Substrate and Inhibitor Specificity of Interleukin-1β-converting Enzyme and Related Caspases*

(Received for publication, July 22, 1996, and in revised form, January 10, 1997)

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Interleukin-1β-converting enzyme (ICE) is a novel cysteine protease responsible for the cleavage of pre-interleukin-1β (pre-IL-1β) to the mature cytokine and a member of a family of related proteases (the caspases) that includes the Caenorhabditis elegans cell death gene product, CED-3. In addition to their sequence homology, these cysteine proteases display an unusual substrate specificity for peptidyl sequences with a P1 aspartate residue. We have examined the kinetics of processing pre-IL-1β to the mature form by ICE and three of its homologs, TX, CPP-32, and CMH-1. Of the ICE homologs, only TX processes pre-IL-1β, albeit with a catalytic efficiency 250-fold less than ICE itself. We also investigated the ability of these four proteases to process poly(ADP-ribose) polymerase, a DNA repair enzyme that is cleaved within minutes of the onset of apoptosis. Every caspase examined cleaves PARP, with catalytic efficiencies ranging from $2.3 \times 10^5$ $M^{-1}$ s$^{-1}$ for CPP32 to $1.0 \times 10^3$ $M^{-1}$ s$^{-1}$ for TX. In addition, we report kinetic constants for several reversible inhibitors and irreversible inactivators, which have been used to implicate one or more caspases in the apoptotic proteolysis cascade. Ac-Asp-Glu-Val-Asp aldehyde (DEVD-CHO) is a potent inhibitor of CPP-32 with a $K_i$ value of 0.5 nM, but is also potent as inhibitor of CMH-1 ($K_i = 35$ nM) and ICE ($K_i = 15$ nM). The x-ray crystal structure of DEVD-CHO complexed to ICE presented here reveals electrostatic interactions not present in the Ac-YVAD-CHO co-complex structure (Wilson, K. P., Black, J.-A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 270–275), accounting for the surprising potency of this inhibitor against ICE.

ICE is the prototypical member of a new family of mammalian cysteine proteases (the caspases) that is distinct from cysteine proteases in the papain superfamily (1–3). The mutagenesis experiments and crystal structure reported by Wilson et al. (4) revealed a different active site geometry and catalytic mechanism for ICE than observed for papain. The structure of the ICE active site contains a Cys-His catalytic diad, and two Arg residues that confer high selectivity for peptidyl substrates with Asp residues at the P1 position (N-terminal to the scissile bond) (4, 5). Although ICE has recently been reported to cleave other proteins in vitro (6, 7), it was identified from its essential role in processing the inactive 31-kDa precursor of interleukin-1β (pre-IL-1β) to the mature 17-kDa cytokine (8).

In 1993, Yuan et al. (9) reported the sequence of the Caenorhabditis elegans programmed cell death gene ced-3. This gene is 29% identical to human ICE. Due to the central role of the CED-3 protein in C. elegans apoptosis, Yuan and colleagues deduced that ICE or ICE homologs might play a similar role in mammalian apoptosis. Overexpression of ICE in rat fibroblast, mammalian COS cells, and neuronal cell lines demonstrated that this protease can indeed induce apoptosis (6, 10, 11). Subsequently, Kuida et al. (12) confirmed an in vivo role for ICE in Fas-mediated apoptosis by disruption of the murine ICE gene.

A family of ICE-related proteases (the caspases) was discovered by searching human cDNA libraries for sequences homologous to ICE or ced-3 (13, 14). At present, at least 10 human homologs of ICE possessing cysteine protease activity have been identified. These homologs can be grouped by sequence similarity into three subfamilies. The most closely related homologs to ICE are TX (caspase-4, also denoted ICH-2 or ICEcasp32), which has 20% identity to ICE and belongs to a distinct subfamily. A third group of homologs comprises proteins that show a higher sequence similarity to CED-3 than to ICE. These include CPP32 (caspase-3) (7, 21), MCH-2 (caspase-6) (22), and CMH-1 (caspase-7, also called MCH-3) (23, 24). Based on in vivo similarity to the C. elegans gene and inhibition by a tetrapeptide aldehyde based on the PARP cleavage sequence (DEVD-CHO), CPP32 was claimed to be the caspase responsible for apoptosis in mammalian cells (25). The dependence of apoptosis in vivo on the presence of CPP32 remains to be confirmed, however, and the inhibition of other caspases by DEVD-CHO has not been addressed. Subsequent work has shown that pro-CPP32 is activated by other caspases during apoptosis (26, 27).

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1 The following abbreviations are used: ICE, interleukin 1β-converting enzyme; IL-1β, interleukin 1β; PARP, poly(ADP-ribose) polymerase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; pDNA, p-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; IVTT, in vitro transcription translation; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; Ac-YVAD-AMC, Ac-Tyr-Val-Ala-Asp-aminomethylcoumarin; Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-CHO, Ac-Tyr-Val-Ala-Asp-aldehyde; HPLC, high performance liquid chromatography.

2 Caspase denotes the cysteine protease subfamily that includes ICE and its human homologs. For a discussion of the nomenclature of ICE and its homologs, please see Ref. 1.
Although a thorough investigation of the kinetics of ICE has been reported (28), little kinetic information on ICE homologs exists in the literature. A rigorous kinetic analysis of these proteases is thus essential to evaluate the putative differential substrate specificity of these proteases (24, 25). For example, in contradistinction to the claim by Nicholson et al. (25) that ICE is unable to cleave poly(ADP-ribose) polymerase (PARP, a cellular enzyme cleaved during apoptosis), we recently reported that ICE is fully competent to cleave this substrate, albeit at enzyme concentrations higher than those required for cleavage of pre-IL-1β (6). Indeed, every ICE homolog identified is able to cleave PARP as a substrate (14), and the MCH-3 homolog appears to be more active in PARP cleavage than CPP32 itself (24). Similarly, TX is able to cleave pre-IL-1β (16), but does so inefficiently.

We have selected for our kinetic studies two members from the ICE subfamily (ICE and TX) and two members of the CPP32 subfamily (CMH-1/MCH-3 and CPP32). We report a kinetic analysis of substrate hydrolysis by these proteases and the inhibition constants for both reversible and irreversible peptidyl inhibitors. Such compounds have been used widely to probe the catalytic mechanism of proteases (25). We chose inhibitors from the ICE subfamily (ICE and TX) and two members of the CPP32 subfamily (CMH-1/MCH-3 and CPP32). We report a kinetic analysis of substrate hydrolysis by these proteases and the inhibition constants for both reversible and irreversible peptidyl inhibitors. Such compounds have been used widely to probe the catalytic mechanism of proteases (25).

**EXPERIMENTAL PROCEDURES**

**Recombinant ICE and ICE Homologs**

ICE—Recombinant human interleukin-1β convertase was expressed from a p30 construct containing an N-terminal T7 tag in *Trichoplusia ni* insect cells using a baculovirus expression system. Active T7 ICE containing p20 and p10 subunits was purified from the medium by affinity chromatography using an immobilized T7 antibody column according to the manufacturer’s protocol (Novagen). The expression, purification, and characterization of this protein has been published elsewhere (33).

Alternatively, ICE was expressed as a p32 construct in *Escherichia coli* and purified as the inactive p30 precursor from inclusion bodies using size-exclusion chromatography. Refolding and subsequent auto-processing was performed at a protein concentration of 3 mg/ml in 25 mM Tris-HCl, 1 mM DTT, pH 7.5, at 25 °C to give the active enzyme, which is immediately frozen in 10% glycerol at −78 °C (4). The kinetic parameters of the *E. coli* and baculovirus expressed ICE were similar, regardless of the purification method used.

Quantification of active enzyme was performed with the irreversible inhibitor Ac-DEVD-CHO. Kinetic parameters for the Ac-DEVD-AMC substrate under standard assay conditions are: *Km* = 12 μM and *kcat* = 1.0 s⁻¹.

**Purification of Pre-IL-1β**

Recombinant pre-IL-1β was cloned and expressed in *E. coli*. Cell pellets (20 g) were resuspended in 100 ml of 10 mM Tris, pH 8.0, containing 0.05 M NaCl, 10% glycerol, 2 mM EDTA, and lysed by microfluidization. Cell extracts were centrifuged at 4 °C, for 30 min at 35,000 × g. Pre-IL-1β was extracted from the cell pellet with 100 ml of 10 mM Tris, pH 8.0, containing 0.05 M NaCl, 1 mM PMSF, 8 M urea and subsequently dialyzed against the lysis buffer without PMSF. The urea extract was loaded on a (3.5 × 26-mm) DEAE-Sepharose column, equilibrated in the same buffer, washed with five column volumes of buffer, and the bound protein was eluted with a linear gradient from 0.05 to 1 M NaCl in 10 mM Tris, pH 8.1, 10% glycerol. Pre-IL-1β was detected in these fractions by SDS-PAGE and by Western blotting using a monoclonal antibody generated against IL-1β (from M. DeCenzo, Vertex). Pre-IL-1β-containing fractions were concentrated by 40% ammonium sulfate precipitation. The subsequent pellet was solubilized in 10 mM Tris, pH 8.0, containing 2% (w/v) EDTA, 2 mM DTT, 0.5 mM NaCl, 5% glycerol and loaded on a Sephadex G-75 column (100 × 26 mm), calibrated, and eluted in the same buffer. Pre-IL-1β eluted at an apparent molecular mass of approximately 40 kDa. The sample was dialyzed against 10 mM Tris, pH 8.0, containing 0.1 M NaCl, 1 mM EDTA and concentrated on a YM10 Amicon membrane. The yield of purified material was 70%, and on Coomassie-stained gel reduced pre-IL-1β migrated as a single band with a mobility corresponding to 33 kDa.

**Purification of Truncated Poly(ADP-ribose) Polymerase**

The DNA binding domain of PARP containing an N-terminal T7 tag was expressed in *E. coli*. Cell pellets from 100-ml cultures were resuspended in lysis buffer containing 50 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaH₂SO₄, 0.2 mM NaCl, 10% glycerol, 5 mM DTT, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 2 mM PMSF, and 1 mM benzamidine and disrupted in microvibrations. This suspension was centrifuged at 4 °C, 30 min at 15,000 × g. The cell pellet was solubilized in buffer (9 ml), containing 8 M urea, 50 mM Tris, pH 8.0, 50 mM NaH₂SO₄, 0.2 mM NaCl, 10% glycerol, and 5 mM DTT, and dialyzed stepwise against the same buffer without urea in 100-ml increments. The dialyzed sample was applied to a (1.8 × 26-mm) heparin-Sepharose column. The bound protein was eluted with a linear gradient from 0.2 to 1.2 M NaCl in the dialysis buffer at 0.35 M NaCl. Fractions were collected and analyzed by Western blotting using a monoclonal antibody against the T7 tag. The protein migrated as a single band of apparent molecular mass 45 kDa. The total amount of pure PARP recovered was 3 mg, which corresponds to 30% yield of purification.

**In Vitro Cleavage of Pre-IL-1β and PARP by ICE and Homologs**

35S-Labeled pre-IL-1β and PARP proteins, which contain 12 and 11 methionines, respectively, were prepared by in vitro transcription-translation (IVTT) using the TNT T7-coupled reticulocyte lysate system (Promega) and [35S]methionine (500 Ci/mmol, Amersham Corp.). Cleavage experiments were performed using one or both of two methods. The first method, in which the amount of radioactivity per assay was kept constant, [35S]-labeled substrate (40 nM/0.5 μl) was incubated in reaction mixtures of 25 μl containing 10–40 nM enzyme.
and varying amounts (100 nM to 10 μM) of unlabeled protein substrate in 10 mM Tris-HCl buffer, pH 7.5, 0.1% CHAPS, 1 mM DTT, 37°C. Aliquots were removed, quenched by the addition of sample buffer, and applied to Novex Tris-glycerine 4–20% gradient denaturing gels. After gel electrophoresis and autoradiography, the concentrations of cleavage products were determined by densitometry. Alternatively, the specific activity of the substrate was kept constant and a stock containing both 35S-labeled IVTT substrate and unlabeled protein substrate was prepared and used identically in reaction mixtures as described above. For both methods, calculation of kinetic parameters was performed by nonlinear least squares fitting of the rate versus concentration data using the commercial program Enzfitter (Biosoft).

Spectrophotometric Assays

Synthetic peptidyl substrates were purchased from Bachem and corrected for purity by HPLC analysis on a Vydac C18 column (4.5 x 250 mm) using a water/acetonitrile gradient in 0.1% trifluoroacetic acid. Purity was also assessed by exhaustive enzymatic digestion, followed by quantification of the chromatographic leaving group by comparison to a standard curve of either p-nitroaniline or aminomethylcoumarin under identical assay conditions.

Assays were conducted in 96-well microtiter plates as described previously and contained: 65 μl of assay buffer (10 mM Tris, 1 mM DTT, 0.1% CHAPS, pH 7.5), 10 μl of enzyme solution (final concentration 2–40 nM), 5 μl of Me2SO containing the inhibitor and 20 μl of substrate (34). Production of p-nitroaniline (pNA) from reaction mixtures containing Suc-YVAD-pNA was measured by following the absorbance at 405 nm. Product was quantified with correction for the inner filter effect using standard curves from mixtures of 4-(4′-dimethylaminophenylazo)benzoic acid and 5-[[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (sodium salt).

Peptidyl inhibitors used in this study were purchased from Bachem and synthesized in-house and had a purity of >95% as quantified by HPLC. Ac-YVAD-AMC or Suc-YVAD-pNA at a concentration of 2–4 μM were present.

Assays containing reversible inhibitors were conducted as above in duplicate with the inhibitor and enzyme preincubated in the microtiter plate for 15 min at 30°C. The assay was started by the addition of substrate, and the initial rates were determined from progress curves at early reaction times. K_i values were calculated from rate versus inhibitor inhibition curves with nonlinear least squares fitting to the tight binding equation of Morrison (35). A commercial program, KineTic, was used for this purpose. Reported values are from at least two replicate determinations, and standard errors are not larger than 20%.

Second order rate constants (k) for reversible inhibitors of ICE homologs were determined from assays where the reaction buffer and containing inhibitor and substrate were preincubated at 37°C and the reaction initiated by the addition of enzyme warmed to 37°C. Progress curves of product versus time were fit using the commercial program Enzfitter (Biosoft) to the Morrison equation for time-dependent-binding (36).

\[ P = v_o + (v_o - v_i)(1-exp(-k_{cat} t))/K_m \] (Eq. 1)

Second order rate constants (k) were then calculated from linear plots of k_cat versus [I] fitting to the equation: \[ k = (k_{cat}/[I]) \times (1 + [S]/K_m) \]. These methods for the analysis of irreversible inhibitors of ICE have been described previously by Thornberry et al. (37).

RESULTS

Kinetic Characterization of ICE and TX—Cleavage of pre-IL-1β (45 kDa) by ICE to the two processed forms of 28 and 17 kDa (mature IL-1β) is easily observed by SDS-PAGE and autoradiography of reaction mixtures using 35S-labeled IVTT substrate. To obtain rate profiles, substrate concentrations were varied by mixing unlabeled purified pre-IL-1β with the labeled material generated by the IVTT system. Quantification of the autoradiographs by densitometry reveals that production of mature IL-1β under these conditions was linear with time up to at least 25% conversion (data not shown). Steady state rates were calculated from the extent of conversion at early reaction times. Saturation kinetics were observed when the substrate concentration was increased, and the kinetic parameters of K_m = 4.0 μM and k_cat = 1.2 s^-1 could be determined by least squares fitting of the data to the Michaelis-Menten equation (Fig. 1).

Three synthetic substrates containing a cleavage sequence similar to the 17-kDa cleavage site are commercially available to measure ICE activity. Using standard spectrophotometric methods, kinetic parameters for the ICE catalyzed cleavage of these substrates were determined and are reported in Table I. The K_m and k_cat values for the aminomethylcoumarin substrate are in good agreement with the values of 11 μM and 0.89 s^-1 reported using ICE (purified from THP-1 cells) (28).
Kinetic Analysis of ICE and ICE Homologs

### Table I

Comparison of kinetic parameters for pre-IL-1β and synthetic substrates for ICE and TX

| Enzyme       | Substrate          | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------|--------------------|--------|----------|---------------|
| ICE          | Pre-IL-1β (YVHDPVR) | 4.0 1.2 | 3.0×10²  |               |
| ICE          | Ac-YVAD-AMC        | 11.5 1.0 | 9.1×10⁴  |               |
| ICE          | Suc-YVAD-pNA       | 21.5 1.6 | 7.7×10⁴  |               |
| ICE          | DABCYL-YVADAPV-EDANS | 13.4 0.41 | 3.1×10⁴  |               |
| TX           | Pre-IL-1β          | ND ND   | 1.2×10⁴  |               |
| TX           | Ac-YVAD-AMC        | 35 0.5  | 1.4×10⁴  |               |
| TX           | Suc-YVAD-pNA       | 48 1.1  | 2.3×10⁴  |               |

### Table II

Comparison of kinetic parameters for PARP cleavage by ICE homologs

| Enzyme | $k_{cat}/K_m$ a $M^{-1}sec^{-1}$ |
|--------|---------------------------------|
| CPP32  | 2.3×10⁶                         |
| CMH-1  | 3.3×10⁴                         |
| ICE    | 2.0×10⁴                         |
| TX     | 1.0×10⁴                         |

a Value determined from the linear part of the rate versus concentration profile as described under "Experimental Procedures."

b ND, not determined.
c Value determined from the linear part of the rate versus concentration profile.

dimethylaminoethylamino-3-phenylpropionic acid-

octapeptide substrate, the catalytic constants we measured are similar to those of 1.4 μM and 0.79 s⁻¹ reported by Pennington and Thornberry (39).

TX, an ICE homolog that shares 50% sequence identity, was also observed to catalyze cleavage of synthetic substrates containing the YVAD sequence, with similar efficiency. Turnover numbers reported in Table I were slightly lower than the corresponding ICE values, and $K_m$ increased by only a factor of 2–3. TX could also cleave pre-IL-1β to the mature form, but only at higher enzyme concentrations (20 nM) and lower (<500 nM) substrate concentrations. As the substrate concentration increased, apparent substrate inhibition was observed and the reaction rate slowed. As a consequence, only the selectivity constant $V/K$ could be determined from the linear part of the rate versus concentration profile. The value obtained, 1.2×10⁴ M⁻¹ s⁻¹, is 2 orders of magnitude lower than that measured for ICE and pre-IL-1β.

**Kinetic Characterization of PARP Cleavage by ICE Homologs**—A second substrate for ICE and its homologs, PARP, was chosen for kinetic investigation based on our earlier observation that ICE and TX as well as the reported CPP32 are able to cleave PARP. In an extension of this earlier study, a truncated version of PARP containing only the DNA binding domain was chosen for kinetic analysis of processing. Reaction mixtures contained various amounts of purified PARP and 35S-labeled IVTT product in addition to enzyme, and were again analyzed by SDS-PAGE and densitometry. In reaction mixtures containing 2 nM CPP32 and low (<500 nM) substrate concentrations, clean conversion to two products of 31 and 12 kDa was observed, consistent with cleavage at the Asp214-Gly215 site. Again analyzed by SDS-PAGE and densitometry. In reaction mixtures containing 2 nM CPP32 and lower (<500 nM) substrate concentrations, the rate was significantly decreased. This value, 2.3×10⁴ M⁻¹ s⁻¹, is the highest measured for PARP cleavage by any of the ICE homologs reported in Table II.

**Analysis of Inhibitors of ICE and ICE Homologs**—The inhibition constants ($K_i$) for several competitive inhibitors of ICE and its homologs were measured using steady state kinetic methods (Table III). The ICE tetrapeptide aldehyde (Ac-YVAD-CHO) is an effective inhibitor of ICE and TX, and is consistent with the nanomolar value reported earlier for ICE. Ac-YVAD-CHO is a potent inhibitor of either CPP32 or CMH-1. The corresponding tetrapeptide aldehyde based upon the PARP cleavage site is a potent subnanomolar inhibitor of CPP32 and an excellent inhibitor of its near homolog CMH-1. Ac-DEVD-CHO, however, is also a potent inhibitor of ICE and TX (Table III). Removal of the P3 Asp residue to generate the tripeptide Glu-Ala-Asp-CHO significantly decreases affinity for CPP32 and CMH-1, but this tripeptide binds to ICE and TX with a $K_i$ of 300 nM.

Second order rate constants ($k$) for the two irreversible inhibitors shown against ICE in Table III agree with those reported previously (28, 40). Both the tetrapeptide dichlorobenzoyl and diazomethyl ketone are also effective inhibitors of TX; the second order rate constants differ by only a factor of 2. No measurable effect can be seen with the diazomethyl ketone against the other two homologs in this study. The Z-Val-Ala-Asp-dichlorobenzoylate, however, is an irreversible inactivator against CPP32 and CMH-1; the second order rate constant for inactivation is 2 orders of magnitude lower for these homologs than for ICE or TX.

**X-ray Crystal Structure of ICE Inhibited by Ac-DEVD-CHO**—We solved the crystal structure of Ac-DEVD-CHO with ICE to explore the molecular basis of the surprising potency of Ac-DEVD-CHO for ICE. The structure of Ac-DEVD-CHO bound to ICE reveals many interactions between the inhibitor side chains and enzyme active site binding pockets (Fig. 2), which help explain effectiveness of Ac-DEVD-CHO ($K_i = 15$ nM) in binding to the ICE active site.

The inhibitor makes three main chain to main chain hydrogen bonds with residues from the p10 subunit of ICE in an anti-parallel $β$-sheet arrangement. This hydrogen bonding pattern was also observed in the Ac-YVAD-CHO/ICE inhibitor complex (4). Ac-YVAD-CHO is also a potent ICE inhibitor ($K_i = 6$ nm), and this structure is shown for comparison (Fig. 2). The largest differences between the two structures occur at the P$_1$ and P$_2$ positions of the inhibitor. The aldehyde oxyanion of Ac-DEVD-CHO is shifted 1.0 Å from its location in the Ac-YVAD-CHO/ICE complex, and is positioned to form hydrogen bonds with the side chain imidazole ring of His$^{237}$ as well as the backbone amide nitrogen atoms of Cys$^{295}$ and Gly$^{298}$. In this location, the oxyanion of Ac-DEVD-CHO more closely matches the expected position of the oxyanion in an enzyme-substrate tetrahedral intermediate than was observed in the crystal structure of Ac-YVAD-CHO bound to ICE (Fig. 2). This is a significant difference between the two inhibitors, as the oxyanion of Ac-DEVD-CHO can make three hydrogen bonds with the enzyme while the oxyanion of Ac-YVAD-CHO makes only one with the side chain of His$^{237}$. The shift in the position of the oxyanion in the Ac-DEVD-CHO inhibitor has an effect on the orientation of the P$_1$ aspartic acid side chain. In the Ac-YVAD-CHO structure, the P$_1$ aspartic acid interacts most strongly with the Arg$^{279}$ side chain. On the basis of hydrogen bonding distance and geometry, the interactions between the P$_1$ aspartic acid in the Ac-DEVD-CHO inhibitor and ICE are more equally divided between Arg$^{279}$, Arg$^{341}$, and Gly$^{285}$. The valine residue in P$_3$ of Ac-DEVD-CHO makes van der Waals contacts with Val$^{338}$ and Trp$^{340}$ of ICE. The bulkier valine side chain, as compared to alanine in the Ac-YVAD-CHO inhibitor, requires a shift in the backbone of the inhibitor at the P$_2$ and P$_3$ positions, but the main chain to main chain hydrogen bonds are preserved. The P$_3$ glutamic acid side of Ac-DEVD-CHO forms a
of the S3 pocket of ICE. A number of new hydrogen bonds present which were not observed in the structure of ICE with Ac-YVAD-CHO. These new hydrogen bonds as well as the hydrogen bond with the guanidino group of Arg^{341}. This is a new interaction in comparison to the Ac-YVAD-CHO complex structure, which cannot form a hydrogen bond between the P3 valine side chain and ICE. The interactions between the P2 aspartic acid in Ac-DEVD-CHO and ICE are more difficult to analyze. The electron density for this portion of the inhibitor is not well defined (data not shown), which suggests that there is no single favorable set of interactions between the P4 aspartic acid of Ac-DEVD-CHO and ICE. In contrast, electron density for the P4 tyrosine residue in Ac-YVAD-CHO is well defined.

**FIG. 2.** X-ray structure of Ac-DEVD-CHO complexed to ICE. Ac-DEVD-CHO (in violet) is shown bound to the active site of ICE via covalent attachment to Cys^{285} forming a thiohemiacetal. The structure of the Ac-YVAD-CHO/ICE complex (in yellow) has been overlaid for comparison (4). Active site residues and the peptidyl side chains P1 through P4 are labeled. Dashed lines depict selected hydrogen bond interactions between the enzyme and Ac-DEVD-CHO that are highlighted in the text.

The selectivity of ICE for pre-IL-1β is reflected by the rate constants for the synthetic substrates listed in Table I. Both the p-nitroanilide and aminomethylcoumarin substrates are good mimics for the natural cleavage sequence. Furthermore, the \( K_m \) values for ICE catalyzed cleavage of the tetrapeptide substrates and pre-IL-1β are similar, indicating that most of the binding energy can be attributed to interactions in the S2–S4 binding pockets. Kinetic selectivity of the closest ICE homolog, TX in cleaving pre-IL-1β is lower by 2 orders of magnitude. This result is consistent with the generally accepted hypothesis that ICE and only ICE is responsible for the processing of interleukin 1β to the mature form. However, we do observe similar kinetic profiles for ICE and TX when synthetic substrates and inhibitors are used, arguing that the peptidyl pockets S1–S3 of both enzymes are comparable. A model of TX built from the ICE structure indicated that residues forming the S1–S3 pockets were quite similar, the largest differences occurring in the S4 pocket (15).

Poly(ADP-ribose) polymerase has been implicated as a substrate for CPP32-like proteases during apoptosis (14). This protein is of special interest, as PARP is a DNA repair enzyme that is cleaved into two fragments at the onset of apoptosis. The identification of PARP as a potential physiological substrate for CPP32-like proteases provides an appealing link between an apoptotic event and a member of the caspase family. Of the ICE homologs tested, we found CPP32 to be the most efficient at PARP cleavage. Other homologs including ICE and TX will cleave PARP, but at much higher protein concentrations and much more slowly, consistent with the previous report of Gu et al. (6). The biological relevance of this finding is unclear at the present time, as the role of PARP (and its subsequent cleavage) in apoptosis remains to be proven, although it is cleaved early on during the apoptotic sequence of events (41). Mice deficient in the PARP gene are healthy and fertile and show no overt abnormalities, indicating that this gene does not play an essential role in cellular proliferation, differentiation, or development (42, 43).

Based on these two substrates, tetrapeptide inhibitors have been devised as reagents to block and thereby implicate the presence of certain ICE homologs in cellular functions. Ac-YVAD-CHO, based upon the pre-IL-1β cleavage site, is an effective inhibitor of proteases with the highest homology to ICE itself, with \( K_i \) values of 6 and 14 nM against ICE and TX respectively. It is not effective against either CPP32 or CMH-1. In a similar fashion, Ac-DEVD-CHO was designed from the PARP cleavage sequence to be a potent inhibitor of CPP32 (25). This is indeed the case, as the compound inhibits the target protease with \( K_i \) of 0.5 nM and is also effective against the closest relative CMH-1. Most surprising is the potency of this inhibitor against both ICE and TX, revealing this compound to be one of the broadest caspase-reversible inhibitors described to date. The x-ray crystal structure of Ac-DEVD-CHO complexed to ICE reveals an additional hydrogen bond between the glutamic acid residue of the inhibitor and an arginine residue in the S3 pocket of ICE. A number of new hydrogen bonds between the oxanion of Ac-DEVD-CHO and the enzyme are present which were not observed in the structure of ICE with Ac-YVAD-CHO. These new hydrogen bonds as well as the flexibility afforded by the small spatial requirements of the aldehyde group may help explain the surprising potency of the inhibitor relative to the poor cleavage efficiency of ICE for the corresponding substrate, PARP.

The irreversible inhibitors based upon the ICE cleavage sequence afford at least 10-fold selectivity for ICE and TX over the CPP32 class of proteases. The increase in potency for the dichlorophenylloxymethyl ketones over the diazomethylketones is seen for ICE and all homologs in this study and may be due to increased interactions of the aromatic moiety on the prime side of the enzyme for all members of the ICE family. It should be noted that the 10-fold difference in activation rates for these irreversible inhibitors is easily compensated for by either increasing the concentration of the inhibitor or prolonging the

**TABLE III**

| Peptidyl inhibitors of ICE and its homologs | ICE (caspase-1) | TX (caspase-4) | CPP32 (caspase-3) | CMH-1 (caspase-7) |
|--------------------------------------------|-----------------|----------------|------------------|------------------|
| Reversible \( K_i \) (nM)                      |                 |                |                  |                  |
| Ac-Tyr-Val-Ala-Asp-CHO                     | 6               | 14             | 500,000          | >500,000         |
| Ac-Asp-Glu-Val-Asp-CHO                     | 15              | 135            | 0.52             | 35               |
| Glu-Val-Asp-CHO                            | 360             | 340            | 65,000           | 200,000          |
| Irreversible \( k (\mu\text{M} \cdot \text{s}^{-1}) \) |                 |                |                  |                  |
| Z-Val-Ala-Asp-DCB                          | 700,000         | 450,000        | 13,000           | 3600             |
| Ac-Tyr-Val-Ala-Asp-CHN<sub>2</sub>         | 15,200          | 6300           | <500             | <200             |

\( \text{Km} \) values for ICE catalyzed cleavage of the tetrapeptide inhibitors and the peptidyl side chains P1 through P4 are labeled. Dashed lines depict selected hydrogen bond interactions between the enzyme and Ac-DEVD-CHO that are highlighted in the text.
reaction time. Thus at conditions of 20 μM or greater and for reaction times greater than 1 h, as are commonly used for whole cell studies of apoptosis, these irreversible inactivators can be expected to show little discrimination among members of the ICE family of proteases. This caveat also applies to caspase (29–32). Additional caution is urged in using reversible caspase inhibitors as "selective" reagents when a thorough study of the inhibition constants against multiple caspases has not been performed. As the present work indicates, such compounds should not be assumed to be selective, and specific inhibitors of CPP32, CMH-1, or MCH-2 have yet to be reported.

Acknowledgments—We thank Robert Aldafe for providing pre-IL-1β and PARP cDNAs; Judith Lippe for providing CMH-1 and CPP32 cDNAs; John Fulghum and Stephen Chambers for assistance in baculovirus protein expression; Jo-Anne DeCenzo for providing the pre-IL-1β antibody; Michael Mullican, Scott Harbeson, and Stuart Jones (Rousell Uclaf) for the synthesis of ICE inhibitors; and Cameron Stover and Stephen Chambers for careful reading of the manuscript.

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