentrations of the enzyme are shown in the inset. (B) Activity of wild-type caspase-8 as a function of the concentration of caspase-8 after equilibration for 3.5 h in low-salt assay buffer (□) or high-salt assay buffer (■). The data were fit with eq 6 (low salt) or a standard linear equation (high salt). (C) Cross-linking of Casp8-D_2A with dimethyl pimelymidate preincubated in the indicated concentrations of sodium citrate (lane ★, no addition of cross-linker). Samples were precipitated with TCA and analyzed by SDS−PAGE.

unaffected by the mutations. However, the Casp8-TEV mutant exhibits higher K_M values probably due to the mutagenesis in the intersubunit linker. With the exception of the thrombin mutant, the specificity constants of the uncleaved mutants are ∼8−10-fold lower than of those of the cleaved enzymes. Overall, the results demonstrate that cleavage does not have the potential to organize the catalytic loops for activation in the same way that sodium citrate does.

Figure 2B shows the gel filtration analysis of the Casp8-TEV mutant (MW ∼ 32 kDa) before and immediately after cleavage with TEV protease. At least in the time frame of the experiment (1.5 h), the change in the elution peak was insignificant. The apparent molecular masses for uncleaved and cleaved Casp8-TEV were ∼22.4 and ∼26.1 kDa, respectively, suggesting that the protein remains a monomer after proteolysis. In addition, we could not detect any activity in the collected fractions when measured in low-salt assay buffer, but substantial activity was measured in the presence of sodium citrate. Similar results were obtained for the Casp8-THR mutant (not shown). Therefore, no dimeric or active species could be detected by size exclusion chromatography following cleavage of caspase-8.

Monomer−Dimer Transition of Caspase-8. Freshly purified recombinant caspase-8 at ∼18 μM occurs in an approximately equal mixture of monomer and dimer (*6*), which loses enzymatic activity over time (*24*). To examine whether the activity decay is due to dimer dissociation, the enzyme was diluted in low-salt assay buffer to the concentrations shown in Figure 3A and incubated at 37 °C. The activity of caspase-8 at the equilibration time of 3.5 h (Figure 3A) was plotted against the concentration, and the results are shown in Figure 3B. There is no clear linear dependence between activity and enzyme concentration, which suggests that the caspase-8 dimer is in equilibrium with the monomer. The data were fit with an equation that assumes that only the dimeric species is active (see eq 6 in the Experimental Procedures), giving a K_d of ∼3.3 μM, close to the published K_d of ∼5 μM for caspase-8 in the presence of the inhibitor (*7*). We call this value the K_d in the presence of the substrate, because the latter influences caspase oligomerization during the activity assays (*19*).

Notice that this is not the case for caspase-8 activity measured in sodium citrate [Figure 3B (■)], for which the dependence is linear. Sodium citrate changes the catalytic properties of caspase-8 by influencing both k_{cat} and K_M (*6*). In Figure 1A of the Supporting Information, we show that the k_{cat} increases linearly with sodium citrate concentration by ∼23-fold from 0 to 1 M sodium citrate, while the K_M decreases ∼8-fold. Overall, caspase-8 is more efficient by ∼180-fold in high-salt assay buffer than in low-salt assay buffer at the working concentration of 20 nM. This can be interpreted in terms of the transition to a stable dimer, but there is also the possibility that the kosmotrope also orders the monomer into an active conformation.

By using cross-linking experiments, we show in Figure 3C that single-chain caspase-8 dimerizes in the presence of sodium citrate, and the amount of dimer is proportional to the concentration of the kosmotrope, although this technique is not quantitative. This is also true for the two-chain caspase-8 (not shown) and caspase-9 (*5*). In support of this, we demonstrate in Figure 1B of the Supporting Information that salts belonging to the group of chaotropes or "structure disordering salts" disrupt the activity of dimeric caspase-8 without producing protein precipitation (not shown), while NaCl, a "neutral" salt in the Hoffmeister series, has a minimal effect on the activity. Therefore, it seems that sodium citrate produces both dimerization and activation of caspase-8, and below, we provide more evidence that these two events are

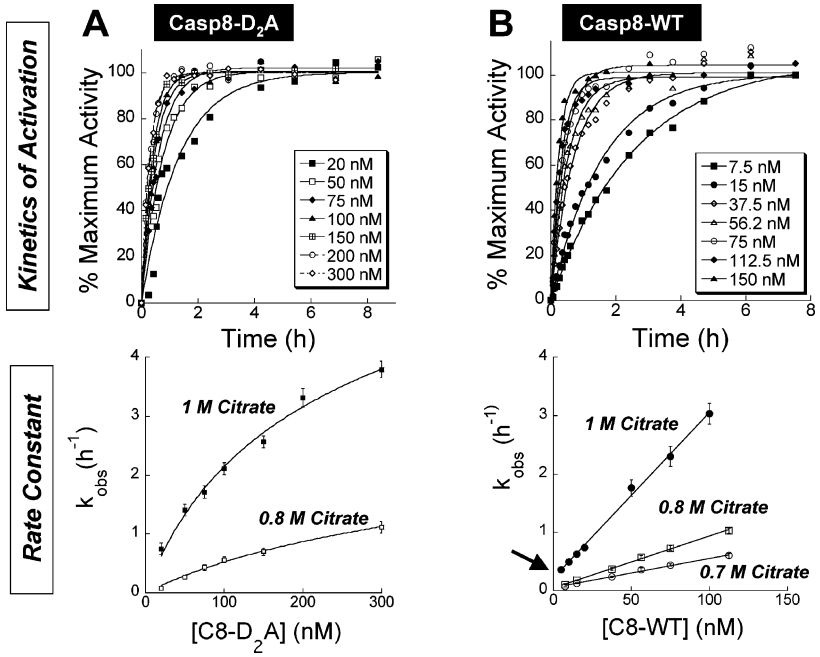

FIGURE 4: Activation of caspase-8 by sodium citrate is a bimolecular process. Kinetics of activation in 1 M sodium citrate for single-chain (Casp8-D_2A, A) and two-chain (Casp8-WT, B) caspase-8 are shown in the top panels after the Ac-IETD-ase activity had been measured as a function of time. The concentrations of proteins used in the study are shown in the legend. The data were fit with a first-order kinetic equation (eq 2; see Experimental Procedures). Bottom panels show the calculation of the second-order rate constant for activation, *k*, at the specified concentrations of sodium citrate. In the case of Casp8-D_2A, the data were fit with eq 4 (see Experimental Procedures), while in the case of Casp8-WT, the data were fit with the linear eq 3*. The arrow points to the intercept on the *Y*-axis of the linear fit (k_{obs} vs concentration) for Casp8-WT activation in 1 M sodium citrate, which corresponds to the value of *k**, the unimolecular rate constant, which is presumed to reflect catalytic loop rearrangements (see Discussion).

correlated. Moreover, caspase-8 can display modest activity subsequent to cleavage if the kinetic barrier for dimer association is reached in time.

Kinetics of Caspase-8 Activation in Sodium Citrate. If sodium citrate activates the monomer of caspase-8 in an allosteric manner, then the kinetics of caspase-8 activation at different protein concentrations would give a first-order response (unimolecular). By contrast, a second-order (bimolecular) activation would describe dimerization. Figure 4 shows the kinetics of activation of single-chain (Casp8-D_2A) and two-chain (Casp8-WT) caspase-8 in kosmotrope buffer. The monomeric form separated by gel filtration was used for all analyses described in this study.

As seen in Figure 4, the activation of caspase-8 in the nanomolar range in 1 M sodium citrate is a slow process, which requires several hours for completion. The kinetics are protein concentration-dependent for both single-chain (Figure 4A) and two-chain caspase-8 (Figure 4B), demonstrating that sodium citrate activates caspase-8 by a bimolecular process. The pseudo-first-order kinetic rate, k_{obs}, generated by the fits was plotted versus the enzyme concentration (Figure 4, bottom panels) to calculate the second-order rate constant. Caspase-8 is activated by 1 M sodium citrate with rate constants of $\sim 8 \times 10^3$ M^{-1} s^{-1} for the two-chain enzyme and $\sim 5 \times 10^3$ M^{-1} s^{-1} for the single-chain enzyme (Table 2). As k_{obs} was a linear function of the protein concentration for the two-chain form, we estimated the dissociation constant (K_d) to be significantly lower than 10 nM for >0.7 M sodium citrate. However, this was not the case for the single-chain form, for which eq 2 was modified so that it takes into account the fact that the dimeric active species occurs at the equilibrium with the inactive monomeric species (eq 4). The estimated K_d in this case was

Table 2: Kinetic Constants for Caspase-8 Activation in Sodium Citrate

parameter	[sodium citrate] (M)	Casp8-WT (two-chain)	Casp8-D_2A (single-chain)
K_d (μM)	0	3.3 ± 0.5	–
	0.7	<0.01	–
	0.8	<0.01	1.3 ± 0.23
	1.0	<0.01	0.51 ± 0.09
k (M^{-1} s^{-1})	0.7	1.35×10^3	–
	0.8	2.25×10^3	0.93×10^3
	1.0	7.87×10^3	5.03×10^3

$\sim$500 nM in 1.0 M sodium citrate and $\sim$1.3 μM in 0.8 M sodium citrate (see Table 2).

Interestingly, the linear distribution of k_{obs} versus enzyme concentration in the case of two-chain caspase-8 reproducibly intersected the *Y*-axis above zero (Figure 4B, bottom panel). The apparent k_{obs} value could therefore incorporate another rate constant that is independent of the protein concentration. One explanation for this is that sodium citrate activates caspase-8 by an additional unimolecular step besides dimerization, for example, by reorganizing the catalytic loops. This hypothesis is supported by the observation that sodium citrate also increases the activity of caspase-3 and caspase-7 by $\sim$3-fold (see Figure 2A of the Supporting Information), although these caspases are already stable dimers, active and cleaved. Consequently, we conclude that dimerization represents the major event during activation of caspase-8 by sodium citrate.

Inactivation of Caspase-8 by Dilution from Sodium Citrate Buffer. The difference in the K_d values for dimer dissociation of the single-chain and two-chain caspase-8 (Table 2) could be due to altered dissociation kinetics. We next monitored the kinetics of inactivation of caspase-8 by dilution from the

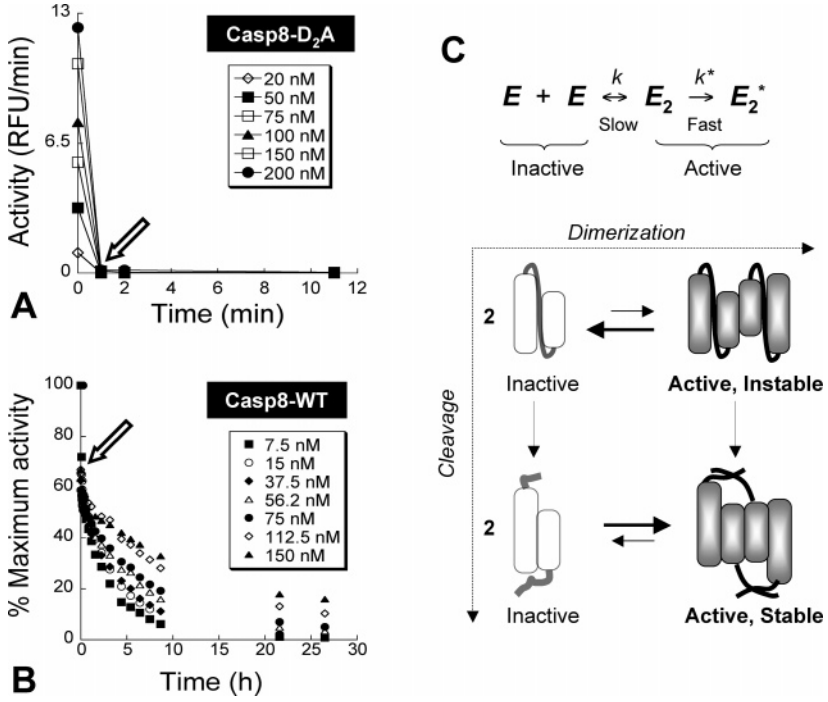

FIGURE 5: Activation in sodium citrate is a reversible process. The kinetics of inactivation of Casp8-D_2A (A) and Casp8-WT (B) were monitored by enzyme dilution from 1 M sodium citrate to the assay buffer. The activity was measured using Ac-IETD-AFC. The concentrations of the proteins used in the study are shown in the legend. The arrows show the first dilution point for which the activity was measured after the dead time of mixing. (C) Proposed model for activation of caspase-8 in sodium citrate. The inactive monomeric enzyme E forms an active dimer E_2 via a slow association step, which then converts rapidly to a more active species E_2*. Cleavage does not activate the enzyme but stabilizes the dimeric conformation.

high-salt assay buffer into the low-salt assay buffer. Figure 5A shows that the dissociation of the single-chain enzyme is too fast to be measured. In contrast, the two-chain enzyme exhibits a rapid initial phase that is concentration-independent, followed by a slower process that is concentration-dependent (Figure 5B). This is consistent with a first-order inactivation of the enzyme (accounting for a 30% decrease in the activity) followed by a dissociation step that accounts for the loss of the remaining activity. Significantly, Figure 2B of the Supporting Information shows that the activity increase of caspase-3 in 1 M sodium citrate is also a fast process, as ~75% of the maximal activation occurs in the mixing time. This is also true for caspase-7 (not shown).

Therefore, it seems that activation of caspase-8 by kosmotropic salt sodium citrate is composed of at least two steps, one involving dimerization and one involving rearrangements within the protein structure, but the order of the two events is still in question (see the model in Figure 5C).

Fluorescence Studies Using Caspase-8. We examined the change in the catalytic loop environment during caspase-8 activation by monitoring the fluorescence emission of the aromatic residues. We took advantage of the fact that the catalytic domain of caspase-8 has only one tryptophan residue, situated on the small subunit, close to the active site. We recorded fluorescence emission spectra via excitation at 295 nm of caspase-8 mutants in the presence of sodium citrate.

Figure 6 shows that both caspase-8 variants undergo a reproducible blue shift (2.5−3 nm) upon incubation in sodium citrate (solid line vs dotted line), suggesting that the tryptophan moves into a more hydrophobic environment during activation (Figure 6A,B). The catalytic mutant (C285A) exhibits a more modest blue shift of only ~1 nm. However, single-chain caspase-8 demonstrates an additional increase in the fluorescence emission versus the background state (~100%), which is not seen for the two-chain enzyme (Figure 6A). This could be due to a change in the ionic environment as a result of two aspartate substitutions in the intersubunit linker, not present in the case of the single-chain catalytic cysteine mutant (Figure 6C). Sodium citrate has the same effect on the fluorescence emission of caspase-3 (Figure 2D of the Supporting Information) and caspase-7 (not shown), although these caspases contain two Trp residues located near the active site. A control experiment shows that the blue shift is not due to a high salt concentration, because NaCl has a minimal effect on the average emission wavelength of caspase-8 (Figure 3D of the Supporting Information). Additionally, sodium citrate itself does not generate a fluorescence blue shift of free Trp in solution (Figure 2C of the Supporting Information) but actually favors a red shift and a slight increase in the fluorescence.

To determine whether sodium citrate has an effect on the catalytic loop reorganization before or after caspase-8 dimerization, we investigated the fluorescence properties of a mutant altered in the dimer interface (T390D) that is incapable of dimerization. Caspase-8(T390D) lacks enzymatic activity in both low-salt and high-salt assay buffer (*6*). Figure 6D shows that, after excitation at 295 nm, the average emission wavelength of the T390D mutant was essentially unaltered in sodium citrate. It is therefore likely that the fluorescence blue shift measured for caspase-8 mutants is associated with the presence of a dimeric form. There is a formal possibility that the kosmotrope causes a rapid effect on the monomeric form, and in this case, one would expect to see a faster kinetics in the fluorescence change versus activation kinetics. In Figure 3 of the Supporting Information,

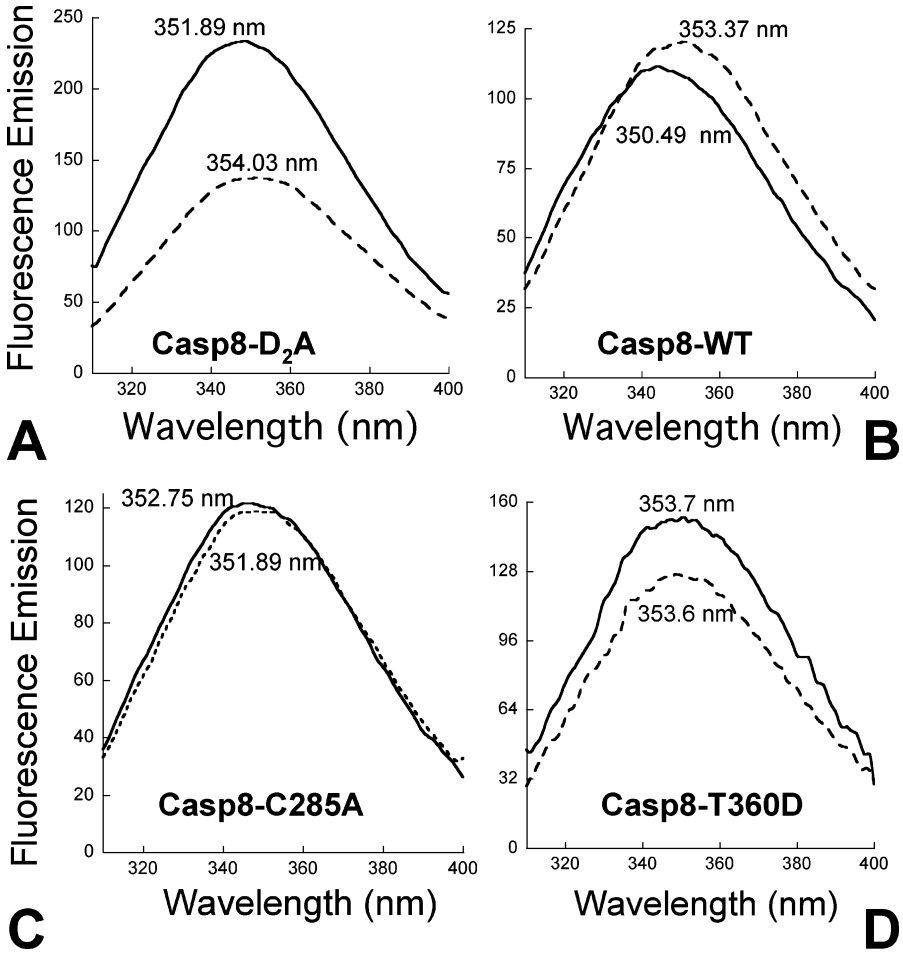

FIGURE 6: Sodium citrate rearranges the catalytic loops of caspase-8. Fluorescence emission after excitation at 295 nm of uncleavable caspase-8 (Casp8-D_2A) (A), wild-type caspase-8 (Casp8-WT) (B), the catalytic caspase-8 mutant (Casp8-C285A) (C), and the interface inactive mutant (Casp8-T390) (D), preincubated in phosphate buffer (- - -) or 1 M sodium citrate (—). The average emission wavelength was calculated using eq 7 and is shown for each curve.

we show that the changes in the maximum fluorescence emission, the average emission wavelength, mirror the changes in enzymatic activity in 1 M sodium citrate, ruling out the latter possibility.

We conclude that the sodium citrate-induced dimerization of caspase-8 transmits a signal to the catalytic loops, during which the active site is developed and becomes capable of hydrolyzing the substrate. The mechanism of activation depicted in Figure 5C supports our experimental outcomes.

DISCUSSION

Although numerous studies show that dimerization of caspase-8 is sufficient for generation of enzymatic activity in vitro (*4*, *6*, *25*), most of the time caspase-8 also appears to be cleaved within cells undergoing apoptosis, presumably as a consequence of activation. Activity-based probes are diagnostic for active enzymes (*26*), and the probe B-VAD-FMK is able to capture the presence of the unprocessed active caspase-8 before autocleavage occurs in vitro (*6*) and even in intact cells (*13*, *15*). As the intersubunit linker of caspase-8 contains the recognition motif for autocleavage, and also cleavage by caspase-6 (*27*) and Granzyme B (*28*), a natural question is what will happen to caspase-8 if other proteases cleave it in the absence of a dimerization stimulus. Using B-VAD-FMK, it is possible to demonstrate that the active form of caspase-8 in cells induced to undergo apoptosis can also be observed in the intersubunit cleaved form, and arguments have been made that cleavage can generate active material (*15*). However, a parallel study found that B-VAD-FMK is unreliable when used to address cleavage versus noncleavage in extracts of apoptotic cells, explaining why the studies mentioned above (*13*, *15*) generated contradictory results (*16*). An alternative way to demonstrate whether cleavage of endogenous caspase-8 in cell lysates generates activity utilized specific immunoprecipitation followed by a substrate-based reporter assay (*16*). However, the experimental protocol used in this report can lead to pseudoactivation due to oligomerization-induced clustering on the beads used to immunoprecipitate the protein, a phenomenon noticed before for caspases-2 and -9 (*25*). In all the reports described above, where endogenous caspase-8 from human cells is analyzed by immunoprecipitation or by B-VAD-FMK, there are substantial discrepancies and potential artifacts, which is the main reason we decided to determine the mechanism using a defined system and conditions using recombinant protein produced from *E. coli*.

We show here that the controlled cleavage of caspase-8 does not generate substantial enzymatic activity or change its oligomeric properties (Figure 2A). However, we do observe a small amount of activity in TEV protease-cleaved caspase-8 (~0.03% of the maximal activity). The activity assay was conducted at a final caspase-8 concentration of

50 nM, which exceeds the estimated cytosolic concentration (~25 nM) for this protein (*29*). Consequently, the amount of activity generated by cleavage is so small that it is unlikely to be of sufficient magnitude to drive forward an apoptotic pathway.

Kosmotropic salts led to elucidation of the difference in activation between single-chain and two-chain caspase-8. First, the activation in high concentrations of sodium citrate is dependent on protein association for both forms, suggesting that sodium citrate produces caspase-8 dimerization (Figure 4). In support of the dimer-induced activation of caspase-8 is the finding that a dimeric species can be trapped by covalent cross-linking, and that the degree of trapping is dependent on the kosmotrope concentration (Figure 3C). However, although both single-chain and two-chain forms of caspase-8 are kinetically equivalent for sodium citrate activation via a bimolecular event (see kinetic rates k in Table 2), the degree of stability of the cleaved dimer is substantially higher than that of the single-chain dimer, as shown by their different K_d values (Table 2). The simplest explanation of this behavior is that dimerization is independent of cleavage, but once formed, the cleaved dimer is more stable. Presumably, the single-chain dimer contains fewer contacts in the loop bundle that defines part of the dimer interface produced by interactions with the cleaved interchain linker (*30*). In the executioner caspases-3 and -7, these form important interdomain contacts only after cleavage (*31*−*35*), but in uncleaved caspase-8, because the interchain linker is much longer, the interactions may still form, though presumably with a lower efficiency. The atomic-resolution structure of single-chain caspase-8 is required to test this possibility. Consequently, the instability of the single-chain caspase-8 dimer relative to the two-chain form is presumably due to the low values of k_{off} and not the rate of association into dimers.

The activation kinetics of two-chain caspase-8 in sodium citrate suggests that the kosmotrope has an additional effect on the enzyme besides promoting its dimerization; this process is reflected by the offset of k_{obs} dependence on the protein concentration (Figure 4B, bottom panel), named k^*. The origin of the offset is unclear but could be due to a unimolecular rearrangement event in caspase-8 reflected as a further increase in the enzymatic activity. During the reversible kinetic inactivation assay, a dimeric intermediate was detected (Figure 5B). The simplest explanation for this is that the activation process in sodium citrate represents dimerization as a slow event, followed by a unimolecular ordering of the active site, possibly by loop ordering within the dimer, as a fast event (Figure 5C). Both mechanisms were previously demonstrated for the kosmotrope activation of some viral proteinases (*36*, *37*). The proposed mechanism is supported by several other experimental findings. First, sodium citrate is capable of increasing the activity of the already dimeric active caspases-3 and -7 by ~2−3-fold (Figure 2A of the Supporting Information). Taking into account the fact that this increase reflects only a change in the k_{cat} and not in the K_M (*33*), we are tempted to speculate that sodium citrate facilitates a more efficient attack on the substrate (better positioning of the catalytic cysteine or histidine) than better recognition of the substrate. Second, the unimolecular activation event of caspase-8 is clearly dependent on the sodium citrate concentration. The activation kinetics of caspase-8 in 0.7 and 0.8 M sodium citrate (Figure 4B, bottom panels) generate lower k^* values than in 1 M sodium citrate, in agreement with the increase in activity of dimeric caspases-3 and -7 at the same concentrations of the kosmotrope (Figure 2A of the Supporting Information). The hypothesis regarding loop reorganization does not seem applicable for the single-chain caspase-8 (Figure 4A, bottom panel), for which there is no contribution of an additional unimolecular event during activation. This may explain the difference in the catalytic constant k_{cat} values between the cleaved and uncleaved caspase-8 (Table 1), but other factors can also be part of the cause, such as simply the effect of mutating the intersubunit linker. Interchain mutagenesis may affect dimerization, especially since phosphorylation of Tyr380, which is situated in the linker, hinders caspase-8 activation (*38*). Importantly, it appears that the unimolecular event does not exist in the absence of dimerization, because a caspase-8 mutant that cannot be dimerized does not demonstrate a Trp fluorescence shift characteristic of the single-chain and two-chain forms in sodium citrate (Figure 6).

Finally, there is an important caveat to the interpretation of our study, namely that all the results shown here are for recombinant caspase-8 lacking the tandem DEDs at the N-terminus of the natural protein. DEDs recruit caspase-8 to the DISC, and although DED cleavage is important for the subsequent release of caspase-8 in the cytosol (*39*), we cannot predict how the presence of DEDs before removal affects dimerization. Eventually, it may be possible to produce recombinant full-length caspase-8 without the currently insurmountable problem of aggregation and precipitation. However, there is no mechanism that has been proposed to explain how DEDs would influence dimerization in the absence of DISC recruitment. Consequently, the conclusion of our study is that caspase-8 cleavage is not sufficient for generation of catalytic activity, but once the dimer is formed, the interchain cleavage greatly enhances the stability of the catalytically active dimer. This may have an important role in stabilizing the enzyme once it is released from the DISC following cleavage between the DEDs and catalytic domain (*39*).

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SUPPORTING INFORMATION AVAILABLE

Influence of sodium citrate and chaotropic salts on caspase-8 catalytic parameters (Figure 1), change in activity and fluorescence emission for caspases-3 and -7 in the presence of sodium citrate (Figure 2), and kinetics of activation and change in fluorescence emission of Casp8-D_2A in presence of 1.0 M sodium citrate (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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