Structure of Recombinant Human CPP32 in Complex with the Tetrapeptide Acetyl-Asp-Val-Ala-Asp Fluoromethyl Ketone*

(Received for publication, September 18, 1996, and in revised form, December 18, 1996)

Peer R. E. Mittl‡‡, Stefania Di Marco‡‡, Joseph F. Krebs‡, Xu Bai‡, Donald S. Karanewsky‡, John P. Priestle‡, Kevin J. Tomaselli‡, and Markus G. Grütter‡

From ‡Core Drug Discovery Technologies, Ciba-Geigy AG, CH-4002 Basel, Switzerland and §IDUN Pharmaceuticals, Inc., La Jolla, California 92037

The cysteine protease CPP32 has been expressed in a soluble form in Escherichia coli and purified to >95% purity. The three-dimensional structure of human CPP32 in complex with the irreversible tetrapeptide inhibitor acetyl-Asp-Val-Ala-Asp fluoromethyl ketone was determined by x-ray crystallography at a resolution of 2.3 Å. The asymmetric unit contains a (p17/p12)2 tetramer, in agreement with the tetrameric structure of the protein in solution as determined by dynamic light scattering and size exclusion chromatography. The overall topology of CPP32 is very similar to that of interleukin-1β-converting enzyme (ICE); however, differences exist at the N terminus of the p17 subunit, where the first helix found in ICE is missing in CPP32. A deletion/insertion pattern is responsible for the striking differences observed in the loops around the active site. In addition, the P1 carbonyl of the ketone inhibitor is pointing into the oxyanion hole and forms a hydrogen bond with the peptidic nitrogen of Gly-122, resulting in a different state compared with the tetrahedral intermediate observed in the structure of ICE and CPP32 in complex with an aldehyde inhibitor. The topology of the interface formed by the two p17/p12 heterodimers of CPP32 is different from that of ICE. This results in different orientations of CPP32 heterodimers compared with ICE heterodimers, which could affect substrate recognition. This structural information will be invaluable for the design of small synthetic inhibitors of CPP32 as well as for the design of CPP32 mutants.

Genetic and biochemical studies have established the importance of the CED3/ICE proteases in programmed cell death or apoptosis (1–4). Of the known mammalian CED3/ICE proteases, CPP32 is the most similar in sequence homology and substrate specificity to the CED3 protease (2, 5). Differences in the active sites of CPP32 and ICE, which have only a 50-fold selectivity for CPP32 (23), result in differences in their recognition by CPP32 and ICE, we have solved the x-ray structure at 2.3 Å of recombinant CPP32 complexed to an irreversible tetrapeptide inhibitor, acetyl-Asp-Val-Ala-Asp fluoromethyl ketone (Ac-DVAD-fmk), and compared it with two previously published ICE structures (24, 25) and a recently published 2.5 Å structure of CPP32 (23).
Crystal Structure of CPP32 with an Irreversible Inhibitor

TABLE I

| Data collection statistics |
|---------------------------|
|                         | Data set 1 | Data set 2 |
| Space group              | P2₁       | P2₁       |
| Unit cell parameters (Å) |           |           |
| a = 50.9, b = 69.1, c = 93.8 |           |           |
| α = 90°, β = 101.2°, γ = 90° |           |           |
| Wavelength (Å)           | 0.873     | 1.5418    |
| Temperature (°C)         | 20        | 4         |
| Crystal to detector distance (mm) | 350 | 110 |
| Frame size/exposure (%e) | 1/0.180   | 0.5/600   |

**EXPERIMENTAL PROCEDURES**

Materials—Ac-YVAD-aldehyde and Ac-DEVD-aldehyde were purchased from Bachem Bioscience Products (King of Prussia, PA). Aceltyl-Asp-Glu-Val-Asp aminomethylcoumarin (Ac-DEVD-ame) was synthesized as described (12).

CPP32 Production—The cloned full-length CPP32 gene (14) was inserted into the BamHIXhoI sites of the pET21b plasmid (Novagen, Madison, WI), which fused a His₆ tag to the CPP32 C terminus. Escherichia coli BL21(DE3) cells containing this plasmid were grown to a density of A₆₀₀,ₘₐₓ = 1.9 at 37 °C in 3 liters of induction medium (20 g/liter Tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 0.5 M × 9 salts, 0.4% glucose, 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.1 mg/ml ampicillin, pH 7.4). Isopropyl-1-thio-β-β-galactopyranoside (1 mM) was added, and the culture was shaken at 25 °C for 3 h. Cells were pelleted and resuspended in 100 ml of binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, pH 8.0) containing 0.1 mg/ml lysozyme and 0.1% Triton X-100. After incubation on ice for 40 min, the cells were flash-frozen and stored at −80 °C. After centrifugation, lysate supernatant was loaded onto a 12-ml Ni²⁺-charged HisBind metal affinity column (Novagen) equilibrated with binding buffer. The purified CPP32 protein was eluted from the column using a linear 60–1000 mM imidazole gradient (Novagen) equilibrated with binding buffer. The purified CPP32 protein was eluted from the column using a linear 60–1000 mM imidazole gradient (total volume of 100 ml). Fractions containing CPP32 activity were pooled for further purification. The inhibited enzyme was concentrated by ultrafiltration and buffer-exchanged into 25 mM HEPES, 50 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 0.02% NaN₃, pH 7.5. All procedures were done at 4 °C.

Inhibitor Synthesis—Ac-DEVD-fmk was prepared via a Swern oxidation of Ac-Asp(OBu)²-Val-Ala-NHCH₂CH₂CO₂Bu/CH(OH)CH₂F with oxalyl chloride/triethylamine in dimethyl sulfoxide/CH₃Cl, followed by deprotection with 50% trifluoroacetic acid in CH₂Cl₂. The synthesized tripeptide was purified by column chromatography on a phenylthiohydantoin-derivative analyzer (Model 120A, Applied Biosystems Inc.) equilibrated with 25 mM HEPES, 50 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 0.02% NaN₃, pH 7.5. The enzyme was chromatographed in the same buffer at a flow rate of 0.2 mg/ml. The elution volume was determined by a comparison with a standard curve of the column calibrated with molecular weight markers (Bio-Rad).

Enzymatic Assay—The enzymatic activity of CPP32 was determined from the initial rate of Ac-DEVD-ame substrate hydrolysis in 25 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS, and 10% sucrose, pH 7.5. Aminomethylcoumarin product formation was detected by the increase in sample fluorescence (λₑₓ = 360 nm, λₑₓ = 460 nm) for 1 h at room temperature using a Cytofluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA). For kinetic and inhibitory measurements, the final substrate concentration range was 1–200 µM, while the enzyme concentration was kept at 32 µM. For inhibition constant (Kᵢ) determinations, the enzyme and inhibitor were preincubated for 30 min at room temperature prior to the addition of the substrate.

Dynamic Light Scattering—Light scattering was performed with a Dynapro-801 molecular sizing instrument (Protein Solutions, Inc., Charlottesville, VA). Data analyses were performed with Protein Solutions Auto Pro Data analysis software. The purified protein was injected into a cell equipped with a 0.02-µm syringe filter at room temperature into the Dynapro-801 detector. The sample was measured at a protein concentration of 2.6 mg/ml.

Cryocrystallization and Data Collection—The hanging and sitting drop methods of vapor diffusion at 4 °C were used (30). CPP32 protein (2–4 mg/ml) mixed with 2–4 µl of precipitant was incubated at 4 °C for 1 week. Crystals were grown by the hanging drop vapor diffusion method at 4°C using 3 vol% precipitant and 1 vol% protein.

Crystal Structure Solution and Refinement—The synchrotron data set was obtained from a small crystal collected at room temperature using monochromated synchrotron radiation at the Swiss-Norwegian Beam Line, European Synchrotron Radiation Facility (Grenoble, France). Data collection and processing were performed using MAR Research imaging plate detector system. Graphite-monochromated CuKα radiation was provided by an Enraf Nonius FRG91 rotating anode x-ray generator operated at 45 kV and 90 mA. A preliminary native data set from a small crystal was collected at room temperature using monochromated synchrotron radiation at the Swiss-Norwegian Beam Line, European Synchrotron Radiation Facility (Grenoble, France). Data collection and processing were performed using MAR Research imaging plate software (31–33). Processing statistics for both data sets are given in Table I.

Crystallographic Data Statistics

|                          | Data set 1     | Data set 2     |
|--------------------------|---------------|---------------|
| Resolution (Å)           | 30.0 to 2.6   | 2.80 to 2.60  |
| Completeness (%)         | 94.4          | 96.7          |
| R_max (%)                | 8.1           | 39.6          |
| I vs. σ (%)              | 70.6          | 41.7          |
| Unique reflections       | 18,690        | 3780          |
| Multiplicity             | 2.7           | 2.7           |

The coordinates for the translation (28) were deposited with Coomassie Blue G-250 (29). Gel filtration analysis of the CPP32-inhibitor complex was performed at 4 °C using a 1.6 × 70-cm Sephadex G-75 column (Pharmacia Biotech Inc.) equilibrated with 25 mM HEPES, 50 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 0.02% NaN₃, pH 7.5. The protein was chromatographed in the same buffer at a flow rate of 0.2 mg/ml. The elution volume was compared against a standard curve of the column calibrated with molecular weight markers (Bio-Rad).

The enzymatic activity of CPP32 was determined from the initial rate of Ac-DEVD-ame substrate hydrolysis in 25 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS, and 10% sucrose, pH 7.5. Aminomethylcoumarin product formation was detected by the increase in sample fluorescence (λₑₓ = 360 nm, λₑₓ = 460 nm) for 1 h at room temperature using a Cytofluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA). For kinetic and inhibitory measurements, the final substrate concentration range was 1–200 µM, while the enzyme concentration was kept at 32 µM. For inhibition constant (Kᵢ) determinations, the enzyme and inhibitor were preincubated for 30 min at room temperature prior to the addition of the substrate.

The synchrotron data set was obtained from a small crystal collected at room temperature using monochromated synchrotron radiation at the Swiss-Norwegian Beam Line, European Synchrotron Radiation Facility (Grenoble, France). Data collection and processing were performed using MAR Research imaging plate detector system. Graphite-monochromated CuKα radiation was provided by an Enraf Nonius FRG91 rotating anode x-ray generator operated at 45 kV and 90 mA. A preliminary native data set from a small crystal was collected at room temperature using monochromated synchrotron radiation at the Swiss-Norwegian Beam Line, European Synchrotron Radiation Facility (Grenoble, France). Data collection and processing were performed using MAR Research imaging plate software (31–33). Processing statistics for both data sets are given in Table I.

Structure Solution and Refinement—The synchrotron data set was used to solve the structure by molecular replacement with the program AOMoRe (34). As a template, a p17/p12 heterodimer of a previously constructed homology model, created on the basis of the ICE structure (25), was used. Two peaks were found in the cross-rotation function for the rotation angles α = 76.1°, β = 149.6°, γ = 56.2° (peak height = 5.8σ, 8.0 to 3.0 Å resolution, and 25-A Patterson radius and α = 323.1°, β = 52.6°, γ = 186.7° (peak height = 5.5σ). The two-dimensional translation function, calculated with the search model rotated according to the first set of angles in the resolution range 8.0 to 3.0 Å, yielded a solution for the translation α = 0.4396, β = 0.0000, and ε =
Crystal Structure of CPP32 with an Irreversible Inhibitor

| TABLE II | Characterization of recombinant human CPP32 |
|-----------|---------------------------------------------|
| **N-terminal sequence analysis (40 steps)** | **SGISLDSNYKMD. (residues 29–175)** |
| p17 | SGVDDDMACHKI. (residues 176–277 plus LEHHHHHHH) |
| p12 | |

Molecular mass (MALDI/TOF mass spectrometry)

| p17 | 12,983 Da (data not shown) |
| p12 | 12,972 Da |

Purity by RP-HPLC

- 95% purity

Purity by SDS-PAGE

- 90% purity

* MALDI/TOF, matrix-assisted laser-desorption ionization/time of flight; RP-HPLC, reversed-phase high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

K_{m} \text{ Ac-DEVD-amlc (μm)} = 9.7 \text{ for natural enzyme (Table III).}

K_{m} \text{ Ac-DEVD-aldehyde (μm)} = 0.2 \text{ for natural enzyme (Table III).}

K_{m} \text{ Ac-YVAD-aldehyde (μm)} > 10 \text{ for natural enzyme (Table III).}

RESULTS AND DISCUSSION

Expression of Pro-CPP32 in Bacteria Yields Processed Active Enzyme That Is Indistinguishable from Native Enzyme—Soluble active recombinant CPP32 was produced in E. coli using a T7 expression system. The enzymatic activity present in the induced cell lysate indicated that 46 mg of active CPP32 were produced from a 3-liter culture. The enzyme was readily purified from the lysate using metal chelate affinity chromatography. Although CPP32 was expressed as the 32-kDa proenzyme, purified CPP32 is composed of two subunits in a 1:1 molar ratio with molecular masses of 17 and 12 kDa, which indicates that the proenzyme has been processed to mature enzyme in E. coli (Table II). Like the native enzyme (8), active recombinant CPP32 is generated by cleavage of the proenzyme at Asp-28 and Asp-175, as determined by N-terminal sequencing and matrix-assisted laser-desorption ionization/time of flight mass spectrometry (Table II). Two possible explanations for the CPP32 proenzyme processing are that CPP32 processes itself, or a bacterial protease processes CPP32. We favor the former possibility for two reasons. First, there is some evidence that in vitro translated CPP32 can cleave its own pro-domain after the p17/p12 cleavage is made; and second, pro-CPP32 in the bacterial lysate is converted to p17/p12 during the Ni^{2+} affinity chromatography step, possibly on the column itself (data not shown). The K_{m} values for Ac-DEVD-amlc and the inhibition constants for Ac-DEVD-aldehyde and Ac-YVAD-aldehyde for recombinant CPP32 are nearly identical to the published values for the natural enzyme (Table III).

Processed CPP32 Exists as a Homo-heterodimer in Solution—Purified CPP32 was inactivated with the potent irreversible inhibitor Ac-DVAD-fmk (K_{i} = 5.5 \times 10^{-6} \text{ M s}^{-1}). The quaternary structure of purified inhibited CPP32 was analyzed by gel filtration chromatography and dynamic light scattering. The processed CPP32 protein eluted from the column as a single peak at a position consistent with a molecular mass of 52 kDa (data not shown), which is 1.7 times the predicted molecular mass of the inhibited p17/p12 heterodimer (30 kDa). This result was confirmed by light scattering. Assuming the protein to be globular in nature, molecular size estimation showed recombinant CPP32 to have a hydrodynamic radius of 32.5 Å, with a standard deviation of the spread of particle sizes about the reported average radius of 49 Å, corresponding to a monodisperse solution (<15% polydispersity). Software-based conversion of hydrodynamic radius measurements estimated a molecular mass of 52 kDa, confirming that CPP32 exists as a (p17/p12)_{2} tetramer in solution. Empirical observations suggest that macromolecules that are monodisperse in solution (i.e. are all the same size) crystallize readily, whereas randomly aggregating or polydisperse systems usually do not yield crystals (37). Recombinant CPP32 was readily co-crystallized with the Ac-DVAD-fmk inhibitor.

Topology of CPP32—The structure of CPP32 contains two copies each of the p17 and p12 chains that contain residues 35–173 and 185–277, respectively. The four polypeptide chains associate to form a (p17/p12)_{2} tetramer, which folds into one compact structure with overall dimensions of ~50 \times 60 \times 30 Å^{3}. The p17 and p12 subunits interact extensively with each other, and the structure is better described as a homodimer of p17/p12 heterodimers. A further subdivision of the p17/p12 dimer into separate domains is impossible since it appears as a single folding unit (Fig. 1). The core of the enzyme is formed by a central 12-stranded β-sheet. Each p17/p12 dimer donates six strands. The first five strands are all parallel. Only the innermost strand f is directed antiparallel to strands e and f (denotes parts from the non-crystallographic symmetry-related dimer). The central β-sheet shows a left-handed 50° twist and is surrounded by 10 α-helices. Looking down the 2-fold symmetry that relates the two p17/p12 dimers, helices 1, 4, 5, 1', 2', 4', and 5' are located below the central β-sheet, and helices 2, 3, 2', and 3' are located above. Helices 1–3 and strands a–d
Crystal Structure of CPP32 with an Irreversible Inhibitor

The C terminus of p17 and the N terminus of the non-crystallographic symmetry-related p12 subunit are in close contact (Fig. 2). Since the two dimers are related by a 2-fold axis, the C terminus of p17 and the N terminus of p12 from the same p17/p12 dimer are 50 Å away from each other. If both subunits in one p17/p12 dimer would come from the same polypeptide chain, larger structural rearrangements during the maturation of CPP32 would have to take place. It is therefore quite unlikely that these subunits were generated by cleaving a single polypeptide chain in the proenzyme, as suggested by the recently published CPP32 structure (23) and by the two ICE structures (24, 25). The close proximity of the p12 N terminus and the p17 C terminus coming from different dimers (Fig. 2) implies a different folding pathway. It seems to be more likely that two different proenzymes associate to form a proteotramer. The maturation of the protease involves the subsequent cleavage of the proteotramer by CPP32 itself (38) or by other proteases at multiple sites, like cytotoxic T cell serine protease granzyme B (39) or other ICE-like proteases. Consequently, two different proenzymes associate to form a proteotramer. The maturation of the protease involves the subsequent cleavage of the proteotramer by CPP32 itself (38) or by other proteases at multiple sites, like cytotoxic T cell serine protease granzyme B (39) or other ICE-like proteases, in an ordered signal transduction cascade (15, 16).

The overall topology of CPP32 is very similar to that of ICE (24, 25). In fact, the two structures can be superimposed with a root mean square deviation of 1.3 Å for C-α atoms (residues 35–40, 45–57, 60–107, 108–131, 132–170, 188–245, 258–266, and 267–276 from CPP32 and the corresponding residues from ICE). Striking differences exist at the N terminus of p17, where the first α-helix found in ICE is missing in CPP32 (Fig. 3).

The residues present no significant sequence homology to ICE. Although purified CPP32 contains Ser-29–Asn-35, they are not defined in the electron density, indicating that they are disordered. N-terminal sequencing of redissolved crystals confirmed the presence of these residues. Striking differences are also observed for the loops around the active site and at the interface between the two p17/p12 dimers. In CPP32, 3 residues are inserted into the loop between strand a and helix 1 (denoted loop 1), and 10 residues into the loop between helix 5 and strand f (denoted loop 3). In ICE, the loop between strand c and helix 3 (denoted loop 2) is 6 residues longer.

In CPP32, there is also a single residue deletion within strand f. In ICE, this residue (Arg-391) forms a β-bulge that is absent in CPP32. From the sequence alignment shown in Fig. 4, it becomes evident that these insertions and deletions are conserved in many other CED3/ICE-like proteases. Particularly, the insertion into loop 3, the deletions of the residues in loop 2, and the absence of a β-bulge are specific for the CED3-like subclass. The insertion into loop 1 is also found in many other proteases from the CED3-like subclass, but not in CED3 itself. The conservation of this deletion/insertion pattern implies that the other members of the CED3-like subclass could have structures that are more similar to CPP32 than to ICE. Since most of these differences appear around the active site, they would be expected to affect the specificities of the ICE- and CED3-like subclasses.

Inhibitor Binding—The inhibitor Ac-DVAD-fmk binds in a narrow cleft across the C-terminal end of the central β-sheet. All atoms are well defined in the electron density (Fig. 5). The active-site cysteine (Cys-163) resides on the elongation of strand d that makes a sharp 90° turn at the end of the β-sheet (Fig. 1). The N terminus of the inhibitor binds between the N terminus of helix 4 and the C terminus of helix 5. As in many other proteases that are totally unrelated to CPP32, the inhibitor is recognized in a β-sheet conformation (40). The main chain of the inhibitor is aligned antiparallel with residues 205–209, and the polar main chain atoms from P1, P3, and the acetyl protection group (denoted P5) form a β-sheet-like hydrogen-bonding network (Table IV). The inhibitor is irreversibly bound through a thioether bond to the side chain of Cys-163 (Fig. 6A). The side chain of the inhibitor P1 Asp points into a deep pocket, forming salt bridges with Arg-64 and Arg-207 and a hydrogen bond with Glu-161. The binding of a small acidic residue in S1 is supported by the strong positive electrostatic potential exerted by the basic residues nearby (Figs. 6A and 7).

Fig. 8 shows a superposition of the ICE inhibitor Ac-YVAD-aldehyde with the CPP32 inhibitor Ac-DVAD-fmk. Both inhib-
itors differ at P4 and the fmk/aldehyde. Although the residue at P1 is thought to be the most rigid, there is some flexibility. The different conformations of the aspartic acid at P1 are due to the different conformations of the P1 carbonyl. In contrast to the structure of CPP32 in complex with Ac-DEVD-aldehyde (23), we can clearly identify the carbonyl oxygen pointing into the oxyanion hole and forming a hydrogen bond with the Gly-122 nitrogen. A similar interaction is observed in the crystal structure of ICE in complex with Ac-YVAD chloromethyl ketone (24). In the ICE-Ac-YVAD-aldehyde structure (25), the P1 carbonyl forms a hydrogen bond with the active-site histidine rather than pointing into the oxyanion hole. Rotaonda et al. (23) proposed a similar interaction in the CPP32-Ac-DEVD-aldehyde structure. From the set of four structures of CED3/ICE-like protease-inhibitor complexes that are now available, we conclude that in the case of ketone inhibitors, the carbonyl interacts with the amide proton in the oxyanion hole. These results are in agreement with early structural studies on papa

Crystal Structure of CPP32 with an Irreversible Inhibitor

FIG. 3. Stereo plot of the superposition of ICE and CPP32 based on the Cα atoms from one p17/p12 dimer. Cα atoms from the CPP32 structure are indicated by black dots. All residue numbers refer to the CPP32 structure, except Arg-391. The numbers indicate regions where deletions/insertions occur.

FIG. 4. Multiple partial sequence alignment of CED3/ICE-like proteases. All proteins are human except those indicated by the number sign and the asterisk, which come from mouse and C. elegans, respectively. The active-site residues are boxed. Insertions that are specific for the ICE and CED3 subclasses are highlighted in black and gray. Loops 1–3 refer to the loops between β-strands and α-helices a/1, c/3, and f/5, respectively. The black bars at the bottom indicate the subunits in CPP32.

FIG. 5. Stereo plot of the electron density in the region of the bound inhibitor. The inhibitor is shown as a ball-and-stick model. The σA-weighted (F – F) omit map was contoured at 2.5σ. Residues 53–66, 119–124, 161–169, 202–210, and 248–293 from the protein are shown as a Cα model. Mainchain breaks are marked by black dots.
resent different states of the same tetrahedral intermediate illustrated in Fig. 6B. In the case of aldehyde inhibitors, the oxyanion is substituted by a hydrogen, and in the case of ketone

inhibitors, the active-site histidine does not activate the water molecule that normally attacks the carbonyl carbon. Indeed, CED3/ICE-like proteases seem to have a catalytic Cys-His dyad rather than the classical (Cys/Ser)-His-(Asn/Asp) triad (23–25).

Residues 248–259 from the loop between helix 5 and strand f create a flap that constricts the active-site pocket. The methyl side chain of Ala (P2) is pointing into a shallow depression that is created by the side chains of Tyr-204, Trp-206, and Phe-256 from the flap. Val (P3) is recognized only by its main chain interactions with the protease. There are no specific side chain interactions that discriminate between different residues at this site.

The P4 Asp side chain is buried in a narrow pocket. This pocket is rather hydrophilic and can accommodate small acidic side chains. The aspartic acid side chain forms hydrogen bonds with the side chains of Asn-208 and Trp-214 and with the main chain of Phe-250 from the flap. CPP32 favors binding of small acidic residues at P4 and rejects large aromatic residues. The differences in selectivity between ICE and CPP32 can be attributed to the size of the S4 pocket. In CPP32, the flap constricts the S4 pocket. This effect is supported by the substitution of Val-348 in ICE with Trp-214 in CPP32. The large

$\text{TABLE IV}$

| Binding site | Atom in Ac-DVAD-fmk | Atom in CPP32 | Distance $Å$ |
|--------------|---------------------|---------------|--------------|
| P1           | Asp-993 O-δ1        | Arg-64 N-ε   | 2.8          |
|              | Asp-993 O-δ1        | Arg-64 N-γ2  | 3.3          |
|              | Asp-993 O-δ1        | Arg-207 N-γ1 | 2.9          |
|              | Asp-993 O-δ2        | Arg-64 N-γ2  | 2.8          |
|              | Asp-993 O-δ2        | Gln-161 N-ε1 | 3.0          |
|              | Asp-993 O           | Gly-122 N    | 3.0          |
|              | Asp-993 N           | Ser-205 O    | 2.8          |
| P2           | Val-991 N          | Arg-207 O    | 2.7          |
| P3           | Val-991 O          | Arg-207 N    | 2.7          |
| P4           | Asp-990 N          | Phe-250 O    | 3.4          |
|              | Asp-990 O-δ2        | Trp-214 N-ε1 | 3.1          |
|              | Asp-990 O-δ2        | Asn-208–N-δ2 | 3.4          |
| P5           | Ac-989 O-α          | Ser-209 N    | 3.1          |
|              | Ac-989 O           | Ser-209 O-γ  | 2.8          |

*Ace, acetyl group.*

$\text{Fig. 6. A, hydrogen bonds and salt bridges in the CPP32-Ac-DVAD-fmk complex. The inhibitor is covalently linked to the active-site Cys-163 via a thioether bond. The inhibitor is shown by thick lines. Hydrogen bonds are represented as dashed lines. B, binding modes of aldehyde (23, 24) and chloro/fluoromethyl ketone (c/fmk) (Ref. 25 and this work) inhibitors in ICE-like proteases. Both binding modes are different states of the tetrahedral intermediate.}$
tryptophan side chain prevents binding of a residue with a bulky aromatic side chain at P4.

The Ac-DVAD-fmk inhibitor carries an acetyl protection group at the N terminus. Since an acetyl group resembles a glycine lacking the amino group, it can be considered as a P5 residue. The bond between Asp (P4) and the acetyl group is trans, like a normal peptide bond, but in the ICE structure, the corresponding bond adopts a cis conformation (25). Whether this difference is real is difficult to state with confidence because, at 2.3-Å resolution, the distinction between an oxygen and a methyl group is dubious. In CPP32, the acetyl oxygen (P5) participates in hydrogen bonds with Ser-209. In ICE, this residue is substituted with Pro-343, which is unable to form similar hydrogen bonds with the protection group. It is therefore likely that the conformational differences described above are real.

Considering all deletions/insertions occurring in the ICE-and CED3-like subclasses discussed above, only the long insertion into loop 3 creating the flap interacts with the small molecular mass tetrapeptide inhibitor. Nevertheless, the deletions/insertions into loops 1 and 2 are well conserved throughout the members of the subclass, indicating that they have a certain function. Since these loops do not interact with P1–P5, they might have a function in the recognition of larger substrates or inhibitors. The 38-kDa cowpox virus serpin CrmA, for example, inhibits ICE, but has almost no effect on CPP32 (8).

Quaternary Structure—The structure of CPP32 contains two copies of the p17/p12 heterodimer. The 2-fold symmetry axis relating the two copies intersects the central β-sheet between Val-266 and Val-266# and is oriented perpendicular to strand f. The interface between the two dimers covers an area of 2000 Å². ICE is also a tetramer in the active form. Structural and mutagenesis data suggest that the integrity of the dimer/dimer interface is indispensable for ICE proteolytic activity (25). The CPP32 dimer/dimer interface contains four hydrogen bonds formed between main chain atoms from strands f and f# and between the C terminus of p17 and the N terminus of p12#, respectively (Fig. 9). Residues from helixes 5 and 5# make side chain interactions across the interface. In particular, Glu-231, His-234, Arg-238, and Glu-272 create a network of salt bridges at the bottom of the interface (data not shown). On the opposite side of the central β-sheet, there is a deep cavity with dimensions of ~17 × 7 × 11 Å³ (Fig. 7). This cavity encompasses the
structures were superimposed based on all residues from the first p17/p12 dimer (denoted ... from the first p17/p12 dimer (denoted e and f). In ICE, Arg-391 creates a β-bulge (indicated by a black dot) and perturbs the regular hydrogen bonding pattern observed in CPP32.

The topology of the interface in ICE differs from that in CPP32. None of the side chains that form hydrogen bonds across the interface are conserved, and the hydrogen bonding pattern between main chain atoms is different (Fig. 9). These differences are mainly caused by the insertion in ICE of a single residue (Arg-391) into strand f. In ICE, this residue forms a β-bulge, which disturbs the regular hydrogen bonding pattern of the antiparallel β-strands f and f' and affects the quaternary structure of the enzyme. Due to this insertion and the changes at the interface, the respective relative orientation of the p17/p12 dimer is different in ICE and CPP32. A superposition of the whole tetramer based on the residues from a single dimer reveals that the second dimer of CPP32 is rotated 13° relative to the second dimer of ICE (Fig. 10).

2-fold axis. The bottom of the cavity is hydrophobic, but the residues along the walls are hydrophilic. The cavity is filled with well ordered water molecules.

The topology of the interface in ICE differs from that in CPP32. None of the side chains that form hydrogen bonds across the interface are conserved, and the hydrogen bonding pattern between main chain atoms is different (Fig. 9). These differences are mainly caused by the insertion in ICE of a single residue (Arg-391) into strand f. In ICE, this residue forms a β-bulge, which disturbs the regular hydrogen bonding pattern of the antiparallel β-strands f and f' and affects the quaternary structure of the enzyme. Due to this insertion and the changes at the interface, the respective relative orientation of the p17/p12 dimer is different in ICE and CPP32. A superposition of the whole tetramer based on the residues from a single dimer reveals that the second dimer of CPP32 is rotated 13° relative to the second dimer of ICE (Fig. 10).

REFERENCES

1. Yuan, J. Y., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) Cell 73, 641–652
2. Xue, D., Shaham, S., and Horvitz, H. R. (1996) Genes Dev. 10, 1073–1083
3. Gagliardini, V., Fernandez, P.-A., Lee, R. K. K., Drexler, H. C. A., Rotello, R. J., Fishman, M. C., and Yuan, J. (1994) Science 263, 826–828
4. Steller, H. (1995) Science 267, 1445–1449
5. Fernandez-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) J. Biol. Chem. 269, 30761–30764
6. Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994) Cold Spring Harbor Symp. Quant. Biol. 111, 377–385
7. Thornberry, N. A., Bull, H. D., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molinexaux, S. M., Weidner, J. R., Aunins, J.,
