Crystal Structure of an Invertebrate Caspase*

Caspases play an essential role in the execution of apoptosis. These cysteine proteases are highly conserved among metazoans and are translated as inactive zymogens, which are activated by proteolytic cleavages to generate the large and small subunits and remove the N-terminal prodomain. The 2.3 Å resolution crystal structure of active Sp-caspase-1, the principal effector caspase of the insect Spodoptera frugiperda, is presented here. The structure represents the first nonhuman caspase to be resolved. The structure of the cleaved and active protease was determined with the tetrapeptide inhibitor N-acetyl-Asp-Glu-Val-Asp-chloromethylketone covalently bonded to the active site cysteine. As expected, the overall fold of Sp-caspase-1 is exceedingly similar to that of the five active caspases from humans solved to date. The overall structure and active site arrangement of Sp-caspase-1 is most comparable with that of the human effector caspases, with which it shares highest sequence homology. The most prominent structural difference with Sp-caspase-1 is the position of the N-terminal region of the large subunit. Unlike the N-terminus of human caspases, the N terminus of Sp-caspase-1 originates from the active site side where it interacts with active site loop L2 and then extends to the backside of the heterodimer. This unusual structural arrangement raises the possibility that the N-terminal prodomain plays a regulatory role during effector caspase activation or enzyme activity in insects.

Apoptosis is a cellular pathway that eliminates damaged, potentially dangerous, superfluous, or unwanted cells in metazoan organisms. This programmed cell death pathway is a naturally occurring physiological process that is vital to normal organismal development and tissue homeostasis. Abnormalities in the regulation of apoptosis can trigger many diseases including cancer, neurodegenerative disorders, autoimmune disorders, and ischemic injury. The apoptotic pathway is highly conserved in the Metazoa kingdom and can be triggered by both intracellular and extracellular stimuli. Apoptosis is executed through the activity of the caspases that are aspartyl-specific proteases (1–6). Because of their central role in apoptosis, these cysteine proteases are attractive targets for therapeutic intervention.

Synthesized as dormant single-chain zymogens, the caspases are activated by a hierarchical series of proteolytic cleavages. The apical initiator caspases typically contain a large N-terminal prodomain that interacts with cellular factors that initiate apoptosis. These initiator caspases oligomerize through their prodomains that also associate with other proteins and promote enzyme activation in the absence of proteolytic processing (7). The activated initiator caspases subsequently cleave the proform of downstream effector caspases, which usually possess a short N-terminal prodomain. The active effector caspases proteolytically cleave cellular substrates that produce the morphological events associated with apoptosis, including cytoplasmic shrinkage, membrane blebbing, DNA fragmentation, and chromatin condensation (8). Effector caspase activation requires a minimum of two cleavages of the zymogen proform; the first cleavage yields the large and small catalytic subunits (~20 and 10 kDa, respectively), and the second cleavage removes the N-terminal prodomain from the large subunit. The order of these activation cleavages appears to be conserved among caspases (9–13).

The x-ray crystal structure has been determined for several activated human caspases, including caspase-1 (14, 15), caspase-3 (16, 17), caspase-7 (18), caspase-8 (19, 20), and caspase-9 (21, 22). Thus, structures have been solved for human initiator and effector caspases. These structures reveal that the heterodimer of large and small subunits associates with another heterodimer to form a tetramer (or dimer-of-heterodimers). Both the large and small subunits contribute residues that form the active site pocket. Residues from the small subunit shape the binding pocket for the substrate residues P2–P4. Both subunits contribute to the selective recognition of aspartate at the P1 position. The carboxylate group of the substrate P1 aspartic acid salt-links with two conserved arginines, one from the large subunit and one from the small subunit, respectively. The P4 subsite in human caspase-1 is a large hydrophobic pocket that accommodates the preferred tryptophan side chain of the substrate. The corresponding pocket in caspase-3 is narrower and smaller, which thereby accommodates the smaller aspartate side chain. The conserved catalytic cysteine residue, which attacks the carbonyl carbon of the scissile bond, is situated at the end of β-strand 4. The...
imidazole side chain of conserved active site histidine is adjacent to cysteine and likely plays a role in catalysis (14, 15).

Since discovery of the Caenorhabditis elegans caspase CED-3 (23), numerous caspases have been identified in diverse metazoan organisms, including invertebrates (24–31). Sf-caspase-1 is the principal effector caspase of Spodoptera frugiperda, a nocturnal moth infected by the large DNA baculoviruses. This invertebrate caspase of interest because it is the major target of the baculovirus apoptosis suppressor P35 (13, 26), a pan-
caspase inhibitor with a novel mechanism of stoichiometric inhibition (32–35). Sf-caspase-1 displays 41 and 39% protein sequence identity to human effector caspases-7 and -3, respectively (26). It is most closely related to DrICE and DCP-1 (65 percent identity to human effector caspases-7 and -3, respectively (26). It is most closely related to DrICE and DCP-1 (65 percent identity to human effector and initiator caspases. Because caspase activity and activation are decisive phases in executing apoptosis, a better understanding of the conserved features in caspase structure and function is necessary. Here we report the three-dimensional x-ray crystal structure of Sf-
caspase-1. We compare the structural details of this invertebrate effector caspase with those of human effector and initia-
tor caspases. The observed similarities and differences of these critical enzymes provide new insight into conserved features of the caspases that are expected to contribute to the design of therapeutic strategies for treatment of apoptosis-associated disorders.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—The Sf-
caspase-1 gene was cloned into the pET22b(+) expression vector (Novagen, Madison, WI) for production of Sf-caspase-1 with a C-termi-
nal hexahistidine extension in Escherichia coli. Purified recombinant His-tagged Sf-caspase-1 was fully functional and stoichiometrically in-
hibited by baculovirus P35 as described previously (13). For purifica-
tion, bacterial pellets were resuspended in binding buffer (50 mM
NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 10 mM imidazole), lysed, and passed over a metal (Ni^{2+})-chelate column. N-Acetyl-Asp-Glu-Val-Asp-chloromethylketone inhibitor (Ac-DEVD-cmk) (Calbiochem, San Diego, CA) was added, then dialyzed against binding buffer, and purified over a second high pressure metal-chelate column. The purified Sf-
caspase-1, with acetyl-DEVD-methylketone inhibitor bound to the active site, was dialyzed against 25 mM Tris, pH 9.0, and concentrated to 10 mg/ml before crystallization trials.

Sf-caspase-1 with bound inhibitor was crystallized by sitting drop vapor diffusion using 2-μl drops of the protein mixed with an equal volume of reservoir buffer (1.6 mM ammonium sulfate, 2% polyethylene glycol 1000, 0.1 M HEPES, pH 7.5). The crystals grew at room temperature to the size of 0.7 × 0.15 × 0.15 mm by 4 weeks. The crystals were transferred to 1.8 mM ammonium sulfate, 2% polyethylene glycol 1000, 0.1 M HEPES, pH 7.5, 30% ethylene glycol and frozen to −175 °C for data collection. X-ray diffraction data were collected to 2.3 Å resolution at Beamline 9-2 of the Stanford Synchrotron Radiation Laboratory, were processed with DENZO, and were scaled with SCALEPACK (Ta-
ble I) (36). The crystals belong to the tringular space group P3_{1}2_{1}2_{1} with unit cell parameters of a = 151.7 Å, c = 79.5 Å.

Phase Determination, Model Building, and Refinement—The Structure was solved by the molecular replacement method (37). The p17-p12 subunits of human caspase-3 (18), in which all residues were truncated to alanine, were used as a search model in the program AMoRe (38) in the CCP4 package (39). Because it was originally thought that two complete dimer-of-heterodimers were in the crystallographic asymmet-
ric unit (corresponding to a V_{o} of 2.1 Å^{3}/Da), the human caspase-3 dimer-of-heterodimers was used to search for two complete copies in the Sf-caspase-1 crystal asymmetric unit. The rotation function of AMoRe revealed many peaks, which were subjected to the first round of a trans-
lation search to identify the position of the first dimer-of-heterodimers. The first translation search found a peak corresponding to a correlation coefficient and R factor of 25.9 and 51.6%, respectively, with the next highest peak giving values of 20.1 and 53.8% and many other peaks with values of 12.1 and 55.8%. (Searching the enantiomorphic space group, P3_{1}2_{1}2_{1}, resulted in no peaks higher than 12% and 57% for the correlation coefficient and R factor, respectively.) The search model was held fixed at this position, and a second translation search was conducted using the same caspase-3 dimer-of-heterodimers model to locate the second protomer in the asymmetric unit. This search resulted in one peak that stood out among the others (correlation coefficient and R factor of 34.8 and 49.3%, respectively, with all other peaks at ~20 and 59%, respectively). Upon packing inspection of models in the tringular unit cell, it was identified that the second translation search result was positioned on a crystallographic 2-fold axis, which was coincident with the dimer-of-heterodimers 2-fold axis, generating essentially two mol-
ecules superimposed on each other. This signified that there were 1.5 dimer-of-heterodimers in the crystallographic asymmetric unit, with one p17-p12 heterodimer situated at the crystallographic 2-fold axis to generate the functional dimer-of-heterodimers. This corresponded to a more reasonable Matthews coefficient of 2.8 Å^{3}/Da (56% solvent) as-
suming three p19-p12 heterodimers/asymmetric unit (40).

Each of the three p19-p12 heterodimers was subjected to rigid body refinement in the CNS program, which lowered the R factor to 45.1% for all data to 3 Å resolution. The molecular replacement phases were refined by 3-fold molecular averaging and solvent flattening using DM in the CCP4 package (39, 41). One thousand rounds of averaging and phase extension starting at 8 Å resolution resulted in an overall final figure of merit of 0.86 at 3 Å resolution. The 3-fold averaged electron density map was readily interpretable, and a single p19-p12 heterodimer was built into the density with aid of the molecular graphics program O (42). After initial model building, the complete asymmetric unit was generated by rotating and translating the p19-p12 heterodimer built in the averaged map. The model was then subjected to subsequent rounds of simulated annealing with the program CNS (43) applying the maximum likelihood as a refinement target. Initially, high restraints were placed on the noncrystallographic symmetry between the three p19-p12 heterodimers in the asymmetric unit. During stages of refinement the weight placed on the noncrystallographic symmetry restraints was re-evaluated to give the lowest R_{free} value. As the model improved, the noncrystallographic weight that gave the lowest R_{free} decreased until the noncrystallographic symmetry restraints were re-

TABLE I

| Data collection and refinement statistics |  |
|----------------------------------------|--|
| Rmerge (%) (32,331 reflections) | 14.1 |
| Resolution (Å) | 3.7 |
| No. of reflections | 32,331 |
| No. of unique reflections | 10,984 |
| Completeness (%) | 97.2 (97.0) |
| Rmerge (%) | 12.7 (2.98) |
| Refinement | |
| Resolution (Å) | 3.0–2.0 |
| R factor (%) (43,689 reflections) | 18.3 |
| Rmerge (%) (2,190 reflections) | 23.2 |
| r.m.s for bond distances (Å) | 0.017 |
| r.m.s for bond angles (deg) | 1.88 |
| No. of non-hydrogen protein atoms | 6,085 |
| No. of water molecules | 318 |
| No. of ethylene glycol atoms (8 molecules) | 32 |

X-ray source | SSRL BL 9-2 |
| Space group | P3_{1}2_{1}2_{1} |
| Cell parameters (Å) | a = b = 151.7, c = 79.5 |
| Resolution (Å) | 0.09 |
| No. of unique reflections | 32,331 |
| Completeness (%) | 97.2 (97.0) |
| Rmerge (%) | 12.7 (2.98) |
| Refinement | |
| Resolution (Å) | 3.0–2.0 |
| R factor (%) (43,689 reflections) | 18.3 |
| Rmerge (%) (2,190 reflections) | 23.2 |
| r.m.s for bond distances (Å) | 0.017 |
| r.m.s for bond angles (deg) | 1.88 |
| No. of non-hydrogen protein atoms | 6,085 |
| No. of water molecules | 318 |
| No. of ethylene glycol atoms (8 molecules) | 32 |
Structure of Sf-caspase-1, an Insect Effector Caspase

Fig. 1. Sf-caspase-1 structure. Stereo ribbon drawing of the insect effector caspase determined at 2.3 Å resolution. Shown is one biologically active dimer of p19-p12 heterodimers. The p19 large subunits are colored blue and red on the outside. The p12 small subunits are colored cyan and pink and lie adjacent to the 2-fold, which is approximately perpendicular to the page. The secondary structural elements are labeled in pink on the left. p19-p12 heterodimer (β-strands with numbers, α-helices with capital letters) along with the termini. The position of the p19 N-terminal region, which forms a short β-strand (βα), is seen interacting with the C terminus of the 2-fold-related p19 subunit. The two active sites are identified by the bound Ac-DEVD-cmk tetrapeptide inhibitor are shown in ball-and-stick. The inhibitor is covalently bound to the active site Cys178 located in the β4-β5 loop. The four loops that define the active site pocket are labeled L1-L4 (the loop designation is that found in Ref. 46). L2 consists of the loop between the C-terminal p19 and N-terminal p12 that is cleaved upon activation to form L2 and L2'. Figs. 1–3 were generated with BOBSCRIPT (59) and rendered with RASTER3D (60).

RESULTS

Overall Structure—The asymmetric unit contains three large subunits (p19)-small subunit (p12) heterodimers. The p19-p12 heterodimer designated A-chain combines with the B-chain heterodimer to form one biologically functional dimer-of-heterodimers. The designated C-chain p19-p12 heterodimer is positioned next to a crystallographic 2-fold axis, which places a symmetry related C-chain p19-p12 heterodimer from the neighboring unit cell adjacent to it, thus generating the functional dimer-of-heterodimers. The final model consists of residues 40–191 (p19) and 201–295 (p12) for the A chain; residues 41–191 and 200–296 for the B chain; and residues 40–191 and 201–300 for the C chain. Sf-caspase-1 expression and purification in E. coli can produce a mixture of large subunits, in which some of the p19 subunits are cleaved after Asp184, generating a composite of p19/p18 large subunits with the p12 small subunit (13, 26). However, our preparations were homogenous with respect to the large subunit as indicated by SDS-PAGE (not shown). In addition, clear electron density was observed in the crystal structure for residues Asp184 to Arg191 in all three p19 subunits in the crystallographic asymmetric unit. The Ac-DEVD-cmk inhibitor is clearly defined in all three active sites of the asymmetric unit. However, the chlorine atom is displaced by the active site Cysγ178, in which Sγ is covalently bonded to the methyl group. The average temperature factor (B) for all atoms is 31.3, 29.8, and 26.1 Å² for the A-, B-, and C-chains respectively. The C-chain likely has an overall lower average B value because it makes more lattice contacts than either the A- or B-chain. Because the C-chain heterodimer residues were the most ordered, and the overall average temperature value is lower than the A- or B-chain, most of the following discussion will concentrate on the C-chain heterodimer.

In Sf-caspase-1, two p19-p12 heterodimers assemble to form the biologically active dimer-of-heterodimers producing a central 12-stranded β-sheet. A total of ~9,400 Å² of surface area is buried between the two p19-p12 heterodimers. The two active sites in the dimer-of-heterodimers are separated by ~35 Å. The overall topology of Sf-caspase-1 (Fig. 1) is very similar to that of the human caspases. The overall shape of the dimer-of-heterodimers is roughly a compact cylinder (~60 Å × 40 Å wide) consisting of a twisted 12-stranded β-sheet running down the axis of the cylinder surrounded by 10 α-helices. Each p19-p12 heterodimer folds into a single-domain α/β motif with a central six-stranded β-sheet (β2, β1, β3, β4, β5, and β6 from outside to inside) sandwiched by five α-helices, two on one side of the β-sheet (αC and αD) and three on the other (αB, αE, and αF) (Fig. 1). The secondary structure designations follow that of the human caspase-1 structure (14), and the active site loop designations (L1–L4) correspond to that previously reported (46). The first α-helix in human caspase-1, αA, is missing because the structural element is not conserved in Sf-caspase-1, as well as the human effector caspases. The p19 large subunit of Sf-caspase-1 contributes the outer four parallel β-strands and three helices, whereas the p12 small subunit adds two α-helices and two antiparallel β-strands adjacent to the 2-fold axis, which forms the central four antiparallel β-strands in the dimer-of-heterodimers (Fig. 1). Following strand β3, three short β-strands (β3α, β3b, and β3c) form a β-meander motif before the short helix αD (Fig. 1). The β7-αE loop (L3, Fig. 1) contains a short β-strand (β7α) that hydrogen bonds to the main chain of the tetrapeptide inhibitor. The αF-β8 loop (L4) adopts a hairpin loop conformation containing two antiparallel β-strands (β7b and β7c), which forms part of the active site pocket. The first ordered N-terminal residues of the p19 subunit (residues 40–44) forms a β-strand (βα), which together with β6 clamps β5 from the 2-fold related p19-p12 heterodimer (Fig. 1). The location of these N-terminal residues of the p19 in Sf-caspase-1 differs significantly from the human caspases (see below). The active site Cysγ178 is located at the end.
of β4, and the active site His106 is situated at the start of β3a. The active site pocket, which binds the tetrapeptide substrate recognition sequence, is formed by loops L1–L4 from both the p19 and p12 subunits.

**Structural Comparison with Human Caspases**—The overall structure and topology of Sf-caspase-1 is very similar to that of the human caspases. A stereo superposition (Fig. 2) demonstrates the similarities of the Sf-caspase-1 p19-p12 heterodimer (in red) with the heterodimers of activated human caspases-1, -3, -7, and -8 (cyan), which exhibit rms deviations of 1.22, 0.61, 0.59, 0.87, and 1.05 Å, respectively, to Sf-caspase-1. Note the difference in location of the N-terminal p19 segment in Sf-caspase-1 compared with all of the human caspases.

**Fig. 2. Structural comparison of insect Sf-caspase-1 to all known activated human caspase structures.** Stereo superposition of Sf-caspase-1 p19-p12 heterodimer colored in red on human caspases-1 (green), -3 (blue), -7 (magenta), -8 (cyan), and -9 (yellow), which exhibit rms deviations of 1.22, 0.61, 0.59, 0.87, and 1.05 Å, respectively, to Sf-caspase-1. The most striking difference observed in the superposition is the position of the N-terminal end of the Sf-caspase-1 large subunit starting at residue 40 (residues 29–39 are disordered). When compared with the human caspases, the large subunit N-terminal end of Sf-caspase-1 extends in the opposite direction (Fig. 2). The point of divergence (from the C to N termini) occurs at Pro51, which corresponds to Glu151 of human caspase-1 in the structural alignment. The main chain conformation for Pro51 in Sf-caspase-1 is the normal trans-peptide configuration, suggesting that the proline is not required for the different disposition of the N terminus.

In human caspases-1 and -9, the N terminus of the large subunit forms an α-helix that extends toward the back of the dimer as viewed in Fig. 2. In the structure of human caspase-3 complexed with isatin sulfonamide inhibitor, the N terminus extends down where it interacts with a symmetry related molecule. In the caspase-3 structures complexed with tetrapeptide inhibitor (16, 17), the first residue observed is Asn35, which corresponds to Pro51 of Sf-caspase-1. The first residue of the human caspase-7 and -8 structures also corresponds to Pro51 of Sf-caspase-1. In all of these activated caspases, the N terminus of the large subunit is preceded by the N-terminal prodomain in the dormant proenzyme, which is cleaved off upon activation. The first few residues of the N terminus in the processed large subunit (after the Asp cleavage site) of all these caspases are also disordered. In the crystal structure of the inactive proenzyme of caspase-7, the prodomain is disordered, but the N terminus follows a path similar to that of the large subunit N terminus of human caspase-1 and -9 (48), which extends in the opposite direction of the N-terminal Sf-caspase-1 large subunit (Fig. 2). Thus, the disposition of the N terminus of the large subunit is unique to Sf-caspase-1.

Because the Sf-caspase-1 prodomain is processed at Asp28, residues 1–28 are absent in the active protease. Additionally, because of their disorder, residues 29–39 are also missing in our Sf-caspase-1 structure. In the crystallographic asymmetric unit, unambiguous electron density clearly defines the N-terminal end starting at residue 40 for all three large subunits, all of which have identical conformations and positions. The large subunit N terminus adopts a β-strand structure (βa) and together with the N terminus of small subunit (β6) clamps the C terminus from the 2-fold related large subunit, forming a short three-stranded antiparallel β-sheet (Fig. 1). Residues 40–44 of the N terminus large subunit form main chain β-structure hydrogen bonds to residues 187–190 of β6 from the 2-fold related large subunit C terminus (active site loop L2), which also hydrogen bonds to residues 201–204 within the N terminus of the small subunit (active site loop L2’).

In Sf-caspase-1, the N terminus of the large subunit interacts with the C terminus of the 2-fold related large subunit from the other heterodimer in the functional dimer-of-heterodimers (Fig. 1). The C terminus of the large subunit corresponds to part of the p19-p12 loop of the inactive proenzyme, which is cleaved after Asp195 upon activation (13, 26). This observation raises the possibility that the N-terminal prodomain (residues 1–28) interacts with the p19-p12 loop of the Sf-caspase-1 proenzyme and thus plays a role in regulating the activation cleavage.

**Active Site**—There are two active sites in the biologically functional dimer of Sf-caspase-1 heterodimers. Each active site is defined by a surface cleft between the large and small subunits of each heterodimer (Fig. 1). Residues from both subunits interact with the bound tetrapeptide inhibitor. A close-up view of the active site (Fig. 3) displays the observed density around the bound tetrapeptide inhibitor and all the amino acid residues interacting with the inhibitor. The distance between the active site Cys175 Sγ and the methyl carbon from the methylketone of the inhibitor is 1.8 Å, illustrating the covalent modification of the active site cysteine. The cysteine thiolate displaces the chloride (in an Sn2 reaction) in the chloromethylketone functional group at the C-terminal end of the tetrapeptide Ac-DEVD-cmk. Electron density clearly defines the covalent bond between the active site cysteine and the inhibitor nature in all three active sites in the crystal asymmetric unit.
The orientation of the inhibitor ketone oxygen is also clearly defined in the electron density map for all three subunits. The carbonyl oxygen is 2.7, 2.8, and 2.8 Å from the main chain amide nitrogen of Gly137 (following the active site His136) in the A, B, and C subunits, respectively, and 3.0, 3.1, and 3.1 Å from the backbone amide nitrogen of the active site Cys778 in the three respective subunits. These amide nitrogens define the "oxyanion hole" that stabilizes the negative charge build-up during the tetrahedral intermediate of the S N2 catalysis. Unlike the caspase structures determined with aldehyde inhibitors, there appears to be no interaction between the inhibitor carbonyl oxygen and the active site histidine imidazole ring in Sf-caspase-1. For the caspase structures determined with the aldehyde form of the inhibitor, the carbonyl oxygen of the thiohemiacetal hydrogen bonds to the active site histidine side chain in a nontransition state conformation (14–16, 18, 49, 50). In Sf-caspase-1, the ketone carbonyl oxygen is 3.4, 3.5, and 3.5 Å from Nδ1 of active site His136 for the three active sites in the crystallographic asymmetric unit. The ketone carbonyl oxygen seen here is in a similar orientation as observed in other human caspase structures with halogen-methylketone inhibitors (17, 19, 20).

The S1 binding pocket is defined by the guanido groups of Arg79, Arg224, and the amide nitrogen of Gin176 that are conserved in all metazoan caspases sequenced to date. This arrangement creates a highly positive potential that defines the effector caspase P1 residue preference of aspartate. The tetrapeptide P1 aspartate carbonyl side chain salt bridges and hydrogen bonds to all three caspase residues, respectively. The valine from the tetrapeptide inhibitor (P2) is partially buried in the hydrophobic S2 pocket defined by Phe221, Trp224, Leu176, and Met274 (Fig. 4). The P3 glutamate of the inhibitor is more solvent exposed and only forms an ionic interaction with Arg224, which also defines the S1 pocket. The S4 pocket is the most variable of the human caspases, which characterizes the different P4 residues preferred among the human caspase: Trp for caspases-1, -4, and -5; Asp for caspases-2, -3, and -7; and Leu/Val for caspases-6, -8, and -9. As expected for an effector caspase, the S4 pocket in Sf-caspase-1 more closely resembles that of the human effector caspases-3 and -7. The carbonyl group of the tetrapeptide inhibitor P4 aspartate is tethered between the main chain amide nitrogen of Asn267 (of the β-hairpin formed by β7b and β7c) to Oδ2 and Oδ1 of the P4 aspartate hydrogen bonds to the amide of side chain Asn225 (Figs. 3 and 4). The carbonyl oxygen of the N-acetyl group of the tetrapeptide inhibitor hydrogen bonds to the main chain amide nitrogen of Thr226.

The backbone of the tetrapeptide inhibitor adopts an extended β structure that forms a β-strand antiparallel to β7a of Sf-caspase-1 (Fig. 1). Four hydrogen bonds are found between these main chains: P4 N-acetyl oxygen to main chain nitrogen of Thr226, P3 main chain nitrogen to carbonyl oxygen of Arg224, P3 peptide carbonyl oxygen to main chain nitrogen of Arg224, and P1 main chain nitrogen to carbonyl oxygen of Ser222 (Figs. 3 and 4). The two main chain hydrogen bonds between the P3 residue and Arg224 may help fasten and orient the Arg side chain that defines both the S1 and S3 pocket. Similar interactions occur in all other caspase structures (51).

Comparison of Human and Insect Caspase Active Sites—Structural comparisons of the human and invertebrate caspases should identify conserved residues across species that are important for functional activity. As expected, the conformation of the active site cysteine and histidine of Sf-caspase-1 are almost indistinguishable from those of the human caspases. The two active site residues flank either side of the methyl ketone group in the inhibitor, which would correspond to the scissile peptide bond. The active site cysteine forms a covalent bond to the methyl group. The residues that shape the S1 pocket, including Arg79, Gin176, and Arg224, are strictly conserved among caspases from diverse species. The conforma-
tions of these side chains, as defined by the torsion angles, are nearly identical to the equivalent residues in the human enzyme. The contacts and hydrogen bonds between these residues and the tetrapeptide inhibitor in Sf-caspase-1 are essentially the same as those found in the human caspases.

The S2 pocket has more variation between the insect and human caspase when compared with the S1 pocket. However, there is more variation between the S2 pockets of human effector and initiator caspases than between the S2 pockets of insect and human effector caspases. Residues that define the S2 pocket in Sf-caspase-1 more closely resemble the human effector caspases-3 and -7. In Sf-caspase-1, the conformation of Phe221 is similar to Tyr203 and Tyr230 in human caspases-3 and -7, respectively. For the initiator caspases, this residue is valine. In the effector caspases, the size of the S2 pocket is also restricted by contributions from residues in the αF-β8 loop (L4), which is truncated in caspases-1, -8, and -9 and does not contribute to the S2 pocket (Figs. 1 and 2). In Sf-caspase-1, Met274 from loop L4 occupies a position comparable with Phe256 and Phe282 in human caspase-3 and -7, respectively. Thus, the S2 pocket is smaller and more restricted in the effector caspases when compared with the initiator caspases. This observation also explains the preference of the initiator caspases for larger residues in the P2 position (S2). Trp223 in Sf-caspase-1 forms the last side of the S2 pocket, and yet this residue (and conformation) is more highly conserved among caspases. This tryptophan has a conformation nearly identical to that of human caspases-1, -3, -7, and -9. Only human caspase-8 contains a tyrosine, but the aromatic group is in the same conformation as the tryptophans. This tryptophan precedes conserved Arg224, which defines the S1 and S3 pocket, indicating that Trp is more conserved in the S2 pocket because it helps position Arg224 for the S1 pocket.

The bottom of the S3 pocket of Sf-caspase-1 is Arg224, which is completely conserved among caspases because it also interacts with Asp in the P1 position of the substrate. The side of the S3 pocket in Sf-caspase-1 is Thr226, which makes a weak hydrogen bond (3.3 Å) with the N-acetyl carbonyl oxygen of the tetrapeptide inhibitor. A similar interaction is seen in human caspase-3 between the N-acetyl carbonyl oxygen and Ser209. However, this residue is proline in human caspase-1, -7, -8, and -9, which slightly pushes the N-acetyl group out of the pocket, suggesting a lower binding affinity toward the substrate in these caspases. In Sf-caspase-1, the bottom of the S4 pocket is formed by Trp231, which is conserved in human caspases-3, -7, -8, and -9, but not caspase-1, where a valine allows for a larger preferred P4 residue. The side of the S4 pocket of Sf-caspase-1 is formed by Asn225 and residues in the αF-β8 loop (L4), which are not well conserved among caspases, and explains the largest divergence in specificity of the P4 residue among the caspases (S2).

**DISCUSSION**

We have determined the crystal structure of Sf-caspase-1, the principal effector caspase from the insect S. frugiperda (order Lepidoptera). As such, the 2.3 Å resolution structure represents the first three-dimensional glimpse of a nonhuman caspase. Sf-caspase-1 is synthesized as a dormant zymogen.

**FIG. 4. Detailed inhibitor-Sf-caspase-1 interactions.** Shown is a flattened representation of the tetrapeptide inhibitor Ac-DEVD-cmk (thick gray bonds) and all of the caspase residues from the p19 large subunit (blue bonds) and p12 small subunit (teal-green bonds) that interact with bound inhibitor. The magenta dashed lines show potential hydrogen bonds between atoms with the numbers expressing the average distance (in Å) among the three active sites in the crystallographic asymmetric unit. Inhibitor atoms with short red lines represent hydrophobic interactions pointing toward the caspase residues also outlined with red lines. This figure was generated with LIGPLOT (61).
that is activated by distinct caspase-mediated cleavages at aspartate residues. The active form of Sf-caspase-1 crystallized here is composed of two subunits, large (p19) and small (p12), which assemble together to generate a biologically active dimer-of-heterodimers. This configuration is identical to that of the human caspases. Thus, this unique arrangement appears to be conserved in all metazoan organisms.

Biochemical and genetic analyses indicate that Sf-caspase-1 is an effector caspase (13, 26, 53). Consistent with this role in apoptosis, Sf-caspase-1 displays the highest sequence similarity with other effector caspases, including human caspases-7 and -3 with amino acid identities of 41.0 and 39.3%, respectively, and Drosophila DrICE and DCP-1 (65 and 61% identical, respectively). Our study here shows that the overall three-dimensional structure of Sf-caspase-1 (Fig. 2) is strikingly similar to that of the human effector caspases. When compared with human caspases-7 and -3, rms deviation differences of 0.59 and 0.61 Å, respectively were observed over 225 equivalent α-carbons. The overall shape of both active sites of Sf-caspase-1 (Fig. 3), including the configuration of the active site cysteine (Cys178) and histidine (His136), is nearly indistinguishable from that of the human effector caspases when bound to substrate inhibitors. Moreover, the contacts and hydrogen bonds formed between the tetrapeptide inhibitor and the residues that define the substrate pocket of Sf-caspase-1 (Fig. 4) are essentially the same as those of the human caspases.

The most striking structural difference between Sf-caspase-1 and the human caspases is the position of the N-terminal segment of the large subunit. Unlike the N terminus of human caspases, the N terminus of Sf-caspase-1 starts from the active site side (front) and extends to the backside of the heterodimer (Figs. 1 and 2). Because prodomain residues 1–28 are removed during activation, and residues 29–39 are disordered within the crystal, the first N-terminal residue observed in the p19 large subunit is Arg40. Nonetheless, this N-terminal segment of Sf-caspase-1 clearly interacts with the C-terminal segment (loop L2) of the 2-fold related large subunit within the dimer-of-heterodimers (Fig. 1). The p19 N terminus together with the p12 N terminus (loop L2′) clamp the C terminus of p19 (loop L2) into position through numerous hydrogen bonds (Fig. 1), possibly strengthening the interaction between heterodimers. This arrangement is unprecedented for the human caspases. It is not yet clear whether the clamping interaction occurs before or after the activation cleavage at Asp195 or after substrate binding (see below).

Current models explaining the molecular mechanism of proteolytic activation for the effector caspases from their proform include dramatic conformational changes in several loops that define the active site in the active enzyme (54). In particular, the recently solved crystal structures of human pro-caspase-7 suggest that proteolytic cleavage of the activation loop (L2) that joins the large and small subunits (interdomain sequence) allows repositioning of essential residues in the active site, which primes caspase-7 for substrate binding (46, 48). In active Sf-caspase-1, only nine residues of the activation loop (TETD195GSDST) are disordered and thus invisible in the crystal structure. Nonetheless, the N-terminal and C-terminal ends at this cleavage site (Arg40 and Ser263, respectively) are separated by >55 Å. Thus, in a model wherein the adjacent large and small subunits of Sf-caspase-1 are derived from the same polypeptide, cleavage within the activation loop also induces a dramatic conformational rearrangement in which the cleaved ends reposition to opposite poles of the dimer. Moreover, these newly generated ends subsequently interact with the N terminus of the large subunit forming a tight β-strand bundle at each pole in a configuration that is expected to stabilize the complex and thus contribute to the conformational change.

Whereas the longer prodomains of the initiator caspases associate with factors required for enzyme activation through specific folded domains (54), the function of the smaller prodomains of effector caspases is unclear. For all effector caspases studied to date, the N terminus is disordered and not visible in the crystal structure. Even in the crystal structure of pro-caspase-7, the prodomain (residues 1–23) and adjacent residues (residues 24–55) are disordered (46, 48). Thus, the disposition of the prodomain of effector caspases with respect to the body of the enzyme is unknown. Nonetheless, several studies suggest that the prodomain of effector caspases have specific roles. Proteolytic removal of the prodomain of human effector caspase-6 contributes to cleavage and activation of human caspase-8 (55), and the prodomain of Xenopus caspase-7 regulates nuclear transport (56). The prodomain in human effector caspase-7 regulates both the activation rate and enzyme activity in vivo (57). Lastly, the prodomain of human caspase-3 may act as a silencing element by retaining the caspase in an inactive state (58).

The N-terminal segment of the large subunit of Sf-caspase-1 lies near what would most likely be the p19-p12 interdomain loop (L2) containing the activation cleavage site TETD195G within pro-Sf-caspase-1. This observation suggests that the prodomain (residues 1–28) also lies near the activation loop and thus may directly or indirectly affect the activation cleavage. Thus, the prodomains of Sf-caspase-1 and human caspase-3 may have analogous functions. Pro-Sf-caspase-1 is activated by a P35-resistant apical caspase that recognizes the sequence TETD195G within the p19-p12 activation loop (13). Thus, if the prodomain shields this cleavage site from the apical caspase, then it would negatively regulate effector caspase activation and could represent a fail-safe mechanism for inadvertent activation. Conversely, removal of the prodomain should enhance activation of Sf-caspase-1. Consistent with this later possibility, apoptotic S. frugiperda cells (triggered to undergo apoptosis by diverse signals) contain readily detected levels of a truncated form of pro-Sf-caspase-1 in which only the prodomain has been removed (13). This prodomainless Sf-caspase-1 may represent an activated intermediate that is more readily cleaved at Asp195 to accelerate the production of active Sf-caspase-1 during apoptosis. Additional studies are needed to test this interesting possibility.
Crystal Structure of an Invertebrate Caspase
Charles M. Forsyth, Donna Lemongello, Douglas J. LaCount, Paul D. Friesen and Andrew J. Fisher

J. Biol. Chem. 2004, 279:7001-7008.
doi: 10.1074/jbc.M312472200 originally published online November 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M312472200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 23 of which can be accessed free at http://www.jbc.org/content/279/8/7001.full.html#ref-list-1