Preparation of an Autolysis-Resistant Interleukin-1β Converting Enzyme Mutant

Luan C. Dang,[‡] Robert V. Talanian,[‡] David Banach,[‡] Maria C. Hackett,[‡] John L. Gilmore,[§] Sheryl J. Hays,[§] John A. Mankovich,[‡] and Kenneth D. Brady*,[‡]

BASF Bioresearch Corporation, 100 Research Drive, Worcester, Massachusetts 01605, and Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105

Received July 19, 1996[⊗]

ABSTRACT: We describe the expression, purification, and characterization of human interleukin-1β converting enzyme (ICE) containing an affinity tag and modified to resist autoproteolysis. The point mutation Asp381 to Glu was added to eliminate the major site of autolytic degradation while maintaining catalytic activity, and an N-terminal polyhistidine tag was added in place of the ICE pro-region to facilitate purification. N-His (D381E) ICE was expressed in *Escherichia coli* and purified by nickel-chelating Sepharose and size-exclusion chromatography (SEC). The enzyme was stabilized greater than 80-fold against autolytic degradation relative to wild-type N-His ICE. SDS−PAGE analysis with silver-staining revealed no impurities, and 85% of the protein was catalytically active as determined by titration with a novel titrant, PD 163594 (3-[2-(2-benzyloxycarbonylamino-3-methylbutyrylamino)propionylamino]-4-oxo-5-(2-oxo-2*H*-chromen-7-yloxypentanoic acid). An oxidized adduct of ICE with glutathione, formed by disulfide rearrangement with oxidized glutathione to inhibit and stabilize the enzyme during purification, was rapidly reduced upon exposure to 5 mM DTT. One mole of glutathione was released per mole of active enzyme. Of the nine cysteines in ICE, eight were present in their reduced form in the glutathione adduct. N-His (D381E) ICE cleaved Ac-YVAD-Amc with the Michaelis−Menten parameters $K_M = 14$ μM and $k_{cat} = 0.7$ s^{-1}, values essentially identical to those reported for enzyme from natural sources.

IL-1β converting enzyme (ICE)[1] is required for the proteolytic activation of the pro-inflammatory cytokine interleukin-1β (Kuida et al., 1995; Li et al., 1995; Thornberry et al., 1992) and represents a promising target for anti-inflammatory drug design (Miller et al., 1993b; Thornberry et al., 1995). A growing body of evidence further implicates ICE and/or its human homologs in programmed cell death, or apoptosis (Martin & Green, 1995; Takahashi et al., 1995; Thornberry & Molineaux, 1995; White, 1996), suggesting additional potential therapeutic applications of ICE or ICE homolog inhibitory drugs.

ICE was first purified from human monocytic THP.1 cells and has been characterized enzymatically (Kronheim et al., 1992; Miller et al., 1993a; Thornberry et al., 1992). Natural sources, however, provide insufficient enzyme for crystallization efforts (Walker et al., 1994; Wilson et al., 1994) or other structural studies requiring large quantities of protein. To overcome this limitation we developed a method for preparing ICE in milligram quantities by refolding proteins corresponding to the p10 and p20 subunits of mature ICE individually expressed in *Escherichia coli* (Talanian et al., 1996; Walker et al., 1994). Refolded ICE (rfICE) complexed with an irreversible tetrapeptide inhibitor yielded crystals which allowed us to solve the structure of ICE to 2.5 Å resolution (Walker et al., 1994). The rfICE structure was essentially identical to that obtained from precursor ICE which had been refolded and autoproteolytically processed (Wilson et al., 1994). rfICE was susceptible to at least two modes of inactivation. At low concentration, the rate and the extent of activity loss were dependent on enzyme concentration, substrate concentration, and pH. This process of decay was understood as a rapid and reversible dissociation of the homodimer (Talanian et al., 1996). At high enzyme concentration, activity loss correlated with proteolytic degradation of the p10 subunit to a p7 fragment.

To improve the stability of ICE to autoproteolysis in studies requiring high enzyme concentrations or long incubations, we prepared an ICE mutant with a conservative substitution at its dominant autolytic site. An N-terminal polyhistidine tag was added to facilitate enzyme purification. The resulting enzyme was easily purified and displayed sufficient stability for detailed studies of ICE oligomerization and stability not possible with rfICE. We describe here expression and purification of N-His (D381E) ICE and an analysis of its conformational purity using a novel active site titrant.

MATERIALS AND METHODS

Materials. Buffer salts, glutathione, oxidized glutathione, 7-hydroxycoumarin, DMSO, 2-mercaptoethanol, trifluoroacetic acid, dimethylformamide, PMSF, leupeptin, pepstatin,

* Corresponding author. Tel: (508) 849-2621. FAX: (508) 754-7784. E-mail: brady@biovax.dnet.basf-ag.de.

[‡] BASF Bioresearch Corporation.

[§] Parke-Davis Pharmaceutical Research.

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: AAA, amino acid analysis; Amc, 7-amino-4-methylcoumarin; βME, 2-mercaptoethanol; BSA, bovine serum albumin; DBD-F, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HBr, hydrobromic acid; HEPES, hydroxyethylpiperazineethane sulfonic acid; HOAc, acetic acid; HPLC, high-performance liquid chromatography; ICE, interleukin-1β converting enzyme; NEM, *N*-ethylmaleimide; PD 163594, 3-[2-(2-benzyloxycarbonylamino-3-methylbutyrylamino)propionylamino]-4-oxo-5-(2-oxo-2*H*-chromen-7-yloxy-pentanoic acid; PMSF, α-toluenesulfonyl fluoride; rfICE, refolded ICE; SEC, size-exclusion chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

S0006-2960(96)01777-1 CCC: $12.00

and glycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Ac-L-Tyr-L-Val-L-Ala-L-Asp-Amc was purchased from Bachem Bioscience (King of Prussia, PA). 7-Amino-4-methylcoumarin (Amc) was purchased from Aldrich Chemical Co. (St. Louis, MO). DTT was purchased from Gibco, BRL (Gaithersburg, MD). 4-(*N*,*N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole was purchased from TCI America (Portland, OR).

Cloning and Expression of N-His (D381) ICE. The D381E mutant was first constructed as the preprocessed p10, ICE 317−404, and then subcloned into the wild-type N-His ICE 120−404. The mutations were generated by a recombination-based site-specific PCR mutagenesis protocol (Jones & Howard, 1991). Two fragments were created that together represented the ICE 317−404 gene with a 28 base pair overlapping sequence where the two can be linked by homologous recombination in *E. coli* and restriction sites for expression vector cloning.

For both fragments, the template was a clone of ICE 317−404 with a Met start codon inserted before Ala317 and with amino terminal codons modified to reflect preferred codon usage of *E. coli*. For fragment 1, the 5′ primer (5′-GGG GAA TTC ATG GCT ATC AAA AAA GCT CAC ATC GAA AAA GAC TTC ATC GCT TTC TGC-3′) complements the ICE 317−404 amino terminus and contains an *Eco*RI site. The 3′ primer (5′-TTC TGG CTG CTC AAA TGA AAA ACG AAC CTT GCG GAA AAT TTC-3′) encodes ICE residues 368−381 with the point mutation D381E and a silent mutation that eliminates a *Taq*I site. PCR amplification using these primers and the ICE 317−404 containing plasmid as template gave a fragment containing the open reading frame of ICE 317−381. For fragment 2, the 5′ primer (5′-GGT TCG TTT TTC ATT TGA GCA GCC AGA AGG TAG AGC GCA GAT G-3′) encodes ICE amino acids 373−386 and contains the mutations described above. The 3′ primer (5′-CCC CAC TAG TCC TCT ATT AAT GTC CTG GGA AGA GG-3′) encodes the amino acid regions ICE 400−404 followed by a stop codon and a *Spe*I site for cloning. PCR amplification using these primers and the plasmid containing ICE 317−404 as template gave a fragment containing the open reading frame of ICE 373−404.

The PCR fragments were ligated to the linearized expression vector pJAM-4, a pBluescript II KS(+) (Stratagene, La Jolla, CA) derivative containing the lambda left promoter (p_L) followed by a polylinker starting with an *Eco*RI site. The ligation products were transformed into competent *E. coli* MV1190 (Δ(*lac-pro*AB), *thi*, *sup*E, Δ(*srl-rec*A) 306::Tn10(Tet^r) [F′:*tra*D36, *pro*AB, *lac*I^q *lac*ZΔM15]) (Bir-Rad, Hercules, CA) carrying a kanamycin-resistant pSC101 derivative containing the temperature sensitive lambda repressor, cI^{857}. Positive clones were identified by restriction site analysis and verified by DNA sequencing.

pJAM-4 His-ICE 120−404 D381E was cloned by replacing the restriction fragment containing the wild-type sequence with that from the pJAM-4 ICE 317−404 D381E mutant. His-ICE 120−404 was from a clone of pJAM-4 His-ICE 120−404. Digestion of both clones, pJAM-4 His-ICE 120−404 and pJAM-4 ICE 317−404 D381E, with *Nde*I and *Cla*I resulted in the liberation of a 193 base pair fragment containing the region of the ICE gene spanning Asp381 or Glu381, respectively.

Fermentations were performed as described (Talanian et al., 1996), except that protein synthesis was induced at 40 °C and cells were harvested after 90 min.

ICE Activity Assay. Enzyme was diluted to 400 μL with HGDE buffer (100 mM HEPES, pH 7.5, 20% glycerol, 5 mM DTT, 0.5 mM EDTA) containing the substrate Ac-YVAD-Amc (15 μM). Activity was measured by following the liberation of 7-amino-4-methylcoumarin (Amc) at 380 nm excitation and 460 nm emission wavelengths (Zimmerman et al., 1976). A standard curve was generated using Amc stocks in assay buffer.

Purification of N-His (D381E) ICE. Frozen cell paste from a 750 mL fermentation (50 g wet weight) was thawed and resuspended in 200 mL of lysis buffer (100 mM HEPES, pH 7.5, 10% glycerol, 0.1 M NaCl, 1 mM PMSF, 2 mM leupeptin, 2 mM pepstatin). Cells were passed through a microfluidizer twice at 4 °C. The lysate was centrifuged at 30000*g* for 30 min at 4 °C. The supernatant was diluted 1:1 (v/v) with Buffer A (50 mM HEPES, pH 7.5, 10% glycerol, 0.1 M NaCl) and filtered at 0.45 μm.

A 5 mL HiTrap chelating column (Pharmacia, Uppsala, Sweden) was charged with 5 mL of 0.1 M $NiCl_2$, washed with 25 mL of Buffer B (Buffer A plus 0.5 M imidazole), and then equilibrated with Buffer A. The lysate was loaded at 4 mL/min and then washed with 10% Buffer B until a steady base line was achieved. The enzyme was eluted with 30% Buffer B. Pooled fractions containing ICE activity were treated with 25 mM oxidized glutathione (GSSG) at room temperature for 45 min or until activity was not detectable when assayed in HGE buffer.

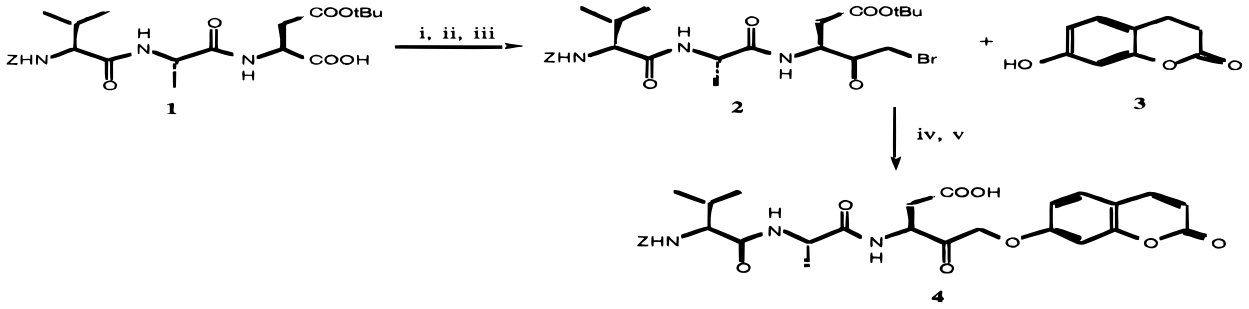
Excess GSSG was removed by passage over a 2.6 × 100 cm (500 mL) Superdex 75 column (Pharmacia), at 2 mL/min in Buffer A. Fractions containing ICE activity were pooled and concentrated using a Centriprep 10 filter (Amicon, Beverly, MA). Enzyme aliquots were stored at −80 °C until use.

Enzyme concentration was determined by amino acid analysis and by Coomassie Plus protein assay (Pierce, Rockford, IL) using BSA as a standard.

Synthesis of Active-Site Titrant PD 163594. The synthesis of 3-[2-(2-benzyloxycarbonylamino-3-methylbutyrylamino)-propionylamino]-4-oxo-5-(2-oxo-2*H*-chromen-7-yloxy)pentanoic acid (PD 168594; **4**) was carried out according to Scheme 1 (Dolle et al., 1994). The tripeptide, Z-Val-Ala-Asp(OtBu)-OH (**1**), was activated as the mixed anhydride using ethyl chloroformate in the presence of *N*-methylmorpholine at −42 °C. This mixed anhydride was treated with a solution of diazomethane in ether followed by treatment with 48% HBr/HOAc (1:1) at 0 °C to give Z-Val-Ala-Asp(OtBu)-α-bromomethyl ketone (**2**). **2** was then coupled with 7-hydroxycoumarin (**3**) using potassium fluoride and DMF to yield **4**.

Active-Site Titration Assay. Fluorescence measurements were performed using an Aminco AB2 fluorimeter (LSI North America, Rochester, NY) using excitation and emission wavelengths of 370 and 450 nm, respectively. Calibration curves were prepared using known stocks of 7-hydroxycoumarin (0.05−0.3 μM) or PD 163594 (1−4 μM) in HGE buffer. Fluorescence of a solution containing titrant (350 μL of 6 μM HGE) was monitored to ascertain signal stability. Enzyme (50 μL) was activated by adding 0.5 μL of 1 M DTT and incubating for 1 min at 4 °C. The activated enzyme was added to the titrant solution, and fluorescence was

Scheme 1[a]



[a] Legend: (i) EtO(CO)Cl, *N*-methylmorpholine, THF, −42 °C. (ii) CH_2N_2, ether. (iii) 48% HBr/HOAc (1:1) (86% yield for i−iii). (iv) KF, DMF, 58%. (v) TFA/CH_2Cl_2 (1:3), 78%.

monitored for 4800 s. The plot of fluorescence *vs* time was fitted to eq 1a (Tian & Tsou, 1982):

$$F(t) = F_o + \frac{V_i(1 - e^{-kt})}{k} \quad (1a)$$

where F_o is the fluorescence at $t = 0$, V_i is the initial rate of fluorescence generation, and k is the pseudo-first-order time constant. The quality of the fit in all cases was excellent. Total released fluorescence was evaluated as

$$\Delta F = \frac{V_i}{k} \quad (1b)$$

In a typical experiment, the fluorescence of PD 163594 was 40 rfu/μM, and fluorescence of 7-hydroxycoumarin was 1641 rfu/μM, so that the total 7-hydroxycoumarin released upon reaction with enzyme was evaluated as

$$\Delta[\text{7-OHC}]\ (\mu\text{M}) = \frac{V_i}{1601k} \quad (1c)$$

Kinetics of GSH Release. Enzyme (6 μM) was diluted 1:1 with HGE buffer containing 4 mM DTT or 20 mM βME. At various time intervals, 1 μL was withdrawn for activity assay in HGE buffer. Concurrently, 45 μL was added to vials containing 5 μL of 125 mM 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F). The DBD-F labeling reaction was carried out at 37 °C for 30 min and quenched with 450 μL of 0.1% TFA. Samples were centrifuged at 13000*g* for 1 h to remove precipitation caused by the derivatized DTT. For each sample, 250 μL aliquots were chromatographed by HPLC using a 4.6 × 220 nm C_{18} reverse-phase column and a linear gradient of CH_3CN-H_2O with 0.1% TFA. Detection was by fluorescence at excitation and emission wavelengths of 380 and 530 nm, respectively.

To calculate the molar amount of GSH recovered, a calibration curve was generated by reacting known concentrations of GSH (0.1−50 μM) with 12.5 mM DBD-F in HGE buffer containing 10 mM βME or 2 mM DTT at 37 °C for 30 min. Reactions were quenched with 10 vol of 0.1% TFA. The standards were centrifuged and analyzed by HPLC as described above. DBD-F-labeled glutathione peak areas were integrated and plotted against GSH standards.

To determine total releasable GSH, enzyme was first diluted 1:1 into 8 M guanidine buffer (8 M guanidine hydrochloride, 50 mM HEPES, pH 7.5, 0.5 mM EDTA) and incubated at room temperature for 30 min. Enzyme was then reduced with 5 mM DTT and derivatized with DBD-F as described above. A calibration curve was generated by reacting known amounts of GSH (0.1−50 μM) with 12.5 mM DBD-F in 4 M guanidine buffer containing 5 mM DTT.

Quantitation of Free Cysteines. Enzyme (10 μL at 11 μM) was added to 590 μL of 6 M guanidine buffer and incubated at room temperature for 15 min. As background control, one sample was pretreated with 1 mM *N*-ethylmaleimide at room temperature for 30 min. A 5 μL amount of 100 mM DBD-F was then added to 495 μL of enzyme sample, and fluorescence was observed for 1000 s using 380 and 530 nm excitation and emission wavelengths, respectively.

A calibration curve was generated as follows. GSH (50 mg) and DBD-F (10 mg) were reacted in 10 mL of HGE buffer at 37 °C for 30 min. The reaction was quenched by adding neat TFA to 0.5% (v/v) and purified by HPLC using a 10 × 220 mm C_{18} reverse-phase column and a linear gradient of CH_3CN-H_2O with 0.1% TFA. Peak fractions were collected in brown vials and lyophilized. The yellow powder was weighed and resuspended in dimethylformamide to 5 mM. GSH/DBD-F adduct was added to 6 M guanidine buffer to between 0.1 and 5 μM. Fluorescence signals were measured as described above and plotted against concentration.

RESULTS

Mutation of ICE to Remove Autolytic Site. Purified rfICE displayed contaminants of approximately 7 and 3 kDa as judged by SDS−PAGE analysis, which we identified by N-terminal sequencing as a proteolytic cleavage product of the ICE p10 subunit at Asp381 (Talanian et al., 1996). To find a conservative mutation that would eliminate this cleavage site while retaining the catalytic activity of rfICE, we prepared mutants of ICE p10 containing the substitutions D381A and D381E and tested the catalytic activities of rfICE prepared with these mutant proteins. Mutant p10 proteins were expressed in *E. coli*, and purified inclusion bodies containing these proteins were solubilized and refolded with purified ICE p20, as described (Talanian et al., 1996). HPLC-purified wild-type p10 was used as a positive control. The resulting rfICE proteins were assayed for catalytic activity as described in Materials and Methods. Relative activities recovered were 100% (wild-type), 83% (D381E), and 5% (D381A). Thus, the point mutant D381E but not D381A yielded ICE enzyme with activity similar to that of the wild-type. In the crystal structure of ICE inhibited with Ac-YVAD-CMK (Walker et al., 1994), D381 forms a salt bridge with R383. However, in a structure of ICE bound with the inhibitor Ac-YVAD-CHO (Wilson et al., 1994), this salt bridge is absent. This suggests that while the Asp381−Arg383 salt bridge is not essential for ICE activity, an acidic

Table 1: Purification Scheme

purification step	protein	specific activity (s^{-1})	recovery (%)	*n*-fold purification (*n*)
cell lysates	~6 g	2×10^{-4}	100	1
chelating Sepharose	3 mg	4×10^{-1}	90	2000
size-exclusion chromatography	1.2 mg	8×10^{-1}	70	4000

side chain at position 381 may play some role in ICE folding or stability.

Expression and Purification of N-His (D381E) ICE. High cell-density fermentation ($OD_{590} > 60$) yielded approximately 3 mg of ICE protein per liter in the soluble fraction of cell lysates as estimated by Coomassie-Blue SDS−PAGE densitometry (data not shown). Clarified *E. coli* cell lysates were loaded onto a nickel-chelating column directly. Washing the column with 50 mM imidazole (10% buffer B) removed several *E. coli* contaminants, and no ICE activity was detected in this wash step. The enzyme was eluted with 150 mM imidazole (30% B) applied as a step gradient to provide adequate concentration for subsequent SEC. Active fractions were pooled and greater than 90% of starting ICE activity was recovered. Approximately 2000-fold purification was achieved through this single step (Table 1).

Affinity-purified enzyme was inactivated by adding 25 mM GSSG to the pooled active fractions. Aliquots were periodically withdrawn for assay in HGE buffer until no activity could be detected. Catalytic activity was recovered completely upon addition of DTT or βME. Reactivation by 5 mM DTT at 30 °C appeared to be instantaneous, with no observable upward curvature when activity toward the substrate Ac-YVAD-Amc was monitored for 15 min (data not shown).

A further 2-fold purification and removal of excess GSSG were accomplished by size-exclusion chromatography (SEC). High molecular weight impurities as well as degraded ICE protein and other smaller proteins were separated from ICE with near baseline resolution (data not shown). Silver-stained SDS−PAGE analysis showed two bands at 25 and 10 kDa, corresponding to the N-His-tagged 20 kDa and 10 kDa subunits, respectively (Figure 1A). Following SEC, purified enzyme could be concentrated up to 10-fold by ultrafiltration. Aliquots of concentrated enzyme were stored at −80 °C for up to 6 months with no loss of activity.

Resistance of N-His (D381E) ICE to Autolytic Degradation. Wild-type N-His-tagged ICE (without the D381E mutation) was prepared as described above for N-His (D381E) ICE, and the autolytic stability of these enzymes was compared as described in Figure 1. The N-His (D381E) enzyme activity was markedly more stable, with >80% activity remaining after 3 h at 30 °C (Figure 1C), and this increased enzymatic stability correlated with greater stability of the 10 kDa subunit (Figure 1A,B).

Titration of the ICE Active-Site. While metal chelating affinity chromatography was highly efficient in removal of *E. coli* contaminants (Figure 1A), this method cannot distinguish between active and inactive soluble enzyme, since all ICE protein contains the polyhistidine tag. Therefore, an ICE-specific active-site titrant was developed to measure the active ICE in the preparations.

PD 163594 is an ICE-specific peptide ketone bearing a 7-hydroxycoumarin fluorophore which can be displaced by the nucleophilic cysteine residue of ICE. In our experimental conditions, release of 7-hydroxycoumarin from PD 163594 generated a net fluorescence signal of approximately 1600 rfu/μM, allowing reliable detection of ICE at concentrations lower than 20 nM. Details of the mechanism of action of

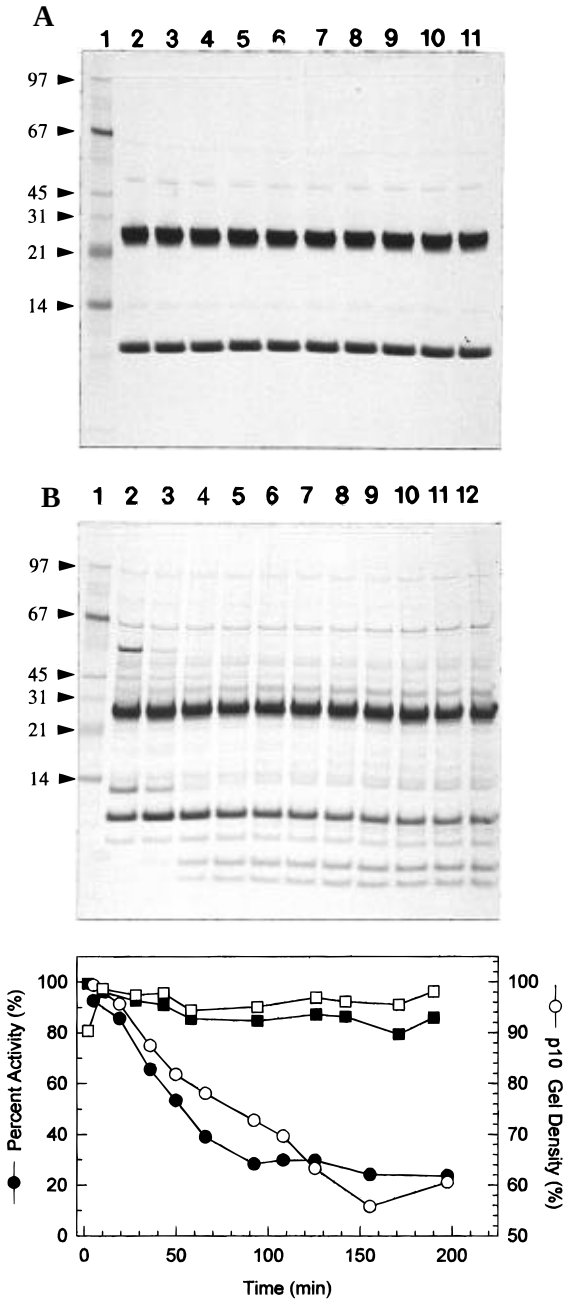


FIGURE 1: N-His (D381E) ICE is resistant to autolytic degradation. N-His (D381E) or wild-type enzyme at 1 μM was preactivated with 5 mM DTT and incubated at 30 °C. At time intervals, 1 μL was withdrawn for activity assay. Concurrently, 10 μL aliquots were removed for SDS−PAGE analysis. (A) Silver-stained gel of N-His (D381E) ICE reveals little degradation of either subunit. (B) Gel of wild-type ICE shows degradation of p10 accompanied by the appearance of 7 kDa band. (C) Activity profiles of N-His (D381E) (solid squares) and N-His wild-type ICE (solid circles) correlated with gel densitometry of the p10 subunits (open symbols). N-His wild-type ICE showed significant activity decay, while N-His (D381E) ICE showed modest loss of activity after 3 h of incubation. In A and B lane 1 contains size-markers, and subsequent lanes correspond to time points shown in 1C.

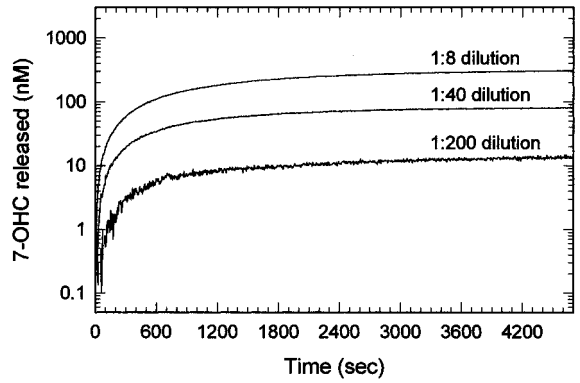


FIGURE 2: Release of 7-hydroxycoumarin from PD 163594 by N-His (D381E) ICE. Fluorescence signal from a cuvette containing 5 μM PD 163594 in HGE buffer was monitored to ascertain signal stability. Enzyme (0.12 mg/mL by AAA; 3.9 μM) was activated by adding DTT to 10 mM and incubating for 1 min at 4 °C, and aliquots (50, 10, and 2 μL) were added to a 5 μM solution of PD 163594 in HGE buffer (final volumes of all reactions were 400 μL); fluorescence was monitored for 4800 s. The active-site concentrations were calculated from these curves as described in Materials and Methods, yielding a mean stock enzyme concentration of 3.1 ± 0.2 μM (mean ± SD).

Table 2: Comparison of Protein Assay, AAA, Titrations, and Total GSH Release

preparation	protein assay (μM)	amino acid analysis (μM)	active-site titrant (μM)	μM GSH recovered
5-30c	7.5	3.9	3.05	–
7-27	7.6	4.4	3.2	3.4
8-3	3.2	1.4	1	1.1
8-3c	16.5	7.0	6.1	6.9
10-24	23	13	11	12

PD 163594 will be described elsewhere. For titration purposes, reactions were run under pseudo-first-order conditions, with the titrant (5 μM) present in great excess over the enzyme. Release of fluorescence was well described by a model of irreversible inactivation (integrated form, eq 1a). The pseudo-first-order time constant was $(9.0 \pm 0.3) \times 10^{-4}$ s^{-1} ($t_{1/2} = 13$ min), and no dependence on enzyme concentration was observed (Figure 2, and data not shown). The total fluorescence change, ΔF, was evaluated from the initial velocity and the time constant (eq 1b), and liberated 7-hydroxycoumarin was evaluated from ΔF and the known net change in fluorescence upon conversion of PD 163594 to 7-hydroxycoumarin, as calculated from the standard curves (eq 1c).

Typical fluorescence *vs*. time traces are shown in Figure 2. For this experiment, a single, concentrated enzyme stock was used at three dilutions. The observed ΔF was proportional to total enzyme concentration over this concentration range. Titration results from several independent N-His (D381E) ICE preparations are compared to protein assay and AAA results in Table 2. On the basis of the titrations, the standard protein assays consistently overestimate the enzyme concentration by greater than 2-fold. Since this discrepancy has not been observed with ICE prepared by refolding (Talanian et al., 1996), we expect that it may be an anomaly due to the polyhistidine tag in this protein assay. The protein concentrations derived by amino acid analysis are in better agreement with the titrations, but still are on average higher by 22%. Thus, either some portion of the protein is inactive and unable to react with the titrant or this difference represents some systematic error between the two techniques.

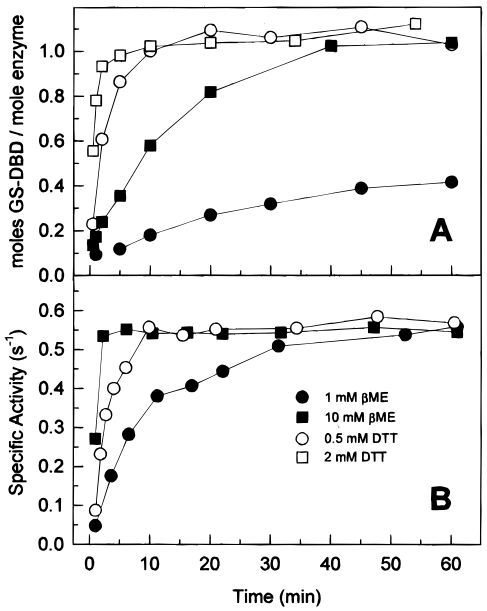


FIGURE 3: Kinetics of GSH release and enzyme activation. Enzyme stock (11 μM) was diluted with an equal volume of HGE buffer containing 1 mM DTT, 4 mM DTT, 2 mM βME, or 20 mM βME and incubated at room temperature. At time intervals, 1 μL was withdrawn for activity assay in HGE buffer. Concurrently, 45 μL of the enzyme sample was withdrawn into vials containing 5 μL of 125 mM DBD-F. Samples were derivatized at 37 °C for 30 min, quenched with 450 μL of 0.1% TFA, and then centrifuged for 1 h. 250 μL aliquots were analyzed by C_{18} reverse-phase HPLC. Fluorescence-labeled GSH was isolated and quantitifed by peak areas. (A) GSH release rates of (D381E) ICE following activation using two reducing agents. DTT (open symbols) completely reduced the enzyme within 2 min at room temperature, releasing 1 molar equiv of total GSH. However, 10 mM βME (closed squares) required 40 min for the complete GSH release with an asymptotic curve toward 1 molar equiv. (B) Activity recovered over time with 0.5 mM DTT (open circles), 1 mM βME (closed circles), or 10 mM βME (closed squares).

Quantitation of GSH Release. Following the affinity purification step, N-His (D381E) ICE was treated with oxidized glutathione (GSSG) to protect the enzyme from oxidative inactivation (Miller et al., 1993a; Thornberry, 1994). Since GSSG could possibly react with any of the nine cysteine residues of ICE, we developed a procedure to quantify the bound glutathione. DBD-F has no fluorescence when excited at 380 nm and monitored at 530 nm; however, under mild conditions, it reacts irreversibly with mercaptans to form stable fluorescent adducts (Imai & Fukushima, 1993; Toyo'oka et al., 1989). To observe the time-dependent release of GSH from N-His (D381E) ICE, the enzyme was treated with DTT or βME for various time intervals and then with excess DBD-F for 30 min at 37 °C. Derivatization reactions were quenched with TFA, and the poorly-soluble DTT/DBD-F adduct was removed by centrifugation. The GSH/DBD-F adduct was separated by HPLC using fluorescence detection, and the peak areas were quantified using a standard curve generated from parallel reactions using GSH stock solutions of known concentration. The kinetics of GSH release from N-His (D381E) ICE are compared to the rate of enzymatic activation in Figure 3.

A 1 M equiv of GSH was released from ICE upon treatment with either 2 or 0.5 mM DTT (Figure 3A). Activation of the enzyme at either DTT concentration was closely correlated with GSH release (Figure 3B). Release of GSH by 10 mM or 1 mM βME was significantly slower than by DTT, and 1 mM βME failed to liberate all of the

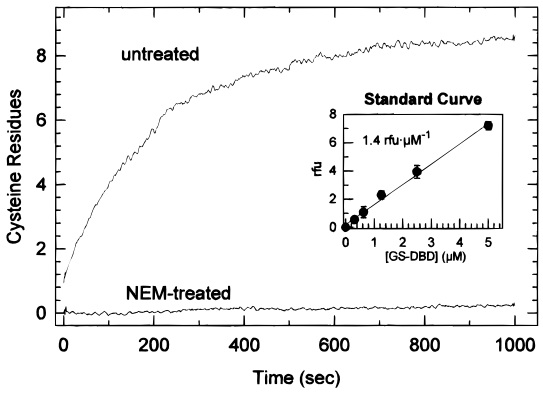


FIGURE 4: Quantification of N-His (D381E) ICE free cysteines. 10 μL of enzyme (11 μM) was added to 590 μL of 6 M guanidine buffer and incubated at room temperature for 15 min. A parallel control sample was pretreated with 1 mM NEM at room temperature for 30 min. 5 μL of 100 mM DBD-F was added to 495 μL of enzyme sample, and fluorescence signal was followed for 1000 s. Inset shows a calibration curve generated as described in Materials and Methods.

GSH, even after 60 min (Figure 3A). Furthermore, kinetics of enzyme activation did not correlate with the kinetics of GSH release by βME (Figure 3B). The simplest explanation is that the βME reacts fairly readily with the enzyme−GSH disulfide bond to form a βME−GSH mixed disulfide but that this mixed disulfide reacts slowly with a second equivalent of βME to generate free GSH. Since the βME−GSH mixed disulfide is not detected by DBD-F, the formation of GS−DBD lags behind the enzyme activation.

When the enzyme was denatured in 6 M guanidine buffer prior to treatment with DTT, a total of 1.1 equiv of glutathione per mole of enzyme was released (Table 2). Since the ICE heterodimer contains a total of nine cysteine residues, eight cysteine residues are expected to be in a reduced state (Cerretti et al., 1994; Thornberry et al., 1992; Walker et al., 1994). When the enzyme was denatured and reacted exhaustively with DBD-F, a fluorescence signal was generated corresponding to 8.4 cysteines per mole of enzyme (Figure 4). We conclude that treatment of the affinity purified N-His (D381E) ICE with excess oxidized glutathione selectively oxidizes a single cysteine residue.

Activity of N-His (D381E) ICE toward Ac-YVAD-Amc. N-His (D381E) ICE displayed normal Michaelis−Menten kinetics toward the tetrapeptide substrate Ac-YVAD-Amc (Thornberry et al., 1992) in HGDE buffer, with $K_M = 14$ μM and $k_{cat} = 0.7$ s^{-1} (data not shown), in agreement with published values (Thornberry, 1994). In addition, Ac-YVAD-CHO (Chapman, 1992) inhibited N-His (D381E) ICE with $k_{on} = 300\,000$ M^{-1} s^{-1} and $K_i = 0.7$ nM, again in agreement with published values (Thornberry et al., 1992). Thus, while D381 of ICE lies within 8 Å of the bound inhibitor and within 17 Å of the catalytic cysteine residue (Walker et al., 1994), no significant changes are observed in the hydrolysis of or inhibition by these small peptidic molecules. The N-terminal polyhistidine tail similarly seems to be without effect on enzyme activity (Gu et al., 1995), a result consistent with the observation that the N-terminus of the refolded enzyme is disordered in the ICE crystal structure (Walker et al., 1994). Importantly, the above enzymatic characterizations have been performed either in assays of short duration (i.e., K_M evaluations) or in the presence of an inhibitor (Ac-YVAD-CHO) with a strong stabilizing effect on the enzyme. As was demonstrated for ICE purified from THP.1 extracts (Thornberry et al., 1992), the N-His (D381E) enzyme is unstable to dilution in the absence of tight-binding ligands and/or stabilizing proteins (e.g., BSA), a property which is likely related to dissociation of the ICE $(p10p20)_2$ tetramer.

DISCUSSION

We describe a simple and reliable method for generating a stable form of recombinant ICE suitable for studies under a variety of conditions. The (D381E) mutant is greatly stabilized toward autolytic degradation compared to wild-type N-His-tagged ICE (Figure 1) or to refolded ICE (Talanian et al., 1996). This stability allowed accurate determination of the active enzyme content of each preparation. For this purpose, we synthesized a novel specific inhibitor used as an active-site titrant for ICE. Through comparison of the active enzyme concentrations evaluated using this titrant to the protein concentrations determined by AAA, we have found that 82% of the total protein is active enzyme.

Having a confident measure of enzyme activity, we next addressed concerns about whether glutathione, added during purification to protect and stabilize the enzyme, might be binding oxidatively to multiple cysteine residues. A total of 1.1 mol of GSH per mole of active-site was releasable from the enzyme by activation with DTT (Table 2). Furthermore, the remaining eight cysteine residues were available in the non-activated enzyme for reaction with the fluorephore DBD-F. Thus, it appears that the inactive protein present in our preparations is inactive toward GSSG, and that only one single cysteine of the active protein is able to react with GSSG. Although our data cannot demonstrate that this single cysteine is, in fact, the active-site residue Cys285, we consider this most likely given the uniquely reactive nature of this residue in the context of the active enzyme (Thornberry et al., 1992). Importantly, the incorporation of the site-directed mutation and the N-terminal polyhistidine tag to the ICE molecule did not interfere with its catalytic activity, as the interactions of N-His (D381E) ICE with the substrate Ac-YVAD-Amc and the inhibitor Ac-YVAD-CHO were indistinguishable from reported values for human ICE purified from THP.1 cells.

ACKNOWLEDGMENT

We thank Cathy Ferenz for assistance in the amino acid analyses.

REFERENCES

Cerretti, D. P., Hollingsworth, L. T., Kozlosky, C. J., Valentine, M. B., Shapiro, D.N., Morris, S. W., & Nelson, N. (1994) *Genomics 20*, 468−473.

Chapman, K. T. (1992) *Bioorg. Med. Chem. Lett. 2*, 613−618.

Dolle, R. E., Hoyer, D., Prasad, C. V., Schmidt, S. J., Helaszek, C. T., Miller, R. E., & Ator, M. A. (1994) *J. Med. Chem. 37*, 563−564.

Gu, Y., Wu, J., Faucheu, C., Lalanne, J. L., Diu, A., Livingston, D. J., & Su, M. S. (1995) *EMBO J. 14*, 1923−1931.

Imai, K., & Fukushima, T. (1993) *Biomed. Chromatogr. 7*, 275−276.

Jones, D. H., & Howard, B. H. (1991) *BioTechniques 10*, 62−66.

Kronheim, S. R., Mumma, A., Greenstreet, T., Glackin, P. J., Van-Ness, K., March, C. J., & Black, R. A. (1992) *Arch. Biochem. Biophys. 296*, 698−703.

Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S., & Flavell, R. A. (1995) *Science 267*, 2000−2003.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F.-Y., Wong, W., Kamen, R., & Seshardri, T. (1995) *Cell 80*, 401−411.

Martin, S. J., & Green, D. R. (1995) *Cell 82*, 349−352.

Miller, D. K., Ayala, J. M., Egger, L. A., Raju, S. M., Yamin, T. T., Ding, G. J., Gaffney, E. P., Howard, A. D., Palyha, O. C., Rolando, A. M., Salley, J. P., Thornberry, N. A., Weidner, J. R., Williams, J. H., Chapman, K. T., Jackson, J., Kostura, M. J., Limjuco, G., Molineaux, S. M., Mumford, R. A., & Calaycay, J. R. (1993a) *J. Biol. Chem. 268*, 18062−18069.

Miller, D. K., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Molineaux, S. M., & Thornberry, N. A. (1993b) *Ann. N.Y. Acad. Sci. 696*, 133−148.

Takahashi, K., Poole, I., & Kinane, D. F. (1995) *Arch. Oral Biol. 40*, 941−947.

Talanian, R. V., Dang, L. C., Ferenz, C. R., Hackett, M. C., Mankovich, J. A., Welch, J. P., Wong, W. W., & Brady, K. D. (1996) *J. Biol. Chem. 271*, 21853−21858.

Tian, W.-X., & Tsuo, C.-L. (1982) *Biochemistry 21*, 1028−1032.

Thornberry, N. A. (1994) *Methods Enzymol. 244*, 615−631.

Thornberry, N. A., & Molineaux, S. M. (1995) *Protein Sci. 4*, 3−12.

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., & Tocci, M. J. (1992) *Nature 356*, 768−774.

Thornberry, N. A., Miller, D. K., & Nicholson, D. W. (1995) *Perspect. Drug Discovery Des. 2*, 389−399.

Toyo'oka, T., Suzuki, T., Saito, Y., Uzu, S., & Imai, K. (1989) *Analyst 114*, 413−419.

Walker, N. P., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuiness, L., Oriewicz, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A., Terranova, M., Welch, J. P., Xiong, L., Möller, A., Tracey, D. E., Kamen, R., & Wong, W. W. (1994) *Cell 78*, 343−352.

White, K. P., Black, J. A., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., & Livingston, D. J. (1994) *Nature 370*, 270−275.

Zimmerman, M., Yurewicz, E., & Patel, G. (1976) *Anal. Biochem. 70*, 258−262.

BI9617771