Cytokine Response Modifier A Inhibition of Initiator Caspases Results in Covalent Complex Formation and Dissociation of the Caspase Tetramer

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Active caspases are generally composed of two catalytic domains, each containing a large (p20) and a small (p10) subunit so that a fully active caspase has the organization (p20-p10)2. The cowpox serpin crmA suppresses host apoptosis and inflammation by inhibiting endogenous caspases. We report on the mechanism crmA uses to inhibit caspases 1, 6, and 8. Native PAGE showed formation of tight crmA-caspase complexes. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry provided evidence for a covalent crmA-p20 thioester linkage. SDS-PAGE of isolated complexes showed near complete loss of the p10 subunit from initiator caspases 1 and 8 but not from the executioner caspase-6. This was confirmed for caspase-1 by sequencing and Western blotting. Size exclusion chromatography indicated a size of ~60 kDa for complexes with caspases 1 and 8, consistent with a crmA-p20 species, suggesting that the p20-p10 interface and possibly the p10-p10 interface had been disrupted. In contrast, crmA-caspase-6 complex behaved as an equilibrium mixture of crmA-(p20-p10)2 and crmA-(p20-p10). Complex deacylation rates were all slow, suggesting effective kinetic trapping of the covalent thioacyl intermediate. These results suggest a novel serpin inhibition mechanism through which crmA down-regulates apoptosis and inflammation. This involves (i) rapid formation of covalent complex with initiator caspases 8 or 1, (ii) very slow deacylation, and (iii) loss of the caspase p10 subunit for initiator but not for executioner caspases, so that any free p20 formed by deacylation of initiator caspases cannot reassociate to active heterotetramer, thus resulting in irreversible inhibition of apoptosis and inflammation.

Caspases are intracellular cysteine proteinases involved in inflammation and apoptosis (1). Caspase-1, also known as interleukin-1β converting enzyme (ICE),3 activates the pro-inflammatory cytokine interleukin-1β by proteolysis (2), whereas caspase-8, itself activated by dimerization through an extrinsic receptor-mediated pathway (3, 4), acts as an initiator of apoptosis through downstream proteolytic activation of so-called “executioner” caspases, such as caspases -3, -6, and -7 (5). Active caspases are obligate homodimers, with each monomer composed of a large (p20, ~17–20 kDa) and a small (p10, ~9–12 kDa) subunit, which are formed after the activation cleavage of the procaspase (6). Structures of several caspases are known, and all show that two p20-p10 heterodimers are associated in an anti-parallel arrangement through the β-sheet of the two p10 subunits (7–11). The p20 subunit, however, contains both the catalytic cysteine and histidine (6).

Orthopox viruses, such as the cowpox virus, enhance their infectivity through specific inhibition of caspases and consequent abrogation of the inflammatory response and of apoptosis (12). They do this using an inhibitor that is a member of the serpin family (13). In the case of cowpox virus, the serpin is crmA (14). crmA can inhibit a range of caspases, although at very different rates, with very high rate constants for the pro-inflammatory caspase-1 and the initiator caspase-8 but much lower rate constants for the downstream executioner caspases -3, -6, and -7 (15). Although crmA can also inhibit caspase-9 with high rate constant in vitro, this is not thought to be an important target in vivo (16).

To better understand how crmA is used by the cowpox virus to abrogate apoptosis and inflammation, we have characterized the complexes formed between crmA and three caspases by SEC, PAGE, mass spectrometry, Western blotting, sequencing, and kinetic assays. The complexes studied are with the initiator caspase-8, the pro-inflammatory caspase-1, and the executioner caspase-6. We found evidence for the presence of a thioacyl intermediate in all three complexes, equivalent to the acyl intermediate formed in serpin complexes with serine proteinases. An unexpected finding was that complex formation appeared to cause dissociation of the caspase tetramer and loss of the small subunit from complexes with caspases 1 and 8. It seems likely that this tetramer disruption is an additional...
means of ensuring irreversible inhibition of apoptosis initiation or inflammation. The complex with the downstream executioner caspase-6, showed much less perturbation of the interface between the p10 subunits, which resulted in an equilibrium mixture of crmA,(p20-p10)2 and crmA+(p20-p10) species.

**MATERIALS AND METHODS**

*Expression and Purification of Caspase-1—* Separate p20 and p10 subunits of caspase-1 were expressed in *Escherichia coli* BL21(DE3), harboring pET11a-ICEp20 or pET11a-ICEp10 as inclusion bodies in LB medium containing 150 μg/ml ampicillin (17). Cells were grown at 37 °C to A600 = 0.6, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, then further incubated overnight and harvested by centrifugation. Plasmids pET11a-p20 and pET11a-p10 were a gift from Dr. Kathy McCusker, Merck. Caspase-1 was refolded and purified as described by Thornberry et al. (18) but with minor modifications. Inclusion bodies of the subunits, separately solubilized in 6 M guanidinium hydrochloride, 50 mM Tris, 1 mM EDTA, pH 8.0, 200 mM DTT at about 3 mg/ml for p20 and 1.5 mg/ml for p10 were combined dropwise in a 1:1 molar ratio and incubated at 37 °C for 1 h. 30 ml of the solubilized subunits was added dropwise with gentle agitation to 3 liters of caspase-1 buffer (100 mM Hepes, 10% sucrose, 0.1% CHAPS, 0.5 mM EDTA, pH 7.5) containing 10 mM DTT and 50 ml of Ac-YVKD-CHO (Bachem) coupled, according to Rano et al. (17), to epoxy-activated Sepharose 6B. Stirring continued overnight to allow the refolded caspase-1 to bind to the aldehyde inhibitor resin. The resin was then packed into a column and washed with 1 liter of caspase-1 buffer without DTT. Caspase-1 was eluted with 50 ml of 100 μM Ac-YVAD-CHO, which is a tighter inhibitor of caspase-1. The exchange reaction was allowed to proceed overnight, then caspase-1 buffer was applied to displace the eluted protein. Caspase-1 was simultaneously activated by adding 100 mM hydroxyamine (final concentration, adjusted to 7.5) and inactivated by adding 10 μM final oxidized glutathione (GSSG). After 4 h at room temperature caspase-1-SG (inactive glutathione adduct of caspase-1) was concentrated and dia-lyzed exhaustively against caspase-1 buffer to remove GSSG and hydroxyamine. Caspase-1-SG was stored at −80 °C in aliquots. The typical yield was 6–7 mg (about 4–5% of the applied inclusion bodies) pure and highly active caspase-1.

*Expression and Purification of Caspases 6 and 8—* Caspase-6 (Mch2) and caspase-8 (FLICE) were expressed as one-chain quots. The typical yield was 6–7 mg (about 4–5% of the applied mixture of crmA2(p20-p10)2 and crmA(p20-p10) species.

*Reaction of crmA and Caspases—* crmA and the caspases were reacted at different molar ratios and in different buffers. The crmA + caspase-1 reaction was examined in more detail.

For preparative scale isolations, the crmA + caspase reaction mixes (20 μM crmA + 5 μM caspase in 0.1–2 ml) were applied to a Superdex 200 column (180 ml, 90 cm column for 0.5–2 ml samples) equilibrated with 10 mM sodium phosphate or 100 mM Hepes buffers containing 100 mM NaCl, 0.1–0.5 mM EDTA, 0.1% CHAPS, pH 7–7.5, and eluted with the same buffer at 0.25 ml/min. Peaks were monitored at 280 nm, and 5–ml fractions were collected and subjected to further analysis. For analytical and small-scale preparative purposes a smaller 24-ml Superdex 200 HR 10/30 column (AP Biotech) was used (as in Figs. 1 and 5) in the same buffer. The applied sample was 100 μl; it eluted at 0.25 ml/min and was detected by protein fluorescence, with excitation at 280 nm and detection at 340 nm. The column was calibrated using myoglobin (17.6 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), IgG (160 kDa), and thyroglobulin (669 kDa), all from Sigma, in the same buffer without DTT.

*Native and SDS-PAGE, Isolation from Native Gel—* Native PAGE was carried out using the standard Laemmli buffer system without SDS on 8–16% precast gels from different manufacturers. To isolate proteins from the native gel for MALDI-TOF MS analysis, the gel-slice, where the protein is expected to migrate, was cut out with a razor blade and extracted with 2 gel volumes of 50 mM NaCl, 10 mM Bicine, 0.5 mM EDTA, 1 mM CHAPS, 10 mM DTT, pH 8.0, overnight at 0 °C. The extracted protein was concentrated when necessary on a small spin col-
The gel was stained afterward to ensure the proper cut. When only SDS-PAGE was performed with the isolated protein, the gel was stained first, then extracted with SDS sample buffer. SDS-PAGE was done on 4–12% Bis-Tris Novex™ gels in combination with MES running buffer (separation range, 2–200 kDa) from Invitrogen. Fluorescein-labeled proteins were visualized using a Fotodyne UV transilluminator.

MALDI-TOF MS Analysis—1 µl of the protein samples (concentration range, 0.05–0.2 mg/ml) in their respective buffers was mixed with 1 µl of the matrix compound solution and analyzed on a Voyager-DE PRO (Applied Biosystems) spectrometer at the University of Illinois at Chicago Research Resources Center facility. The samples were made fresh or thawed on ice just before the analysis.

Amino Acid Sequencing—crmA-caspase-1 complex samples were separated and purified by native gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) in a Bio-Rad Mini Trans-Blot Cell for 1 h at a constant 100 V at 5 °C using 25 mM Tris, 192 mM glycine, 20% methanol buffer. Stained bands were cut from the polyvinylidene difluoride membranes and sequenced by the Macromolecular Structure Facility at Michigan State University.

Western Blotting—Proteins run as above (for sequencing) were transferred to nitrocellulose membranes (Hoefer Scientific) for antibody detection. Western blots were developed using the Super Signal West Pico kit and CL-Exposure film from Pierce. After transfer the nitrocellulose membranes were blocked using 5% dry milk in PBS (20 mM NaPO₄, 100 mM NaCl, pH 7.3), 0.3% Tween 20, 0.02% sodium azide overnight at room temperature. Membranes were incubated with primary antibodies for 2 h at room temperature in PBS, 0.05% Tween 20, 2 mg/ml BSA. The primary antibody for p10 was Oncogene Pan Caspase-1 rabbit polyclonal antibody, diluted 1/1000. After extensive washing in PBS, 0.05% Tween 20, 2 mg/ml BSA, the membranes were incubated with peroxidase-linked secondary antibodies for 2 h at room temperature in PBS, 0.05% Tween 20, 2 mg/ml BSA. The primary antibody for p20 was Santa Cruz Biotechnology goat polyclonal anti-caspase-1 p20, diluted 1/1000. After extensive washing in PBS, 0.05% Tween 20, 2 mg/ml BSA, the membranes were incubated with peroxidase-linked secondary antibodies for 2 h at 25 °C. The secondary antibody for p10 was Cell Signaling anti-rabbit IgG-peroxidase diluted 1/1000 in PBS/ Tween 20/BSA. The secondary antibody for p20 was anti-sheep/goat Ig peroxidase from the Binding Site, diluted 1/10,000 in PBS/Tween 20/BSA. Membranes were washed twice using PBS/Tween 20/BSA and three times using PBS before development using the chemiluminescent substrate.

Measurement of Regenerated Caspase Activity—For measurement of dissociation of in situ-formed complex, complexes were prepared in HSC buffer (100 mM Hepes, 0.1 mM EDTA, 0.1% CHAPS, 20% sucrose, pH 7.5) plus 5 mM DTT at 25 °C with 0.1–2 µM caspase and a sufficient molar excess of crmA to completely inhibit the caspase, based on stoichiometric titrations of the enzyme with the inhibitor. After allowing enough time for the caspase to be fully inhibited, complexes were diluted >100-fold into fluorogenic tetrapeptide substrates (25 µM YVAD-amidomethylcoumarin (AMC) for caspase-1, 50 µM WEHD-AMC for caspase-6, and 50 µM IETD-AMC for caspase-8) in the above Hepes buffer without sucrose plus 5 mM DTT to concentrations of 0.4–12 nM. The initial rate of complex dissociation was then continuously monitored for up to 90 min from the parabolic increase in fluorescence due to substrate hydrolysis by active protease released from the complex, with excitation and emission wavelengths of 380 and 440 nm, respectively, as in previous studies (21). Initial rates were measured by nonlinear least squares fitting of progress curves to a parabolic function and then plotted as a function of the complex concentration for at least three different concentrations of complex. The dissociation rate constant was determined from the slope of this linear plot. The extensive dilution of complex into substrate and the proportional relationship between the initial rate of complex dissociation and complex concentration ensured that a first order rate constant for complex dissociation was being measured, with no significant contribution of complex reformation due to any excess inhibitor present (21). For measurement of dissociation from purified crmA-caspase complexes, complexes purified by size-exclusion chromatography (see above) were diluted into fluorogenic substrates, and complex dissociation was measured as above. Complex concentrations for purified complexes were measured from the absorbance at 280 nm using extinction coefficients for crmA and caspase subunits together with the determined composition of the complexes.

RESULTS

crmA-Caspase-1 Complex Formation—Caspase-1 was incubated with an excess of crmA, and the mixture was fractionated on a previously calibrated Superdex 200 size exclusion column. The elution profile consisted of two major peaks (Fig. 1). When analyzed by SDS-PAGE, the tall, later-eluting peak was found to comprise uncleaved crmA (data not shown), whereas the early eluting peak was taken to be the complex of caspase-1 and crmA, even though it eluted at a position suggesting a size of ~60–70 kDa rather than the ~140 kDa expected for a crmA2(p20-p10)2 species. This was confirmed by native PAGE (Fig. 2A), which showed that the purified complex migrated at the same position (Fig. 2A, lane 4) as the complex band formed by caspase-1 in the presence of excess crmA (Fig. 2A, lane 3). The complex band migrated at a quite distinct
position from that of free caspase-1 (Fig. 2A, lane 2) or crmA (Fig. 2A, lane 1).

The complex was examined first by SDS-PAGE and shown to consist of cleaved crmA, which gives the expected two bands at 34 and 4 kDa, corresponding to the portions of the serpin N- and C-terminal to the cleavage site, respectively, together with the p20 subunit of caspase-1 (Fig. 2B, lanes 5 and 6). Cleaved crmA gives two bands, the larger N-terminal at ~34 kDa (labeled crmA-N) and smaller C-terminal at ~4 kDa (labeled crmA-C).

Presence of a Covalent Linkage between Caspase-1 p20 and crmA and Loss of the p10 Subunit—To independently confirm the loss of the p10 subunit from the crmA-caspase-1 complex and also to determine the nature of the crmA-p20 interaction, MALDI-TOF was run on the crmA-caspase-1 complex. Here the complex was isolated from the discrete complex band that is resolved on a native gel (see Fig. 2A). The most intense peak was at 53.88 kDa (Fig. 3A), corresponding to the sum of the larger crmA fragment and the p20 caspase-1 subunit (calculated 53.68 kDa). A peak of ~40% intensity occurred at an m/z value of 58.12 kDa, which corresponds to both chains of crmA together with caspase-1 p20 subunit (calculated 57.91 kDa). Additional smaller peaks were observed that correspond to m/z values expected for cleaved crmA (both chains, 37.98 kDa; calculated, 37.95 kDa), cleaved crmA (N-terminal fragment, 33.77; calculated, 33.72), caspase-1 p20 (20.18 kDa; calculated, 19.98 kDa), and a small peak for caspase-1 p10 (10.27 kDa, calculated 10.24). No significant peaks at m/z higher than the 58.12 peak were seen. The appearance of pairs of peaks for species containing RCL-cleaved crmA is the same as seen for cleaved crmA itself (Fig. 3B). Species corresponding to z = 2 for complex and for cleaved crmA are also detectable in Figs. 3, A and B.
Although absolute amounts of each species cannot be calculated directly from such a spectrum, the inverse relationship between molecular size and peak intensity that reflects the difficulty of vaporizing larger species allows a crude estimate to be made of relative amounts of different species. Thus, free caspase-1 gave a peak for the p10 subunit approximately twice as intense as for the p20 subunit even though both were present in equimolar amounts (Fig. 3C). The dominance of the highest \( m/z \) peak of crmA + p20 in Fig. 3A, thus, suggests that nearly all of the p20 subunit is very tightly associated with crmA, consistent with the presence of a covalent thioester linkage between the p20 and the larger N-terminal crmA fragment. That the p10 subunit is not present in the complex rather than simply too weakly associated to give higher \( m/z \) species is shown by the negligible intensity for free p10 and the absence of higher \( m/z \) peaks corresponding to the presence of both p20 and p10 in complex with crmA (Fig. 3A). Caspase-1 alone shows some evidence for the ability of p20 and p10 subunits to remain associated during analysis (Fig. 3C). MALDI-TOF of crmA-caspase-1 complex isolated from SEC gave essentially the same results as for complex isolated from the separate band on a native gel (data not shown).

**Western Blotting and Amino Acid Sequencing of the crmA-Caspase-1 Complex**—Two additional approaches were used to confirm the composition of the crmA-caspase-1 complex. Western blotting of reaction mixtures of crmA and caspase-1 using specific anti-caspase-1-p10 and anti-caspase-1-p20 antibodies showed that the complex contained p20 but not p10 subunits (Fig. 4, A and B). Demonstration that the complex contained crmA was carried out using fluorescein-labeled crmA for the reaction (Fig. 4C). Finally, N-terminal sequencing of the complex, extracted from the native gel, was performed and confirmed the presence of caspase-1 p20 (PAMPT) and of the two crmA chains (the original N terminus of MDIFRE and the additional new N terminus from RCL cleavage at P1-P1' DS of SASTVT) but not of the caspase-1 p10 peptide. A control sequence determination of caspase-1 gave both expected sequences for the p20 (PAMPT) and p10 (AIKKA) subunits.

**cramA-Caspase-8 Complex Formation**—Size exclusion chromatography of an incubation mixture of caspase-8 with an excess of crmA gave similar results to those with caspase-1 and crmA. The early eluting peak appeared at a position consistent with a species of \( \sim 60–80 \) kDa rather than \( \sim 140 \) kDa (Fig. 5A).

Native PAGE of crmA-caspase-8 reaction mixtures showed a similar shift in band position as seen with caspase-1, corresponding to complex formation (Fig. 6A). The residual intensity of unreacted caspase-8 resulted from the presence of about 50% inactive caspase, since reactions at different crmA-caspase-8 ratios, but with constant amount of caspase-8, showed the same amount of complex formation (data not shown). Complex excised from the native gel and run on SDS-PAGE showed the presence of cleaved crmA and of the larger, p18 subunit of caspase-8 but with very little detectable intensity for the smaller, p11, caspase-8 subunit (Fig. 6B). The relative intensities were again consistent with the presence of equimolar amounts of crmA and caspase-8 p18 subunit.

MALDI-TOF analysis of this complex isolated from the native gel showed similar intense peaks for crmA-caspase-8 p18 complex without and with the crmA C-terminal 4-kDa peptide, indicating that the complex was composed of p18 covalently linked by thiol ester to the major N-terminal portion of crmA (Fig. 7). Although there is also a somewhat larger peak for p11 than found for the p10 peak of caspase-1, this is still much less intense than expected if p11 were present in a similar molar ratio to crmA (compare with MALDI-TOF of crmA-caspase-6 complex; Fig. 9).
**crmA-Caspase-6 Complex Formation**—The complex formed between caspase-6 and crmA behaved differently from those of caspases 1 and 8 on size exclusion chromatography. Two peaks eluted before the main peak containing unreacted and cleaved crmA (Fig. 5B). The earlier peak, which was about 3 times the size of the later peak, eluted at about the position expected for an intact complex of crmA₂-(p20-p10)₂, whereas the later eluting peak was close to the position for the complexes of caspases 1 and 8 with crmA (Figs. 1 and 5A). SDS-PAGE of each of these species showed the presence of cleaved crmA and both large and small caspase-6 subunits, with intensities consistent with the same 1:1:1 ratio of crmA-p20-p10 in each of the two complex species (Fig. 8B, lanes 3 and 4).

Although native PAGE showed little change in mobility for the caspase-6 band upon incubation with crmA, the two sharp bands visible (Fig. 8A, lane 3), when excised and examined by SDS-PAGE, showed the presence of crmA in addition to both caspase-6 subunits (not shown) in the same way as material from the two peaks separated by SEC. This suggests that the sharpening and splitting of the caspase-6 band on the native gel in the presence of crmA corresponds to the presence of slowly interconverting crmA₂-(p20-p10)₂ and crmA-(p20-p10).

MALDI-TOF was also run on both the early and later peaks of crmA-caspase-6 complex. The spectra of both fractions were similar (one shown in Fig. 9), consistent with the SDS-PAGE analysis and suggesting that they differ in one being a dimer of the other. The quality of the spectra was much lower than for complexes of caspases 1 and 8 as a combined result of lower protein concentration and the presence of solubilizing detergent that was difficult to remove and which adversely affected sample vaporization. Nevertheless, it was possible to detect peaks at high m/z corresponding to the complex between the caspase and crmA. Here the very highest m/z peak corresponded to that expected for a covalently linked complex between the p20 subunit and the large N-terminal fragment of crmA but with the p11 subunit still associated (65.81 kDa, calculated 65.73), whereas the next smaller peak suggested loss of the p11 subunit (53.43 kDa). Only a small peak was seen for free p20, consistent with it being predominantly in covalent linkage with crmA, whereas an extremely large peak was seen for p11.
The presence of this very large peak together with the higher 65.81-kDa peak (i) confirms that all or most of the p11 is still present (in marked contrast to the situation for complexes with caspases 1 and 8), either dissociated under the conditions of the MALDI-TOF or still associated with the complex, and (ii) provides an internal calibration for the size of this peak relative to that of the crmA-p20 species when both are present in equimolar amounts. This is very useful for gauging the negligible amounts of p11/p10 subunits still present in complexes of caspase-1 and caspase-8 with crmA (Figs. 3A and 7), which must be very small.

Complex Stability and Regain of Activity—The complexes formed by crmA with the three caspases, whether isolated by SEC or by elution from native gels, appear to be the same kind of trapped acyl enzyme intermediates that have been well documented for the complexes of serpins with serine proteinases of the trypsin family and which lack enzymatic activity. In the case of trypsin family complexes with serpins, the rate at which the trapped acyl intermediate can be hydrolyzed, i.e. the kinetic stability of the complex, can be determined from the rate of reappearance of enzymatic activity. For the caspase complexes examined here an additional consideration in determining the kinetic stability of the complexes is that if the thiol ester bond between the caspase catalytic subunit and crmA is hydrolyzed when the p10 subunit is absent, the caspase would not be expected to regain catalytic activity, since both large and small subunits are required for activity. Measurement of the regain of activity for crmA-caspase complexes can, therefore, be used both to provide evidence for the presence or absence of the smaller p10 subunits and, if the subunits are present, to calculate the rate of complex hydrolysis.

Regeneration of catalytic activity for the three crmA-caspase complexes was, therefore, carried out by dilution into substrate for complexes isolated by SEC as well as for caspase inhibited in situ, i.e. by mixing of crmA and caspase until inhibition was complete followed by dilution into substrate. In the latter case any small subunit that might dissociate as a result of complex formation would still be present and available for reassociation upon release of the large subunit from complex with crmA, whereas complex isolated from SEC, in which the p10 subunit had been mostly lost, would be largely unable to reform such competent caspase tetramers.

Initial dissociation rates were measured by dilution of in situ-generated complex. These rates showed significant variation between the three caspases, with the crmA-caspase-1 complex the least stable and the crmA-caspase-8 complex having stability similar to that of trypsin family complexes with antithrombin and α1-proteinase inhibitor (Table 1). In contrast, the apparent rates of dissociation measured for SEC-purified crmA-caspase-1 and crmA-caspase-8 complexes were lower by between 8- and 23-fold, presumably reflecting the presence of only very small amounts of p11/p10 subunit (12 and 5%, respectively) that could reassociate with liberated p20 subunit. For both the isolated large and small complexes of caspase-6 with crmA, there was no such apparent reduction in rate of complex dissociation, reflecting the continued presence of stoichiometric amounts of p10, still associated with the p20 subunits in either dimers or tetramers (Table 1).

To demonstrate that the regain of activity was a result of hydrolysis of the major species present, a high concentration (100 mM) of the potent small nucleophile NH2OH was used to cleave the putative thiol ester linkage. Measurement of caspase catalytic activity after such treatment showed that 30–50% activity could be recovered from in situ-inhibited samples.

**DISCUSSION**

Although little is definitively known of how serpins such as crmA inhibit cysteine proteinases of the caspase family, the mechanism by which serpins inhibit the structurally unrelated serine proteinases that possess the trypsin fold is now quite well understood (22). Serpins inhibit trypsin family serine proteinases by a suicide substrate inhibition mechanism in which a massive conformational change occurs in the serpin upon formation of the acyl enzyme intermediate. This involves insertion of the reactive center loop into the central β-sheet of the serpin, the concomitant translocation of the covalently attached proteinase to the distal end of the serpin (23, 24), and the kinetic trapping of the proteinase through a compression-induced distortion of the catalytic apparatus of the enzyme (25, 26). There is some evidence to suggest that an analogous loop insertion and kinetic trapping mechanism is also used for inhibition of caspases. Thus, x-ray structures of cleaved forms of crmA have shown that crmA can undergo the same insertion of the RCL into the catalytic apparatus of the enzyme (25, 26). There is some evidence to suggest that an analogous loop insertion and kinetic trapping mechanism is also used for inhibition of caspases. Thus, x-ray structures of cleaved forms of crmA have shown that crmA can undergo the same insertion of the RCL into the catalytic apparatus of the enzyme (25, 26). There is some evidence to suggest that an analogous loop insertion and kinetic trapping mechanism is also used for inhibition of caspases. Thus, x-ray structures of cleaved forms of crmA have shown that crmA can undergo the same insertion of the RCL into the catalytic apparatus of the enzyme (25, 26). There is some evidence to suggest that an analogous loop insertion and kinetic trapping mechanism is also used for inhibition of caspases. Thus, x-ray structures of cleaved forms of crmA have shown that crmA can undergo the same insertion of the RCL into the catalytic apparatus of the enzyme (25, 26). There is some evidence to suggest that an analogous loop insertion and kinetic trapping mechanism is also used for inhibition of caspases. Thus, x-ray structures of cleaved forms of crmA have shown that crmA can undergo the same insertion of the RCL into the catalytic apparatus of the enzyme (25, 26).
Serpin-Caspase Complexes

ases (27, 28). There is also the same critical dependence of RCL length on inhibitory effectiveness that is seen for inhibition of serine proteinases (29). Mutation of the RCL residue at the hinge point for insertion has also been shown to modulate inhibitory effectiveness of crmA in the same way as for inhibition of serine proteinases (30). Finally, fluorescence resonance energy transfer measurements on crmA-caspase-1 complexes support proteinase translocation to the serpin distal end in a manner analogous to that of serine proteinases inhibited by serpins.4

The findings presented here for complexes of crmA with the caspases 1, 6, and 8 support such a mechanism of serpin-caspase inhibition by kinetic trapping of a thioacyl enzyme intermediate. Because formation of such a thioacyl intermediate necessitates cleavage of the crmA polypeptide and covalent linkage of the larger moiety to the caspase p20, it is expected that species would appear in the MALDI-TOF spectrum with m/z corresponding to the sum of crmA (N terminal) + p20 as well as crmA (N + C-terminal fragments) + p20, with the relative amounts depending on how tightly the small C-terminal fragment of crmA (~4 kDa) remained associated with the rest of the complex. In each of the three complexes examined here this was the case, with a very large peak present at m/z corresponding to crmA (N-terminal) + p20 and with a smaller peak with about 4000 larger m/z, as expected for the entire crmA (N + C-terminal fragments) + p20. The ~53,000 peak is a strong argument for the presence of a covalent linkage between the N-terminal fragment crmA and the caspase p20 subunit, since a nonspecific non-covalent interaction would be unlikely to hold these species together. An analogous fragment was seen for the complex of the caspase inhibitor p35 with the p18 fragment of caspase-8 (32). In that case the accompanying x-ray structure demonstrated that a covalent thioacyl linkage between the two components was present. The lower relative intensity of the higher m/z peak in Figs. 3A and 6, corresponding to crmA (both N + C-terminal fragments) + p20, illustrates how, even for a very tightly but non-covalently associated C-terminal fragment, most of the peptide dissociates during the analysis. Equivalent behavior was seen for the covalent acyl intermediate of the serpin plasminogen activator inhibitor-1 (PAI-1) with the serine proteinase tissue-type plasminogen activator (tPA), examined by electrospray mass spectrometry, where a dominant peak for the N-terminal PAI-1 + tPA was seen, reflecting the covalent linkage between the two species, and a smaller peak for N + C fragments of PAI-1 + tPA (33).

Although none of the SDS-PAGE gels of complexes showed the presence of a covalent linkage between crmA and the larger caspase subunit, this is almost certainly due to the reactivity of the thioacyl linkage once the complex is denatured by SDS. In the study of the p35-caspase-8 complex it was only possible to see small amounts of covalent complex by SDS-PAGE and then only when altered conditions of temperature and pH were used for sample preparation and electrophoresis despite the x-ray evidence that all of the complex involved a covalent thioacyl linkage (32).

Independent evidence that the complexes of caspases with crmA represent kinetically trapped intermediates of the normal substrate cleavage pathway rather than thermodynamically determined, and hence indefinitely stable species, came from measurements on the regain of catalytic activity. Thus, all three crmA-caspases complexes showed slow regain of catalytic activity, which is presumed to result from hydrolysis of the covalent thioacyl linkage and release of the p20 subunit. Although the rate constants for such hydrolysis showed a variation of nearly 80-fold, with a low of 2.6 × 10⁻⁶ s⁻¹ for the crmA-caspase-8 complex to a high of 2.1 × 10⁻⁴ s⁻¹ for the crmA-caspase-1 complex, all represent a significant stabilization over composite k_cat values for caspase substrate hydrolysis.

Whereas it had been predicted previously that caspases might be inhibited by crmA by kinetic trapping of the thioacyl intermediate (22), the present finding that complex formation caused disruption of the caspase tetramer was quite unexpected. For caspases 1 and 8 several pieces of evidence indicate that the smaller p10 subunit has almost completely dissociated and that the resulting complex is composed of only a single cleaved crmA and a single p20 subunit (Fig. 10). Thus, SDS-PAGE analysis of the complex band isolated from native PAGE or of the complex species from SEC showed the presence of only cleaved crmA and p20 subunits in about equimolar amounts. The loss of p10 was further supported by the very low intensity of the m/z peak corresponding to the p10 subunit in MALDI-TOF spectra of these complexes. For the crmA-caspase-1 complex it was also shown by Western blotting and by amino acid sequencing that the p20 subunit was present, whereas the p10 subunit was not. Also, in regain of activity assays there was an 8–23-fold reduction in the apparent rate of reformation of functional caspase when the assay was carried out on purified complex, where the p10 subunit was thought to be almost completely missing, compared with assays on complex generated in situ. This is consistent with 87–95% loss of the smaller caspase subunit in the former case and, hence, the inability to reform functional caspase heterotetramers when the larger subunit is released from complex with crmA by hydrolysis of the thioacyl linkage. Such reassociation of p20 and p10 subunits to generate active caspase has been demonstrated under controlled refolding conditions (34). Because the organization of all three caspases examined here involves association of p20:p10 heterodimers through the p10 interface, although the p10:p10 interface may also be affected. The consequences for caspase-6 of complex formation with crmA were somewhat different from those for caspases 1 and 8 (Fig. 9). Although a thioacyl intermediate was also formed that was quite stable, conformational effects transmitted to the caspase tetramer did not disrupt the p20:p10 interaction and appeared only to weaken the p10:p10 interface that holds the tetramer together, such that two species of complex could be isolated, each containing cleaved crmA, p20, and p10, but differing in size, consistent with them being crmA₃(p20+p10)₂ and crmA₄(p20+p10)₃ forms. The proportion of the latter is much greater than seen for SEC of procaspase-6 under similar conditions (35). In keeping with the retention of the p10 subunit, there was no reduction in the rate of regain of caspase activity subsequent to thioacyl intermediate hydrolysis when

Cleaved crmA was used for crmA covalently linked to caspase. The complex generated by this reaction was purified and used for the assay. The reaction was carried out on purified complexes compared with native complexes. The assay was carried out on purified complexes to ensure that the results were not influenced by the presence of impurities or other proteins.

There are two important questions that arise from the findings. The first is how such perturbations might come about, and the second is what functional role they might play. In vivo, with regard to how such changes might occur, there is precedent for structural perturbations within a serpin-complexed proteinase for complexes of serpins with serine proteinases of the trypsin family. Thus, it has been known for many years that complex formation in such systems can result in an increase in proteolytic susceptibility for some regions of the complexed proteinase, suggesting long range conformational effects (36–42). The means of propagation of such changes is likely to be the direct path through the catalytic mechanism of the enzyme and not by changes in the active site. This has been shown to result in reorganization and displacement of the catalytic residues and extraction of the P1 side chain from the S1 pocket of the serpin (25, 26, 43). In the case of α1-proteinase inhibitor-complexed elastase the more distal propagation of the active site perturbations may be through a disulfide linkage between Cys-191 close to the active site and Cys-220 in an adjacent strand (26).

It has been shown that dimerization via the p10-p10 interface plays a critical role in the activation of initiator caspases such as caspase-8. Whereas executioner procaspases are dimers, initiator procaspases, such as caspase-8, are monomers (44). Activation of casepase-8 depends on dimerization through the p10-p10 interface rather than cleavage of the monomer, such that interface mutants that cannot dimerize but can be cleaved within the monomer are not activated (44). This suggests a linkage between the p10-p10 interface and the active site. By microscopic reversibility it would be expected that structural perturbations in the active site, such as those caused by covalent crmA complex formation, would be manifested in alteration in the p10-p10 interactions. The present observation that such complex formation results in loss of the p10 subunits from the crmA-caspase-8 complex suggests that the p20-p10 interface is also affected. The different behavior seen with caspase-6 is also reconcilable with what is known about activation of such executioner caspases. Here the procaspase is already dimeric, and proteolytic cleavage of the monomer into p20 and p10 subunits is necessary to allow the active site to reorganize into the active species (35). There is, thus, less of a direct functional linkage between the active site conformation and the p10-p10 interface in caspase-6, which makes understandable the less severe perturbations caused by crmA complex formation with caspase-6. Caspase-1, which has anti-inflammatory rather than anti-apoptotic activity, nevertheless requires zymogen dimerization for activity, implying that it should behave with crmA in the same way as caspase-8. This is what was found here with loss of the p10 subunits upon complex formation with crmA.

A possible answer to the second question of why crmA inhibition of an initiator caspase appears designed to induce loss of p10 whereas inhibition of an executioner caspase does not may also lie in the different roles dimerization plays in activation of each of these caspases and the location of these caspases in the apoptotic cascade. From the perspective of shutting down apoptosis, it would be more efficient to inhibit the initiator caspase-8 than the downstream executioner caspase-6. Consistent with this, crmA is a much faster inhibitor of caspase-8 than caspase-6 (200-fold) (15). However, by also causing disruption of the critical subunit interfaces, crmA can further prevent reactivation of caspase-8 upon the release of the p20 subunit by slow hydrolysis and perhaps also prevent activation of procaspase-8 by forming nonproductive heterodimers between the caspase and crmA complex.
Serpin-Caspase Complexes

a procaspase-8 monomer and a dissociated p10 subunit (31). In the case of caspase-6 such heterodimers would still be activable, and so there would be no advantage to their formation and, hence, no advantage in inducing loss of p10 from the crmA-caspase-6 complex. In the case of the crmA-caspase-1 complexes, the pro-inflammatory caspase-1 behaves like the initiator caspase-8, possibly reflecting an analogous “early” involvement, although here in inflammation rather than apoptosis.

Acknowledgments—We thank Yan Wang for carrying out the MALDI-TOF analyses, Dr. John Zhang and Tina Bedsted-Faevang for help with the expression of the caspase-1 subunits and with preparation of caspase-1, and Scott Snipas and Annamarie Price for preparing caspases 6 and 8.

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