Production, purification, and crystallization of human interleukin-1 β converting enzyme derived from an *Escherichia coli* expression system

JOHN J. MALINOWSKI,¹ BRUCE L. GRASBERGER,¹ GARY TRAKSHEL,¹
EDWARD E. HUSTON,¹ CARLA T. HELASZEK,² ANGELA M. SMALLWOOD,³
MARK A. ATOR,² TRACEY M. BANKS,¹ PATRICIA G. BRAKE,¹
RICHARD B. CICCARELLI,¹ BARRY N. JONES,¹ JAMES A. KOEHN,¹
DIANE KRATZ,¹ NICOLE LUNDBERG,¹ TRAVIS STAMS,³ BYRON RUBIN,³
RICHARD S. ALEXANDER,³ AND PANAYIOTIS E. STEVIS¹

¹ Department of Molecular and Cellular Biology,

² Department of Biochemistry, and

³ Biophysics and Computational Chemistry, Sterling Winthrop Pharmaceutical Research Division,
Collegeville, Pennsylvania 19426-0900

(RECEIVED May 30, 1995; ACCEPTED August 1, 1995)

Abstract

Interleukin-1 β converting enzyme (ICE) is a cysteine protease that catalyzes the conversion of the inactive precursor form of IL-1 β to an active mature form. The mature form of IL-1 β is involved in mediating inflammatory responses and in the progression of autoimmune diseases. We recently reported on the production of active human ICE in insect cells using the baculovirus expression system (Wang XM et al., 1994, *Gene* 145:273–277). Because the levels of expression achieved with this system were limiting for the purpose of performing detailed biochemical and biophysical studies, we examined the production of ICE in *Escherichia coli*. By using a *tac* promoter-based expression system and fusion to thioredoxin we were able to recover high levels of active ICE protein. The expressed protein, which was distributed between the soluble and insoluble fractions, was purified to homogeneity from both fractions using a combination of classical and affinity chromatography. Comparisons of ICE derived from both fractions indicated that they were comparable in their specific activities, subunit composition, and sensitivities to specific ICE inhibitors. The combined yields of ICE obtained from the soluble and insoluble fractions was close to 1 mg/L of induced culture. Recombinant human ICE was crystallized in the presence of a specific ICE inhibitor in a form suitable for X-ray crystallographic analysis. This readily available source of ICE will facilitate the further characterization of this novel and important protease.

Keywords: autoimmune disease; autoprocessing; crystallography; cysteine protease; heterodimer; ICE; protein refolding; thioredoxin

Interleukin-1 β converting enzyme is a cysteine protease (Black et al., 1989; Thornberry et al., 1992) with a strict requirement for Asp at the P1 position of substrates (Kostura et al., 1989; Howard et al., 1991). The cDNA for ICE was cloned from a

monocytic cell line and upon sequencing was found to code for a 404-amino acid protein (Cerretti et al., 1992; Thornberry et al., 1992). Characterization of native ICE purified from THP-1 cells (Kronheim et al., 1992; Thornberry et al., 1992; Miller et al., 1993) revealed that the 45-kDa (p45) precursor protein was processed to an active form consisting of 10-kDa (p10) and 20-kDa (p20) subunits (Thornberry et al., 1992). The conversion of the inactive p45 species to the p10/p20 heterodimer is thought to involve an interplay between an uncharacterized protease and autocatalysis (Wang et al., 1994).

The active form of ICE is found predominantly in monocytic cells, where it catalyzes the conversion of the inactive precursor form of interleukin-1 β (preIL-1 β) to the active mature form (Black et al., 1988; Kostura et al., 1989). In this capacity ICE is implicated in the initiation of the inflammatory responses that

Reprint requests to P.E. Stevis at his present address: Women's Health Research Institute, Wyeth Ayerst Research, 145 King of Prussia Rd., Radnor, Pennsylvania 19087.

Abbreviations: Ac, acetyl; AMC, aminomethylcoumarin; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, aldehyde; DCB, dichlorobenzoate; DITC, diisothiocyanate; DMSO, dimethyl sulfoxide; DO, dissolved oxygen; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid); ICE, interleukin-1 β converting enzyme; IL-1 β , interleukin-1 β ; IPTG, isopropyl- β -D-thiogalactopyranoside; PEG8000, polyethylene glycol with an average M_r of 8,000 Da; PMSF, phenylmethylsulfonyl fluoride; TRX, thioredoxin; V_r , column bed volume.

are mediated by IL-1 β . As such, it has become a target for the design of inhibitors aimed at moderating inflammatory and autoimmune diseases such as rheumatoid arthritis (Oppenheim et al., 1986; Dinarello, 1988). More recently, ICE was shown to be homologous to the *Caenorhabditis elegans ced-3* gene (Yuan et al., 1993), suggesting that ICE may play a role in apoptosis (Miura et al., 1993).

Expression of human ICE in a heterologous system has been reported for COS-7 cells (Cerretti et al., 1992; Thornberry et al., 1992) and insect cells (Wang et al., 1994). A truncated form of mouse ICE lacking the N-terminal 119 amino acids was expressed in *Escherichia coli* (Molineaux et al., 1993). Recently, the structure of ICE was solved (Walker et al., 1994; Wilson et al., 1994) and revealed that the active site spans the p10 and p20 subunits, which are associated as a tetrameric complex composed of two p10/p20 heterodimers.

In this paper, we provide a detailed report on the expression and recovery of active ICE from the soluble fraction and by refolding of insoluble ICE derived from induced cultures of *E. coli* transformed with a thioredoxin-ICE fusion plasmid construct. The expressed protein (rhICE) was purified to homogeneity from both fractions by using a combination of classical and affinity chromatography. Characterization of the biochemical properties of the rhICE derived from the two fractions indicated that they were comparable in their specific activities, inhibitor sensitivities, and subunit association. The rhICE derived from refolding was crystallized in the presence of an ICE inhibitor and was found to be suitable for X-ray crystallographic analysis.

Results

Construction of expression vector

The use of TRX as a fusion partner was reported to be effective in maintaining proteins in a soluble form and localizing them to the cell membrane-cell wall junctions of *E. coli* (Lunn & Pigiet, 1982; LaVallie et al., 1993). The construction of the TRX-based expression vector was performed in two parts. First, the thioredoxin gene (*trxA*) was isolated from *E. coli* genomic DNA via PCR using primers TX-1 and TX-2, which were based on the DNA sequence of *trxA* (Wallace & Kushner, 1984). The 3' primer (TX-2) eliminated the native stop codon, to allow for read-through into genes cloned downstream of *trxA*. The resulting fragment was digested with *Nde* I + *Bam*HI and cloned into the corresponding sites of pFLAG1, yielding pFTRXA. The plasmid was transformed into INV1 α for the expression of thioredoxin, in order to demonstrate functionality of the protein. Upon induction with IPTG, a considerable amount of TRX was produced and could be released from *E. coli* cells by osmotic shock (data not shown).

The second step in the construction entailed the cloning of the human ICE cDNA downstream of the *trxA* gene as an *Eco*RI–*Not*I fragment, yielding pFTRXA-ICE (Fig. 1). The rationale for this construction was to impart enhanced solubility and membrane localization of the ICE protein, in order to facilitate processing and reduce toxicity, respectively. The decision to use the full-length cDNA for ICE was based on observations with baculovirus-mediated expression of a truncated ICE derivative (deletion of N-terminal 119 amino acids) in Sf9 insect cells. In this system, where the expression levels of the truncated form were comparable to those obtained with full-length ICE (Wang

Fig. 1. A: Map of ICE expression vector pTRX-ICE. Details of the construction are given in the text. B: Protein and nucleotide sequence of the fusion junction between *trxA* and *hICE*. *Ptac*, tac promoter; *ApR*, ampicillin resistance; *lacI*, gene coding for lac repressor.

et al., 1994), processing to the p10/p20 heterodimer and preIL-1 β conversion activity were considerably reduced, suggesting an important role for the pro region in the efficient folding and processing of ICE in vivo (P.E. Stevis, unpubl. results).

Optimization of ICE expression

In order to determine the optimal conditions for the production of rhICE we examined the effect of induction time and temperature while monitoring soluble ICE activity via the AMC assay (described in the Materials and methods). Using an expression index (units of activity per liter of culture) as a means of standardizing yields, we found that an induction time of 2 h at 30 °C yielded the highest levels of soluble rhICE, with BL21(DE3) as the host. Western analysis of lysates derived from cells grown and induced under these conditions with antibodies specific for the p10 and p20 subunits of ICE indicated the presence of processed ICE (data not shown). Using these conditions the production of rhICE was scaled up to 9.9 L in a Braun fermentor. For the large-scale purification of ICE, cultures were induced at an A_{600} of 6 and grown at 30 °C for 2 h. The pH and DO were maintained at 7.1 and >20%, respectively. The typical yield for this induction regimen was 180 g of packed wet cells.

Isolation of ICE from soluble fraction

Attempts to affinity purify soluble rhICE (s-rhICE) from crude *E. coli* lysates using a peptide aldehyde column (Thornberry

et al., 1992) resulted in a number of contaminating proteins following elution with the free peptide aldehyde (data not shown). As a result it was necessary to perform a two-step purification prior to the use of the affinity column. We found that the inclusion of a (PEG) precipitation (8–25% PEG cut) and an ion-exchange step (Q-Sepharose Fast-Flow) were sufficient in this capacity. Both chromatographic procedures were effective in the enrichment of ICE activity (Table 1).

Application of the concentrated pooled Q-Sepharose material onto the peptide aldehyde affinity column yielded >95% pure ICE protein following elution. The purified soluble protein (s-rhICE) was composed predominantly of p10 and p20 subunit (Fig. 2A,B, lane 2). Native ICE, purified from a monocytic cell line (THP-1), is shown in lane 3 of Figure 2A and B.

Refolding and purification of ICE from inclusion bodies

Although the optimization of expression was aimed at increasing the levels of s-rhICE, we found that a considerable amount of rhICE (p10, p20, and partially processed rhICE) was still present in the insoluble fraction (data not shown). To determine if we could reclaim some of this protein the inclusion bodies were taken through a refolding regimen (described in the Materials and methods). Following refolding, the material was applied directly onto the affinity column and eluted as before. As shown in Figure 2A and B (lane 1), refolding of the insoluble material yielded rhICE (r-rhICE) which bound to the affinity column and could be recovered by elution with the free peptide aldehyde. With the refolded material the majority of the eluted protein corresponded to p10 and p20 subunits. Variant forms of p10 and p20 corresponding to incomplete or aberrant processing were also recovered. The underprocessed forms were effectively removed by using Sepharose-immobilized antibodies generated against synthetic peptides corresponding to the interdomain regions of proICE (Ser¹⁰⁴–Asp¹¹⁹ and Ser²⁹⁸–Asp³¹⁶) (data not shown).

Characterization of purified protein

N-terminal sequencing of the purified protein derived from the soluble and insoluble fractions was performed to determine whether processing in *E. coli* was occurring at the same sites as those observed with ICE derived from THP-1 cells (Thornberry et al., 1992; Miller et al., 1993). The majority of r-rhICE (65%)

Fig. 2. SDS-PAGE analysis of purified rhICE derived from the soluble and insoluble fractions. **A:** Silver-stained 16% Tris-glycine gel. **B:** Western blot with Abp10 and Abp20. Samples shown are: lane 1, refolded rhICE; lane 2, soluble rhICE; lane 3, native ICE, purified from THP-1 cells; M, Bio-Rad low MW markers. Positions of the p10 and p20 subunits are indicated by the arrows.

Table 1. Purification of soluble ICE from BL21(DE3)/pFTRXA-ICE^a

Fraction	Total protein ^b (mg)	Specific activity (units/μg)	Recovery (%)	Fold purification
Lysate	17,163	1.2	100	1.0
8–25% PEG	1,234	2.8	17	2.4
Q-Sepharose	33	55	9.2	47.5
Concentrate	36	86	15.7	74.0

^a Starting cell culture volume was 3.5 L.

^b Protein concentration was determined by the Bradford (1967) method.

was found to be processed at the expected sites for p10 and p20, yielding Ala³¹⁷ and Asn¹²⁰ for the N-termini, respectively. Additional sequences corresponding to p24 (Asp¹⁰³) and an over-processed form (Ala¹²²) were also detected for the p20 subunit. The predominant form of the p20 subunit of s-rhICE was found to be processed at an alternate site (Met¹²³), whereas the p10 was correctly processed.

The specific activities (Table 2) and k_{obs}/I values for specific ICE inhibitors (Dolle et al., 1994) (Table 3) were shown to be comparable for r-rhICE and s-rhICE. The deletion of four N-terminal amino acids from the p20 of s-rhICE appears to have no effect on the enzymological characteristics of the protease.

Enzymological comparison of the rhICE to the native protease is complicated by the difficulty in obtaining significant quantities of homogeneous native ICE. The specific activity of the

Table 2. Comparison of soluble and refolded ICE

	Refolded	Soluble
Specific activity ^a (Fu ^b /min/ng)	2.0	1.9
K_m (Suc-YVAD-AMC) ^b (μ M)	9.5	6.4

^a Activity determined as described in the Materials and methods.

^b Fluorescence units.

rhICE is approximately fourfold higher than that of ICE purified to homogeneity from lysates of THP-1 cells (data not shown). This discrepancy is most likely due to loss of activity of the native protease during its isolation and underscores the importance of having a facile recombinant source of the enzyme. Although a direct comparison of inhibitors with homogeneous native and rhICE has not been accomplished, the k_{obs}/I values obtained with partially purified ICE derived from the baculovirus expression system (Wang et al., 1994) are statistically indistinguishable from those provided in Table 3 (data not shown). The baculovirus-expressed protease has been demonstrated previously to display activity with inhibitors comparable to that of THP-1-derived ICE (C.T. Helaszek and M.A. Ator, unpubl. obs.).

Crystallization of rhICE

Crystals of the ICE-inhibitor complex were obtained and had the shape of tetragonal bipyramids with the widest dimension being 0.8 mm (Fig. 3). Two data sets for the enzyme-inhibitor complex were collected and diffraction intensities were measured to a limiting resolution of 2.9 Å. Raw data frames were processed using the MOSFLM package (Leslie, 1992), and replicate and symmetry-related structure factors were merged using PROTEIN (Steigemann, 1992). The native R_{merge} was 7.7% on intensity and the data set was over 90% complete.

Given the unit cell parameters derived from precession photos and the systematic absences along h and l , it was unambiguous that the space group was either $P4_12_12$ or $P4_32_12$. Although the unit cell parameters that we derived are identical to those reported (Walker et al., 1994; Wilson et al., 1994), our crystals were grown in an ammonium sulfate solution, whereas the two

Fig. 3. Crystal of ICE. Crystal dimensions are approximately 0.8 mm $\times$ 0.3 mm $\times$ 0.3 mm.

others were grown in a PEG solution. Subtle differences may exist between the structures solved in these two media.

Discussion

Previous reports of heterologous expression of human ICE in COS-7 cells (Cerretti et al., 1992; Thornberry et al., 1992) and in insect cells (Wang et al., 1994) indicated that the expression levels would not generate sufficient material for detailed biochemical studies. A report on the expression of a truncated form of mouse ICE in *E. coli* (Molineaux et al., 1993) did not indicate the final yields of purified ICE. We have demonstrated that the *E. coli* expression system is effective in generating sufficient quantities of purified ICE for further detailed studies. The calculated combined yields of purified ICE from the soluble and insoluble fractions were greater than 1 mg/L. There was some heterogeneity observed in the purified fractions that were shown to correspond to partially processed and overprocessed forms of p10 and p20. The inclusion of an additional antibody capture purification step was effective in removing the underprocessed species. Their presence in the eluted material indicates that they constitute an active heterodimer, which is a prerequisite for binding to the affinity column.

Protein sequencing of the individual subunits from the refolded material indicated that processing in *E. coli* was occurring in a similar fashion to what is observed with THP-1 cells. This is not surprising because the processing is believed to be autocatalytic (Thornberry et al., 1992; Wang et al., 1994). On the other hand, the p20 subunit derived from the soluble extract of *E. coli* lysates was found to be processed at an alternate site

Table 3. k_{obs}/I of rhICE for selected inhibitors

	k_{obs}/I ($M^{-1} s^{-1}$) ^a	
	Refolded	Soluble
Z-Asp-DCB	1,940	2,000
Z-Val-Asp-DCB	13,100	13,200
Z-Val-Ala-Asp-DCB	186,000	192,000

^a Values presented here differ from those given in Dolle et al. (1994) due to differences in temperature (ambient versus 37 °C) and reaction conditions, which were required to improve the stability of ICE purified to homogeneity. Standard assay conditions included 10 mM HEPES, pH 7.5, with 25% glycerol (v/v); 0.005% CHAPS (w/v); 3.3 mM DTT; and 10 μ M Suc-YVAD-AMC. Reactions were run at ambient temperature for 30 min. Values listed are corrected for $1 + [S]/K_m$.

(between Ala¹²² and Met¹²³). Because this does not correspond to a preferred cleavage site for ICE (Kostura et al., 1989; Howard et al., 1991), it was most likely a result of an *E. coli* protease. The ability of this species to bind to the affinity column and an unchanged specific activity, relative to the nontruncated form, suggests that the truncation is inconsequential.

There are some discrepancies concerning the importance of the pro region of ICE in mediating the folding and processing of ICE. Although active ICE was obtained in COS-cells (Cerretti et al., 1992) and *E. coli* (Molineaux et al., 1993) using a truncated ICE cDNA, there was no assessment of efficiency via comparison to the full-length molecule. In fact, expression of a similar truncated species in insect cells produced partially processed ICE, which had less than 10% of the activity of ICE derived from the full-length molecule, when normalized for p20 and p10 content (P.E.S., unpubl. results). This observation prompted us to use the full-length proICE for expression in *E. coli*.

Although the expression vector was constructed to produce a fusion between TRX and ICE, there was no fusion protein observed on Westerns. In fact, large amounts of free TRX protein were observed on Coomassie-stained gels (not shown). This suggests that removal of TRX from ICE occurred soon after the synthesis of the fusion protein. Whether this proteolysis is mediated by ICE itself or an *E. coli* protease is not certain.

There was no overt toxicity associated with the high levels of soluble ICE activity in *E. coli*. This supports the notion that ICE recognizes a limited set of substrates (Kostura et al., 1989). However, the expression of ICE in insect cells (Wang et al., 1994) was observed to enhance the lysis of the transfected cells. This might be attributed to the ability of ICE to trigger apoptosis.

Recombinant ICE derived from refolding was crystallized in the presence of an ICE inhibitor under unique conditions. The crystals are suitable for X-ray analysis and may prove useful in the study of enzyme-inhibitor complexes.

In summary, we have described a facile expression-purification system for obtaining rhICE from soluble extracts and by refolding of insoluble material of hICE expressed in an *E. coli* system. ICE derived from these sources demonstrated similar enzymological properties. This readily available source of active ICE will facilitate the analysis of its role in mediating inflammation and apoptosis.

Materials and methods

Chemicals

Media components for the cultivation of *E. coli* were purchased from Difco Laboratories and Sigma Chemical. Restriction endonucleases and modifying enzymes were purchased from BRL, Boehringer Mannheim, or Promega. Reagents for PCR were purchased from Perkin Elmer Cetus or Boehringer Mannheim. Specialty chemicals and protease inhibitors were purchased from Sigma.

E. coli strains, plasmids, and oligonucleotides

The *E. coli* strains used for expression and subcloning experiments were BL21(DE3) (Novagen) and INV α (Invitrogen). Plasmid pFLAG1 (IBI) was used for the construction of the ICE expression vector and contains the *tac* promoter and a plasmid-

borne *lacI* gene for tight regulation of transcription. Oligonucleotides were acquired from Biosynthesis Inc.

Construction of expression vector

The *trxA* gene (Wallace & Kushner, 1984) was isolated from *E. coli* genomic DNA using the PCR primers TX-1 (5'-CGCTCTAGAGCATATGTTACACCAACAACGAAACCAACA CGC-3') and TX-2 (5'-GGAAGATCTCACGTGGAATTCCG ATCCCCGCCAGGTTAGCGTCGAGGAAC-3'). The TX-2 primer eliminated the native stop codon to allow for readthrough into the adjoining gene. The resulting fragment was cloned into pFLAG1 (IBI) as an *Nde*I-*Xba*I fragment, generating pFTRXA (not shown).

The cDNA for proICE, derived from FL1bp/CAV (Cerretti et al., 1992), was subcloned into pSP73 (Promega) as an *Asp*718-*Bgl*II fragment, generating pRC391 (not shown). The cDNA for proICE was amplified with primers IL-1b-2 (5'-GCGAAGCTT CGAGATCTATTTTAATGTCCTGGGAAGAG-3') and IL-1b-3 (5'-GCGAATTCATGGCCGACAAGGTCCTGAAG-3') using pRC391 as the template. The resulting fragment was digested with *Bgl*II + *Eco*R I and cloned into pGEM11 (Promega), which was digested with *Eco*R I + *Bam*H I, generating pGEM11-proICE (not shown).

The construction of the final expression vector was accomplished by isolating the proICE gene from pGEM11-proICE as an *Eco*R I-*Not* I fragment and cloning it into the *Eco*R I-*Not* I sites of pFTRXA, yielding pFTRXA-ICE. The map of the expression vector and the sequence of the fusion junction between *trxA* and *hICE* is shown in Figure 1A and B, respectively.

Fermentation and processing of induced cultures

Overnight cultures of BL21(DE3)[pFTRXA-ICE] were grown in LB++, which consisted of (per L): 15 g tryptone, 10 g yeast extract, 40 g glycerol, 4 g Na citrate, 1 g NH₄Cl, 0.5 g K₂SO₄, 5 g NaCl, 10 mM NaH₂PO₄, 10 mM MgSO₄ and 1 × trace metals (100 × = 6 g CaCl₂ · 2H₂O, 6 g FeSO₄ · 2H₂O, 1.15 g MnCl₂ · 4H₂O, 0.8 g CoCl₂ · 2H₂O, 0.7 g ZnSO₄ · 2H₂O, 0.3 g CuCl₂ · 2H₂O, 0.02 g H₃BO₃, 0.25 g [NH₄]₆Mo₇O₂₄ · 2H₂O, 5 g EDTA, with the volume brought to 1 L and acidified with 1 M HCl until dissolved) in shaking flasks at 30 °C. A 1-L seed culture (grown overnight in LB broth) was used to inoculate a 9.9-L fermentation in LB++, with pH and O₂ controlled at 7.1 and >20%, respectively. The fermentations were grown at 37 °C to an A₆₀₀ of 6, the temperature was then lowered to 30 °C, and induction was initiated by the addition of 1 mM IPTG. The cultures were induced for 2 h, harvested by centrifugation, washed once with PBS, and stored at -80 °C until further use.

Cells were thawed on ice then resuspended in an equal volume of lysis buffer (25 mM HEPES, pH 7.5, 10% glycerol, 0.2 mM PMSF, 4 U/mL DNase, 2.5 mM MgCl₂, 0.7 μg/mL pepstatin, 1 μg/mL aprotinin) and broken using a French press (two passages at 16,000 psi at 4 °C). The lysate was brought to 4 mM EDTA, 1% CHAPS, and centrifuged 30,000 × g for 60 min. The pellets were re-extracted with lysis buffer (containing 1 mM EDTA, 1% CHAPS, and without DNase) and centrifuged a second time (30,000 × g for 60 min). The supernatants were combined and used for purification of the soluble rhICE, as described below. The pellets were extracted with 2 volumes of 5 M guanidine-HCl in the presence of 100 mM DTT, centri-

fused at $30,000 \times g$ for 45 min, and the soluble fraction (guanidine extract) was used for refolding, as described below.

Partial purification of ICE

Stock 50% (w/v) PEG8000 was slowly added to the soluble lysate to reach a concentration of 8% (w/v). After 30 min of mixing, the resulting slurry was centrifuged at $40,000 \times g$ for 30 min. The supernatant layer was decanted from the pellet and further treated with stock 50% (w/v) PEG8000 to reach a final concentration of 25% (w/v). After 30 min of mixing, the resulting slurry was centrifuged at $150,000 \times g$ for 1 h. The supernatant was discarded and the pellet was dissolved in a minimal amount of a buffer that contained 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 25% (v/v) glycerol, 0.1% (v/v) Tween-20, 1 mM PMSF, 1 μ M pepstatin A, 1 μ M leupeptin, 0.25 μ M aprotinin, 5 mM DTT. Following clarification by centrifugation at $150,000 \times g$ for 30 min the 8–25% PEG8000 fraction was applied to a Q-Sepharose Fast-Flow column that was previously equilibrated with 5 V_i of an equilibration buffer (20 mM Tris-HCl, pH 8.1, 0.1 mM EDTA, 25% [v/v] glycerol, 2 mM DTT). After charging the column with sample to reach a protein load of approximately 7.6 mg protein/mL media, the column was washed with 2 V_i of the equilibration buffer, which supported a gradient of NaCl from 0 to 10 mM. Afterward, a complex gradient of NaCl was used for elution: first, a linear NaCl gradient with a slope of 20 mM/ V_i was used from 10 to 110 mM, and then, from 110 to 250 mM NaCl, a concave gradient was used which had a (20 M/ V_i)/ V_i acceleration. The flow rate and fraction size were maintained at 2 V_i /h and 0.1 V_i /fraction, respectively, throughout the chromatographic process.

The fractions containing active ICE were combined, supplemented with DTT to increase its concentration from 2 to 5 mM, and adjusted to pH 7.2 by the dropwise addition of 1 M phosphoric acid. Afterward, the ICE activity was concentrated by ultrafiltration over an Amicon YM3 membrane to reach a minimal protein concentration of 0.2 mg/mL.

Refolding

The guanidine extract was diluted 50-fold in HSC buffer (25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS), centrifuged, and concentrated to 200 mL using a tangential flow concentrator. The sample was then diafiltered with 1 L of HSCD (HSC + 1 mM DTT), concentrated to >2 mg/mL protein, and used for affinity purification.

Affinity purification of ICE

Affinity purification was performed essentially as described in Thornberry et al. (1992). The tetrapeptide Ac-N-Tyr-Val-Lys-Asp-semicarbazone was coupled through the lysine side-chain to epoxy-activated agarose (Sigma). The coupling reaction proceeded for 46 h at room temperature in a buffer of 0.3 M Na_2CO_3 (pH 11). After coupling of the tetrapeptide ligand, remaining reactive sites on the beads were blocked by treatment with 6% ethanolamine (pH 9). The semicarbazone protecting group was subsequently removed by treatment with a solution of methanol/acetic acid/formaldehyde (5/1/1), generating the immobilized tetrapeptide aldehyde Ac-Tyr-Val-Lys-Asp-CHO.

The pooled rhICE from the Q-Sepharose Fast Flow column or the refolded rhICE was mixed with the immobilized inhibitor for 16 h at 4 °C. The beads were packed into a column and washed at room temperature with 100 column volumes of HSCD, then washed with 10 column volumes of HSC buffer. One column volume of 250 μ M AcN-Tyr-Val-Ala-Asp-CHO in HSC buffer was allowed to flow into the column, and it was capped. After standing for 24–40 h at room temperature, the column was eluted with two volumes of HSC buffer. The collected eluate contained purified ICE complexed to the Ac-Tyr-Val-Ala-Asp-CHO inhibitor.

Purified ICE was reactivated by incubation with hydroxylamine (1 M) and oxidized glutathione (100 mM) for 4 h at room temperature, followed by dialysis at 4 °C against 200 volumes of HSC buffer containing 1 mM oxidized glutathione. Active ICE was regenerated by a brief treatment with excess DTT just prior to assay.

Protein analysis

For the detection of ICE by Western blot, antibodies raised against peptides corresponding to amino acids 120–131 (Abp20) and 365–379 (Abp10) of the proICE sequence (Cerretti et al., 1992) were used. The antibodies were affinity purified by passing the serum through a column containing the respective peptides coupled to Sepharose 4B beads. Samples for amino acid sequence analysis were run on SDS-PAGE (Laemmli, 1970), and either electroblotted (Matsudaira, 1987) to Transblot membrane (BioRad Laboratories) or diffusion-eluted (Kurth & Stoffel, 1990) and covalently attached to DITC-derivatized polyvinylidene difluoride membranes (Millipore Corporation). Analyses were done on a MilliGen ProSequencer 6600.

AMC assay

Standard assays contained 10 mM HEPES, pH 7.5, 25% glycerol, 3.3 mM DTT, 0.005% CHAPS, 10 μ M substrate (Suc-Tyr-Val-Ala-Asp-AMC; Bachem). All reactions were initiated by the addition of enzyme and were run in a 30- μ L volume in a 96-well polystyrene microtiter plate at room temperature. For K_m determinations, reactions with various concentrations of substrate were followed for 60 min. Progress curves were linear throughout the reaction. Reactions with varying concentrations of inhibitor included 10% DMSO and were run for 30 min at room temperature. Control curves included 10% DMSO and remained linear throughout the reaction. Kinetic data were obtained as described (Graybill et al., 1994).

Crystallization

A sample of ICE containing a reversible aldehyde inhibitor (Ac-Tyr-Val-Ala-Asp-CHO; Thornberry et al., 1992) at an initial concentration of approximately 0.15 mg/mL was concentrated to a final A_{280} of 4.55 (approximately 5 mg/mL). The concentrated protein solution also contained 25 mM HEPES, pH 7.5, 0.1% CHAPS, and 400 μ M inhibitor. To this solution was added an equal volume of a 50 mM DTT and 20% saturated ammonium sulfate solution. This protein solution was then filtered using a 0.22- μ m Millipore Ultrafree-MC microcentrifuge filter. Hanging drop vapor diffusion experiments were set up by placing 15- μ L drops of the protein solution over 1-mL well solutions

containing 30–50% saturated ammonium sulfate and 25 mM HEPES, pH 7.5. The plate was then moved to 4 °C where, after 1 week, crystals appeared. SDS-PAGE analysis of a dissolved ICE crystal clearly showed the presence of both the 20-kDa and the 10-kDa subunits in the crystal (not shown).

Crystals grown at 4 °C were transferred to room temperature in the original hanging drop. Typically, a crystal of the ICE-inhibitor complex was mounted and sealed in a glass capillary with a small portion of mother liquor. A MAR image plate detector was used for X-ray data acquisition. A Rigaku RU-200 rotating anode X-ray generator operating at 50 kV/100 mA with Charles Supper double X-ray focusing mirrors supplied Cu-K α radiation. All data were collected at room temperature; the crystal-to-detector distance was set at 20 cm. Data frames of one degree rotation about the spindle axis, ϕ , were collected, with exposure times of 20–30 min/frame, for total angular rotation ranges about ϕ of at least 50° per data set.

Acknowledgments

We thank Marian Kelley for generating and affinity purifying antibodies for ICE; T. Graybill for synthesis of the peptide aldehydes; and C.V.C. Prasad, D. Hoyer, and R. E. Dolle for synthesis of ICE inhibitors.

References

- Black RA, Kronheim SR, Cantrell M, Deeley MC, March CJ, Prickett KS, Wignall J, Conlon PJ, Cosman D, Hopp TP, Mochizuki DY. 1988. Generation of biologically active interleukin-1 β by proteolytic cleavage of the inactive precursor. *J Biol Chem* 263:9437–9442.
- Black RA, Kronheim SR, Sleath PR. 1989. Activation of interleukin-1 β by a co-induced protease. *FEBS Lett* 247:386–390.
- Bradford MM. 1967. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA, Huebner K, Black RA. 1992. Molecular cloning of the interleukin-1 β converting enzyme. *Science* 256:97–100.
- Dinareello CA. 1988. Biology of interleukin 1. *FASEB J* 2:108–115.
- Dolle RE, Hoyer D, Prasad CVS, Schmidt SS, Helaszek CT, Miller RE, Ator MA. 1994. P1 aspartate-based peptide α -(2,6-dichlorobenzoyl)oxymethyl ketones as potent time-dependent inhibitors of interleukin-1 β -converting enzyme. *J Med Chem* 37:563–564.
- Graybill TL, Dolle RE, Helaszek CT, Miller RE, Ator MA. 1994. Preparation and evaluation of peptidic aspartyl hemiacetals as reversible inhibitors of interleukin-1 β converting enzyme (ICE). *Int J Pept Protein Res* 44:173–182.
- Howard AD, Kostura MJ, Thornberry N, Ding GJF, Limjuco G, Weidner J, Salley JP, Hogquist KA, Chaplin DD, Mumford RA, Schmidt JA, Tocci MJ. 1991. IL-1-converting enzyme requires aspartic acid residues for processing of the IL-1 β precursor at two distinct sites and does not cleave 31-kDa IL-1 α . *J Immunol* 147:2964–2969.
- Kostura MJ, Tocci MJ, Limjuco G, Chin J, Cameron P, Hillman AG, Chartrain NA, Schmidt JA. 1989. Identification of a monocyte specific pre-interleukin 1 β convertase activity. *Proc Natl Acad Sci USA* 86:5227–5231.
- Kronheim SR, Mumma A, Greenstreet T, Glackin PJ, Van Ness K, March CJ, Black RA. 1992. Purification of interleukin-1 β converting enzyme, the protease that cleaves the interleukin-1 β precursor. *Arch Biochem Biophys* 296:698–703.
- Kurth J, Stoffel W. 1990. A facile method for the isolation and preparation of proteins and peptides for sequence analysis in the picomolar range. *Biol Chem Hoppe-Seyler* 371:675–688.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- LaVallie ER, DiBlasio EA, Kovacic S, Grant KL, Schendel PF, McCoy JM. 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Bio/Technology* 11:187–193.
- Leslie AGW. 1992. Recent changes to the MOSFLM package for processing film and image plate data. CCP4 and ESF-EACMB. *Newsletter on Protein Crystallography* 26.
- Lunn CA, Pigiet VP. 1982. Localization of thioredoxin from *Escherichia coli* in an osmotically sensitive compartment. *J Biol Chem* 257:11424–11430.
- Matsudaira P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 262:10035–10038.
- Miller DK, Ayala JM, Egger LA, Raju SM, Yamin TT, Ding GJF, Gaffney EP, Howard AD, Palyha OC, Rolando AM, Salley JP, Thornberry NA, Weidner JR, Williams JH, Chapman KT, Jackson J, Kostura MJ, Limjuco G, Molineaux SM, Mumford RA, Calaycay JR. 1993. Purification and characterization of active human interleukin-1 β -converting enzyme from THP.1 monocytic cells. *J Biol Chem* 268:18062–18069.
- Miura M, Zhu H, Rotello R, Hartwig EA, Yuan J. 1993. Induction of apoptosis in fibroblasts by IL-1 β -converting enzyme, a mammalian homologue of the *C. elegans* cell death gene *ced-3*. *Cell* 75:653–660.
- Molineaux SM, Casano FJ, Rolando AM, Peterson EP, Limjuco G, Chin J, Griffin PR, Calaycay JR, Ding GJF, Yamin TT, Palyha OC, Luell S, Fletcher D, Miller DK, Howard AD, Thornberry NA, Kostura MJ. 1993. Interleukin 1 β (IL-1 β) processing in murine macrophages requires a structurally conserved homologue of human IL-1 β converting enzyme. *Proc Natl Acad Sci USA* 90:1809–1813.
- Oppenheim JJ, Kovacs EJ, Matsushima K, Darum SK. 1986. There is more than one interleukin 1. *Immunol Today* 7:45–56.
- Steigemann W. 1992. *Protein: A program system for the crystal structure analysis of proteins, version 3.1. User's guide*. Max Planck Institut fuer Biochemie.
- Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding GJF, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin TT, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA, Tocci MJ. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* 356:768–774.
- Walker NPC, Talanian RV, Brady KD, Dang LC, Bump NJ, Ferenz CR, Franklin S, Ghayur T, Hackett MC, Hammill LD, Herzog L, Hugunin M, Houy W, Mankovich JA, McGuiness L, Orlewicz E, Paskind M, Pratt CA, Reis P, Summani A, Terranova M, Welch JP, Xiong L, Moller A, Tracey DE, Kamen R, Wong WW. 1994. Crystal structure of the cysteine protease interleukin-1 β -converting enzyme: A (p20/p10) $_2$ homodimer. *Cell* 78:343–352.
- Wallace BJ, Kushner SR. 1984. Genetic and physical analysis of the thioredoxin (*trxA*) gene of *Escherichia coli* K-12. *Gene* 32:399–408.
- Wang XM, Helaszek CT, Winter LA, Lirette RP, Dixon DC, Ciccarelli RB, Kelley MM, Malinowski JJ, Simmons SJ, Huston EE, Koehn JA, Kratz D, Bruckner RC, Graybill T, Ator MA, Lehr RV, Stevis PE. 1994. Production of active interleukin-1 β -converting enzyme in a baculovirus expression system. *Gene* 145:273–277.
- Wilson KP, Black JF, Thomson JA, Kim EE, Griffith JP, Navia MA, Murcko MA, Chambers SP, Aldape RA, Raybuck SA, Livingston DJ. 1994. Structure and mechanism of interleukin-1 β converting enzyme. *Nature* 370:270–275.
- Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell* 75:641–652.