Inactivation of Interleukin-1β Converting Enzyme by Peptide (Acyloxy)methyl Ketones

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ABSTRACT: Interleukin-1β converting enzyme (ICE) is a cysteine protease in monocytes that is essential for the proteolytic activation of interleukin-1β, an important mediator of inflammation. Peptide (acyloxy)-methyl ketones designed with the appropriate peptide recognition sequence (Ac-Tyr-Val-Ala-Asp-CH_2-OC(O)Ar) are potent, competitive, irreversible inhibitors. Mass spectrometry and sequence analysis indicate that inactivation proceeds through expulsion of the carboxylate leaving group to form a thiomethyl ketone with the active site Cys^{285}. The second-order inactivation rate is independent of leaving group pK_a, with an approximate value of 1×10^6 M^{-1} s^{-1}. This rate constant is directly proportional to the reaction macroviscosity, indicating that the rate-limiting step in inactivation is association of enzyme and inhibitor, rather than any bond-forming reactions. Affinity labeling of THP.1 monocytic cell cytosol with a biotinylated tetrapeptide (acyloxy)methyl ketone for 28 half-lives resulted in labeling of only ICE, demonstrating the selectivity of these inhibitors. These inhibitors are relatively inert toward other bionucleophiles such as glutathione ($<5 \times 10^{-4}$ M^{-1} s^{-1}), making them excellent candidates for *in vivo* studies of enzyme inhibition.

Interleukin-1 (IL-1) has been implicated in the pathogenesis of several acute and chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and septic shock (Dinarello & Thompson, 1991). IL-1β, the predominant form of this cytokine released by human monocytes in culture, is synthesized as a 31-kDa inactive precursor, which is activated to the 17.5-kDa active form by a highly selective protease termed interleukin-1β converting enzyme (ICE) (Black et al., 1989; Kostura et al., 1989). Evidence for a major role for this enzyme in inflammation has recently been provided by the cowpox virus, which facilitates infection through inhibition of the host inflammatory response, at least in part by encoding a potent inhibitor of ICE, crmA (Ray et al., 1992).

The purification, cloning, and characterization of this protease have recently been described (Thornberry et al., 1992). The enzyme is composed of two subunits of 20 (p20) and 10 kDa (p10), both of which are required for catalytic activity, and both of which are proteolytically derived from a 45-kDa proenzyme. The enzyme is a unique cysteine protease, lacking sequence homology to any other known enzyme in this class. It is also atypical with regard to substrate specificity in that aspartic acid is required in the P_1 position for effective catalysis. Exploiting this unusual specificity, potent, highly selective peptide aldehyde inhibitors of ICE have been developed (Chapman, 1992), which were employed to prove that the enzyme is essential to the production of IL-1β in human monocytes and murine peritoneal macrophages (Thornberry et al., 1992; Molineaux et al., 1993).

In efforts to develop more suitable inhibitors for assessing the effects of ICE inhibition *in vivo*, we have investigated peptide (acyloxy)methyl ketones **I**, which Krantz and his colleagues have recently described as potent, selective inactivators of cysteine proteases (Smith et al., 1988a; Krantz et al., 1991). Inhibitors designed with the appropriate active site specificity for cathepsin B have been shown to be extremely reactive toward the active site cysteine, with second-order inactivation constants exceeding 1×10^6 M^{-1} s^{-1} for the best inhibitors, while being relatively inert toward other bionucleophiles. While a detailed mechanism has not been established, it is clear that inactivation of cathepsin B proceeds through expulsion of the aryl carboxylate leaving group to form a thiomethyl ketone **II** with the active site cysteine (Scheme 1).

As described in this paper, (acyloxy)methyl ketones containing a peptide sequence that satisfies the specificity requirements for ICE have been prepared. The compounds are competitive, irreversible inhibitors of the enzyme. The best inhibitors of this class are highly selective and inactivate the enzyme with a rate limited by diffusion, making them the most potent inhibitors of this enzyme yet described.

MATERIALS AND METHODS

Materials. (1) Interleukin-1β Converting Enzyme. Human enzyme from a monocytic cell line, THP.1, or recombinant sources,[1] was purified to homogeneity by affinity chromatography using a peptide aldehyde ligand as described (Thornberry et al., 1992). The enzyme was stored inactive as the mixed disulfide between the active site cysteine and glutathione (γ-L-glutamyl-L-cysteinylglycine). To generate active enzyme, the enzyme–glutathione conjugate was reduced with 10 mM DTT[2] at 25 °C ($t_{1/2} < 1$ min).

(2) THP.1 Cell Cytosol. Human THP.1 cells were obtained from the American Type Culture Collection and were grown

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[2] Abbreviations: DTT, dithiothreitol; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; AMC, amino-4-methylcoumarin; DMF, dimethylformamide; MPLC, medium-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); BSA, bovine serum albumin; HPLC, high-performance liquid chromatography.

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Scheme 1

I II

in suspension in Iscove's modified Dulbecco's medium supplemented with 9% fetal horse serum. The cells were harvested by centrifugation at 1000*g* for 10 min and washed three times with Dulbecco's PBS without $MgCl_2$ and $CaCl_2$ (GIBCO). Cells were resuspended at 10^8 cells/mL in hypotonic buffer containing 25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 1 mM EGTA, and placed on ice for 20 min. Following addition of protease inhibitors (1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL pepstatin), cells were broken with 20 strokes in a tight-fitting Dounce homogenizer. Cell lysates were clarified by centrifugation at 2000 rpm for 10 min, followed by centrifugation at 80 000 rpm for 8 min in a Beckman TL100. The cytosol was dialyzed overnight at 4 °C against 20 mM Tris, pH 7.8, 2 mM DTT, and 0.1% CHAPS. The only ICE proteins detectable in a cell cytosol prepared in this manner are p10, p20, and an alternately processed form of p20, p22 (Thornberry et al., 1992; Miller et al., 1993).

(3) Other Materials. The substrate Ac-Tyr-Val-Ala-Asp-AMC (Thornberry et al., 1992) and the peptide aldehyde inhibitor Ac-Tyr-Val-Ala-Asp-CHO (Chapman, 1992) were prepared as previously described. Amersham was the source of ^{125}I-streptavidin. Sequence-grade trypsin was obtained from Boehringer Mannheim. All other chemicals and solvents were of commercial reagent grade or better.

Syntheses. ^{1}H NMR spectra were recorded on a Varian XL-400 spectrometer and are reported in ppm on the δ scale using CD_3OD (3.30 ppm) as internal standard. DMF was stored over 3-Å and 13× molecular sieves and used without further purification. Tri-*n*-butyltin hydride was purchased and handled under a nitrogen atmosphere after opening.

(Acyloxy)methyl ketones **4–9** were synthesized by the method of Krantz (Krantz et al., 1991) from the appropriate bromomethyl ketone and carboxylic acid or phenol. Peptidyl (acyloxy)methyl ketones **1–3** were prepared from the tripeptide and (allyloxy)carbonyl (Alloc)-protected (acyloxy)methyl ketones **4a** and **6a** using the *in situ* deprotection coupling procedure which we have reported previously (Chapman, 1992). A representative example follows:

(1) N-(Acetyltyrosinylvalinylalaninyl)aspartic Acid [(2,6-Dimethylbenzoyl)oxy]methyl Ketone (**2**). To a solution of *N*-[(allyloxy)carbonyl]aspartic acid [(2,6-dimethylbenzoyl)-oxy]methyl ketone β-*tert*-butyl ester (1.00 g, 2.38 mmol), *N*-acetyltyrosinylvalinylalanine (1.15 g, 2.38 mmol), hydroxybenzotriazole (643 mg, 4.76 mmol), dicyclohexylcarbodiimide (540 mg, 2.62 mmol), and 300 mg of bis(triphenylphosphine)palladium dichloride in 10 mL of anhydrous DMF was added tri-*n*-butyltin hydride (3.21 mL, 11.90 mmol) dropwise over 5 min. The mixture was allowed to stir overnight. The reaction mixture was partitioned between methanol and hexane, and the methanol layer was washed three times with hexane (discarded) and concentrated *in vacuo*. The product was purified by MPLC on Sephadex LH-20 using a 50 × 1000 mm column and eluting with methanol. The tetrapeptide product was further purified by MPLC on silica gel using a 35 × 350 mm column and eluting with a linear gradient of dichloromethane to 20% methanol in dichloromethane to afford 850 mg of the desired product as a colorless solid. The *tert*-butyl ester was removed by treatment with neat TFA for 40 min at ambient temperature. The product was purified by MPLC on silica gel using a 35 × 350 mm column eluting with a gradient of dichloromethane to 6% formic acid and 24% methanol in dichloromethane to afford 560 mg (36%) of the desired product as a colorless solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.92 (d, 2H, *J* = 8.12 Hz), 7.20 (t, 1H, *J* = 7.98 Hz), 7.03 (m, 2H), 6.67 (m, 2H), 5.14 (AB, 2H, *J* = 17.01 Hz), 4.76 (t, 1H, *J* = 6.22 Hz), 4.58 (m, 1H), 4.32 (m, 1H), 4.16 (m, 1H), 3.05–2.70 (m, 4H), 2.34 (s, 6H), 2.04 (m, 1H), 1.90 (s, 3H), 1.38 (d, 3H, *J* = 7.23 Hz), 0.95 (d, 3H, *J* = 6.87 Hz), 0.93 (d, 3H, *J* = 6.82 Hz). FAB mass spectrum *m/z* 655 (M + H), 677 (M + Na), 693 (M + K).

(2) N-(Acetyltyrosinylvalinylalaninyl)aspartic Acid [(2,6-Bis[(trifluoromethyl)benzoyl]oxy]methyl Ketone (**1**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (d, 2H, *J* = 8.107 Hz), 7.89 (t, 1H, *J* = 8.57 Hz), 7.04 (d, 2H, *J* = 8.48 Hz), 6.67 (d, 2H, *J* = 8.48 Hz), 5.23 (d, 1H, *J* = 17.06 Hz), 5.13 (d, 1H, *J* = 17.01 Hz), 4.74 (t, 1H, *J* = 6.22 Hz), 4.57 (m, 1H), 4.31 (m, 1H), 4.14 (m, 1H), 3.05–2.60 (m, 4H), 2.04 (m, 1H), 1.90 (s, 3H), 1.38 (d, 3H, *J* = 7.19 Hz), 0.93 (t, 6H, *J* = 6.65 Hz). FAB mass spectrum *m/z* 763 (M + H), 785 (M + Na).

(3) N-(Acetyltyrosinylvalinyl-Nε-biotinyllysinyl)aspartic Acid [(2,6-Dimethylbenzoyl)oxy]methyl Ketone (**3**). ^{1}H NMR (400 MHz, 1:1 DMF-d_7/CD_3OD) (1:1 mixture of diastereomers at the aspartic acid α-carbon) δ 7.27 (t, 1H, *J* = 8.07 Hz), 7.11 (m, 4H), 6.73 (m, 2H), 5.34 (d, 0.5H, *J* = 17.29 Hz), 5.25 (s, 1H), 5.20 (d, 0.5H, *J* = 17.33 Hz), 4.81 (t, 1H, *J* = 6.27 Hz), 4.64 (m, 1H), 4.6–4.2 (m, 4H), 3.3–2.7 (m, 8H), 2.38 (s, 6H), 2.22 (t, 2H, *J* = 7.33 Hz), 2.12 (m, 1H), 1.92 (s, 3H), 0.98 (d, 3H, *J* = 6.87 Hz), 0.95 (d, 3H, *J* = 6.96 Hz). FAB mass spectrum *m/z* 939 (M + H).

(4) N-[(Allyloxy)carbonyl]aspartic Acid [[(2,6-Bis(trifluoromethyl)benzoyl)oxy]methyl Ketone (**4a**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.10 (d, 2H), 7.88 (t, 1H), 5.92 (m, 1H), 5.4–5.1 (m, 5H), 4.7–4.4 (m, 3H), 2.95–2.62 (m, 2H). FAB mass spectrum *m/z* 472 (M + H), 494 (M + Na).

(5) N-[(3-Phenylpropionyl)valinylalaninyl]aspartic Acid [[(2,6-Bis(trifluoromethyl)benzoyl]oxy]methyl Ketone (**4b**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (d, 2H), 7.87 (t, 1H), 7.3–7.1 (m, 5H), 5.18 (br s, 2H), 4.74 (t, 1H), 4.33 (m, 1H), 4.12 (m, 1H), 3.0–2.70 (m, 4H), 2.57 (t, 2H), 1.99 (m, 1H), 1.34 (br d, 3H), 0.89 (d, 3H), 0.86 (d, 3H). FAB mass spectrum *m/z* 690 (M + H).

(6) N-[(Allyloxy)carbonyl]aspartic Acid [(2,6-Dihydroxybenzoyl)oxy]methyl Ketone (**5a**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.28 (m, 1H), 6.42 (d, 2H), 5.93 (m, 1H), 5.5–5.0 (m, 5H), 4.7–4.4 (m, 3H), 3.0–2.75 (m, 2H). FAB mass spectrum *m/z* 368 (M + H), 390 (M + Na).

(7) N-[(3-Phenylpropionyl)valinylalaninyl]aspartic Acid [(2,6-Dihydroxybenzoyl)oxy]methyl Ketone (**5b**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.30 (t, 1H, *J* = 8.25 Hz), 7.27–7.10 (m, 5H), 6.42 (d, 2H, *J* = 8.21 Hz), 5.37 (d, 1H, *J* = 17.06 Hz), 5.25 (d, 1H, *J* = 17.06 Hz), 4.75 (t, 1H, *J* = 6.18 Hz), 4.30 (m, 1H), 4.10 (m, 1H), 3.0–2.70 (m, 4H), 2.57 (m, 2H), 2.00 (m, 1H), 1.37 (d, 3H, *J* = 7.10 Hz), 0.89 (d, 3H, *J* = 6.78 Hz), 0.86 (d, 3H, *J* = 6.82 Hz). FAB mass spectrum *m/z* 586 (M + H), 608 (M + Na).

(8) N-[(Allyloxy)carbonyl]aspartic Acid [(2,6-Dimethylbenzoyl)oxy]methyl Ketone (**6a**). ^{1}H NMR (400 MHz,

CD_3OD) δ 7.31 (t, 1H), 7.05 (d, 2H), 5.95 (m, 1H), 5.4–5.1 (m, 3H), 5.19 (AB, 2H), 4.55 (t, 1H), 4.49 (d, 2H), 2.93 (dd, 1H), 2.77 (dd, 1H), 2.36 (s, 6H). FAB mass spectrum *m/z* 364 (M + H).

(9) N-[(3-Phenylpropionyl)valinylalaninyl]aspartic Acid [(2,6-Dimethylbenzoyl)oxy]methyl Ketone (**6b**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.3–7.2 (m, 6H), 7.04 (d, 2H), 5.13 (d, 2H), 4.76 (t, 1H), 4.31 (q, 1H), 4.11 (d, 1H), 3.0–2.70 (m, 4H), 2.57 (m, 2H), 2.34 (s, 6H), 2.00 (m, 1H), 1.38 (d, 3H), 0.91 (d, 3H), 0.87 (d, 3H). FAB mass spectrum *m/z* 586 (M + H), 582(M + Na).

(10) N-[(Allyloxy)carbonyl]aspartic Acid (Benzoyloxy)-methyl Ketone (**7a**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.06 (d, 2H), 7.61 (m, 1H), 7.47 (m, 1H), 5.94 (m, 1H), 5.32 (dt, 1H), 5.25–5.1 (m, 2H), 5.19 (AB, 2H), 4.67 (t, 1H), 4.57 (d, 2H), 2.92 (dd, 1H), 2.74 (dd, 1H). FAB mass spectrum *m/z* 336 (M + H).

(11) N-[(3-Phenylpropionyl)valinylalaninyl]aspartic Acid (Benzoyloxy)methyl Ketone (**7b**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (d, 2H), 7.62 (t, 1H), 7.48 (t, 2H), 5.15 (br s, 2H), 4.88 (br m, 1H), 4.32 (br q, 1H), 4.12 (d, 1H), 2.88 (t, 2H), 2.9–2.70 (m, 2H), 2.56 (m, 2H), 2.01 (m, 1H), 1.37 (d, 3H), 0.91 (d, 3H), 0.87 (d, 3H). FAB mass spectrum *m/z* 554 (M + H).

(12) N-[(Allyloxy)carbonyl]aspartic Acid (Pentafluorophenoxy)methyl Ketone (**8a**). ^{1}H NMR (400 MHz, CD_3OD) δ 5.95 (m, 1H), 5.4–5.1 (m, 5H), 4.69 (d, 2H), 4.55 (m, 1H), 2.88 (dd, 1H), 2.74 (dd, 1H). FAB mass spectrum *m/z* 398 (M + H).

(13) N-[(3-Phenylpropionyl)valinylalaninyl]aspartic Acid (Pentafluorophenoxy)methyl Ketone (**8b**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.3–7.1 (m, 5H), 5.17 (AB, 2H), 4.68 (t, 1H), 4.30 (m, 1H), 4.08 (m, 1H), 2.90 (t, 3H), 2.78 (dd, 1H), 2.58 (dd, 1H), 1.98 (m, 1H), 1.36 (d, 3H), 0.91 (d, 3H), 0.86 (d, 3H). FAB mass spectrum *m/z* 616 (M + H), 638 (M + Na).

(14) N-[(Allyloxy)carbonyl]aspartic Acid (p-Nitrophenoxy)methyl Ketone (**9a**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.20(br d, 2H), 7.09 (br d, 2H), 5.94 (m, 1H), 5.4–5.0 (m, 5H), 4.65 (br s, 1H), 4.57 (d, 2H), 2.86 (br m, 2H). FAB mass spectrum *m/z* 353 (M + H).

(15) N-(N-Tyrosinylvalinylalaninyl)aspartic Acid (p-Nitrophenoxy)methyl Ketone (**9b**). ^{1}H NMR (400 MHz, CD_3OD) δ 8.17 (d, 2H), 7.17 (d, 2H), 7.03 (d, 2H), 6.67 (d, 2H), 5.3–5.1 (br m, 2H), 4.7 (br m, 1H), 4.57 (m, 1H), 4.28 (br m, 1H), 4.13 (br m, 1H), 3.00 (dd, 1H), 2.87 (br m, 2H), 2.77 (dd, 1H), 2.01 (m, 1H), 1.90 (s, 3H), 1.38 (d, 3H), 0.93 (d, 3H), 0.91 (d, 3H). FAB mass spectrum *m/z* 628 (M + H), 638 (M + Na).

Continuous Fluorometric Assay. The fluorometric assay used in these studies has been previously described (Thornberry et al., 1992). Liberation of AMC from the substrate, Ac-Tyr-Val-Ala-Asp-AMC, was monitored continuously in a Gilford Fluoro IV spectrofluorometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Data were transferred via RS232 interface by a Sun Microsystems SPARCstation 1 Workstation for analysis. The K_m and k_{cat} for hydrolysis of this substrate by ICE are 14 μM (Thornberry et al., 1992) and 0.89 s^{-1}, respectively. A unit is defined as the amount of enzyme required to liberate 1 pmol of AMC in 1 min using saturating levels of substrate under standard reaction conditions, defined as 100 mM Hepes, 10% sucrose, 0.1% CHAPS, and 10 mM DTT, pH 7.5, and 25 °C.

Data Analysis. All kinetic constants were computed by direct fits of the data to the appropriate equation using a nonlinear least-squares analysis computer program (NLIN) developed in this laboratory. This program, when furnished with the equation and its partial derivatives with respect to each unknown parameter, uses the Marquardt algorithm to converge on the best estimates of the parameters and provides the standard error of each estimate.

Kinetic Studies. The simplest model for irreversible inhibition, shown below, involves formation of an initial reversible complex (EI) followed by irreversible conversion to the inactivated enzyme (EI*).

$$E + I \underset{k_2}{\overset{k_1}{\leftrightarrow}} EI \xrightarrow{k_3} EI^*$$

$$k_{obs} = [k_1 k_3 I_t/(k_2 + k_3)]/(1 + S/K_m) \quad (1)$$

$$k = (k_{obs}/I_t)(1 + S/K_m) \quad (2)$$

The observed rate of disappearance of free enzyme, k_{obs}, is a function of the individual rate constants and total inhibitor concentration, I_t, as shown in eq 1, assuming $I_t > E_t$ in the assay (Mahler & Cordes, 1971). If the rate of inactivation is governed by the irreversible step ($k_3 \ll k_2$), the second-order rate constant is defined by k_3/K_i, where $K_i = k_2/k_1$. Alternatively, if the rate of the inactivation is determined by the association of enzyme and inhibitor ($k_3 \gg k_1 \cong k_2$), as has been demonstrated for the tetrapeptide (acyloxy)methyl ketones, the second-order rate constant is equal to k_1. In both cases the second-order rate constant, referred to simply as k in the text, is computed according to eq 2.

Unless otherwise indicated, all kinetic reactions were performed using standard reaction conditions using homogeneous enzyme from THP.1 cells. Typically, reactions were initiated by adding approximately 25 units of enzyme (0.9 nM) to an assay mixture containing substrate and inhibitor in a total volume of 500 μL. In every case, consumption of substrate during the reaction was <10%. Progress curves were obtained and k_{obs} was computed by a direct fit of the data to

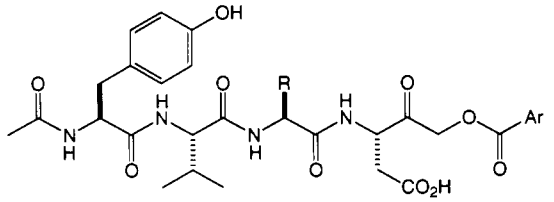
$$[AMC] = v_s t + (v_0 - v_s)[1 - \exp(-k_{obs} t)]/k_{obs} \quad (3)$$

where v_0 is the initial reaction velocity and v_s is the final steady-state velocity. The derivation and applications of this equation have been developed fully elsewhere (Morrison & Walsh, 1988). The appropriate second-order rate constant was then computed as described above. The error in reproducing the rate constant on different days was typically 10%, and never more than 25%.

Viscosity Studies. Reactions were performed as described above using 10 mM Hepes and 10 mM DTT, pH 7.5, in the absence (solution A) and presence (solution B) of 36% glycerol by weight. Viscosities were measured at 22 ± 0.2 °C with an Ostwald viscometer. The viscosity determined for solution A (0.96 ± 0.02 cP) was calculated using a density of 0.999 ± 0.004 g/cm^3. The viscosity determined for solution B (2.36 ± 0.03 cP) was calculated using a density of 1.088 ± 0.001 g/cm^3. Viscosities are reported relative to solution A.

Affinity Labeling of THP.1 Cytosol. Samples were incubated with the biotinylated (acyloxy)methyl ketone **3** (200 nM) for 10 min under standard reaction conditions in a total reaction volume of 20 μL. The peptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO (1 μM) was included in control reactions to protect the enzyme from inactivation. Samples were applied to an SDS–PAGE gel (Novex 10% Tricine), transfered to PVDF membranes (Millipore), and incubated in PBS containing 3% BSA and 0.1% Tween-20 for 2 h at room

Table 1: Tetrapeptide (Acyloxy)methyl Ketones



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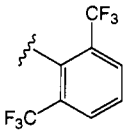
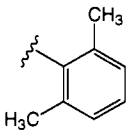
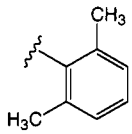
1	CH_3	
2	CH_3	
3	$(CH_2)_4NH$-biotin	

temperature. The blot was washed 2 × 10 min with PBS, 0.1% BSA, and 0.1% Tween-20 at room temperature, followed by incubation with ^{125}I-streptavidin (5 × 10^5 cpm/mL) for 1 h. The blot was then washed 6 × 10 min with a solution containing 50 mM Tris-HCl, pH 7.5, 0.25% gelatin, 0.05% Tween-20, 150 mM NaCl, and 5 mM EDTA, air-dried, and autoradiographed at −70 °C. The Daiichi silver stain kit was used for detection of protein in the samples.

Structural Analysis of Enzyme–Inactivator Complex. Baculovirus-derived recombinant enzyme (480 nM) was incubated with 5 μM of compound **2** under standard reaction conditions in a total reaction volume of 100 μL. Incubation for 5 min at 25 °C resulted in >99% inhibition. Inactivated enzyme (10–20 pmol) was analyzed by capillary HPLC–electrospray mass spectrometry (HPLC-ESI-MS) using a Finnigan TSQ-700 triple-quadrupole mass spectrometer as described in detail (Griffin et al., 1991). The enzyme–inactivator complex was fragmented using trypsin and the resulting peptide mixture analyzed by HPLC-ESI-MS/MS. The peptide containing the inactivator was further analyzed by HPLC-ESI-MS utilizing collision-activated dissociation (CAD) to determine the structure of the labeled peptide.

RESULTS

Irreversible Inhibition by Tetrapeptide (Acyloxy)methyl Ketones. Previous studies of substrate specificity have established the minimum requirements for efficient cleavage of peptides by ICE: four amino acids to the left of the cleavage site are necessary, methylamine is sufficient to the right, and there is a strong preference for aspartic acid in P_1 (Sleath et al., 1990; Howard et al., 1991; Thornberry et al., 1992). The design of the tetrapeptide (acyloxy)methyl ketone, Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CF_3)_2$]Ph (**1**, Table 1), was based on these considerations, and studies with cathepsin B which established that peptide (acyloxy)methyl ketone inactivators containing the 2,6-bis(trifluoromethyl)benzoate leaving group are the most potent inactivators in this class (Smith et al., 1988a; Krantz et al., 1991).

As shown in Figure 1, compound **1** is an efficient, irreversible inhibitor of ICE. Treatment of the enzyme with inhibitor (10 nM) in the presence of 1 × K_m levels of substrate resulted in

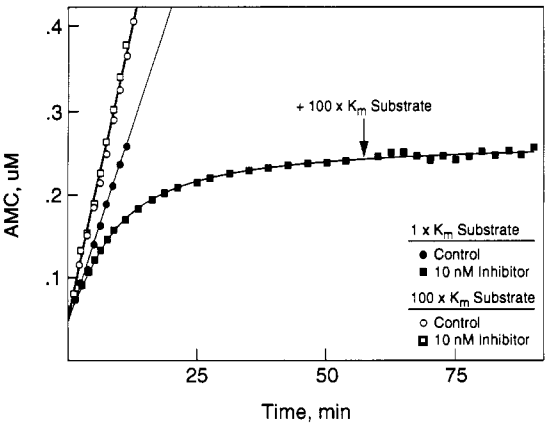


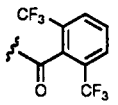
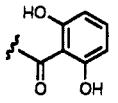
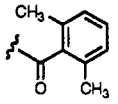
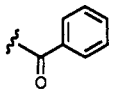
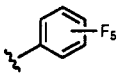
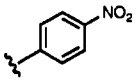
FIGURE 1: Competitive, irreversible inhibition by Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CF_3)_2$]Ph (**1**). Addition of inhibitor (10 nM) to a reaction mixture containing enzyme (1 nM) and 1 × K_m substrate (14 μM) resulted in first-order loss of enzyme activity (closed squares). The solid line is theoretical for a fit to eq 3, which yielded a value for the pseudo-first-order rate constant of 4.8 × 10^{-3} s^{-1}, corresponding to a second-order rate constant of 9.6 × 10^5 M^{-1} s^{-1}. No saturation of enzyme was evident, as indicated by the virtually identical values for the the initial velocity, v_o = 0.042 μM AMC/min, and the control velocity, v_{ctrl} = 0.047 μM AMC/min. Addition of saturating substrate (1.4 mM, 100 × K_m) had no influence on the inhibited velocity, indicating irreversible inhibition. The reaction rate in the absence of inactivator is linear (closed circles). To demonstrate competitive inactivation, an identical experiment was performed at saturating levels of substrate (open symbols). In this case, no time-dependent inhibition was observed, consistent with a competitive mechanism of inactivation.

rapid, time-dependent inhibition of activity. This inhibition was not reversed by the addition of saturating levels of substrate (100 × K_m) to the fully inhibited enzyme, or by prolonged dialysis of the enzyme–inhibitor complex. The inhibition is competitive, as indicated by the protection from inactivation observed in the presence of saturating levels of substrate (100 × K_m). The second-order rate constant for inactivation by this compound is 9.6 × 10^5 M^{-1} s^{-1}. Truncation of this inhibitor to the corresponding single amino acid (acyloxy)methyl ketone, Alloc-Asp-$CH_2OC(O)$-[2,6-$(CF_3)_2$]Ph (**4a**, Table 2), resulted in more than a 100-fold decrease in the second-order inactivation rate constant (5.3 × 10^3 M^{-1} s^{-1}), consistent with the results from substrate specificity studies (Thornberry et al., 1992).

Influence of Leaving Group on Inactivation Rate. We have investigated the influence of leaving group pK_a on the second-order inactivation rate for both phenylpropionyl-Val-Ala-Asp and Alloc-protected Asp acyloxy and aryloxy methyl ketones. The results, shown in Table 2, reveal a striking difference in the effect of pK_a on inactivation rate between the two series. In the case of all of the tetrapeptide inhibitors investigated, the second-order rate constant is virtually identical over a range of pK_a values from 0.6 to 7, with an approximate value of 1 × 10^6 M^{-1} s^{-1}. In contrast, over the same pK_a range, the rate constant for the corresponding single amino acid inhibitors decreases approximately 100-fold, from 5300 M^{-1} s^{-1} (pK_a = 0.58) to 61 M^{-1} s^{-1} (pK_a = 7.16). These results imply that the rate-determining step in inactivation is different in the two series.

Inactivation Is Diffusion Controlled. The observation that the second-order inactivation rate for the tetrapeptide (acyloxy)methyl ketones is independent of leaving group pK_a, and the magnitude of this rate constant (1 × 10^6 M^{-1} s^{-1}), led to the proposal that diffusion is the rate-limiting step in inactivation in this series. This was confirmed with the inhibitor Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**2**, Table 1), where the rate of inactivation was shown to be

Table 2: Influence of Leaving Group pK_a on Inactivation Rate

compd	Ar	pK_a	k (M^{-1} s^{-1}) **a**	k (M^{-1} s^{-1}) **b**
4		0.58[a]	5300	900 000
5		1.22[c]	2700	710 000
6		3.35[a]	812	1 200 000
7		4.20[c]	100	280 000
8		5.53[b]	978	1 100 000
9		7.16[c]	61	1 300 000

[a] Krantz et al., 1991. [b] Smith et al., 1988a. [c] Kortüm et al., 1961.

directly proportional to the macroviscosity of the reaction milieu. As shown in Figure 2A and summarized in Table 3, the second-order rate constant for inactivation in the absence of a viscogenic agent (2.75×10^6 M^{-1} s^{-1}) decreases 2.5-fold to 1.1×10^6 M^{-1} s^{-1} in the presence of 36% glycerol (relative viscosity 2.5), as predicted for a cleanly diffusive process. Experiments designed to control for the influence of glycerol on the enzyme itself employed the corresponding single amino acid (acyloxy)methyl ketone, Alloc-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**6a**), a much slower inactivator. In this case the second-order rate constant was found to be virtually identical in the absence (788 M^{-1} s^{-1}) and presence (745 M^{-1} s^{-1}) of 36% glycerol (Figure 2B).

Active Site Labeling. The results of mass spectrometry and sequence analysis of ICE inactivated with Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**2**) are consistent with the chemical mechanism shown in Scheme 1. According to this mechanism, inactivation proceeds through expulsion of 2,6-dimethylbenzoate to form a thiomethyl ketone with the active site Cys^{285} on p20. The molecular mass of p20 in the inactivated enzyme was determined by HPLC-ESI-MS to be 20 349.6 Da, in excellent agreement with the mass predicted for the thiomethyl ketone (20 349.7 Da). As expected, the molecular mass of p10 is identical (10 242 Da) in free and inactivated enzyme, indicating that no adduct is formed with this subunit.

Analysis of a tryptic digest of the inactivated enzyme by HPLC-ESI-MS localized the inhibitor on a single peptide with the sequence Val-Ile-Ile-Ile-Gln-Ala-Cys-Arg, which corresponds to Val^{279} to Arg^{286}, a region including the catalytic Cys^{285}. Further analysis of this peptide by HPLC-ESI-MS/MS confirmed the covalent attachment of the inhibitor to this cysteine.

Selectivity. The selectivity of the tetrapeptide (acyloxy)-methyl ketones was assessed by affinity labeling crude lysates from a monocytic cell line (THP.1) with a biotinylated tetrapeptide (acyloxy)methyl ketone, Ac-Tyr-Val-(biotin)-Lys-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**3**, Table 1). The design of this affinity ligand was based on previous studies indicating that liberal substitutions, including lysine, are well tolerated in the P_2 position (Thornberry et al., 1992). This compound is an efficient, irreversible inhibitor of the enzyme with a second-order inactivation rate of 5.6×10^5 M^{-1} s^{-1}.

To demonstrate the selectivity of compound **3** for ICE, supernatants from THP.1 cells, where ICE comprises <0.001% of the total protein, were treated with affinity ligand for 28 half-lives (200 nM, 10 min), followed by SDS–PAGE, electroblotting, and visualization by ^{125}I-streptavidin. The results, shown in Figure 3, indicated labeling of only one protein, which comigrates on SDS–PAGE with p20 from affinity-purified ICE. Labeling could be completely prevented by including the potent, selective tetrapeptide aldehyde inhibitor (10 μM) in the reaction mixture, confirming the identity of the labeled band as p20.[3]

DISCUSSION

Peptide (acyloxy)methyl ketones **I**, designed with an appropriate amino acid sequence, are highly selective and potent irreversible inhibitors of ICE. Inactivation proceeds through displacement of the carboxylate leaving group to form a thiomethyl ketone **II** with the active site Cys^{285}, as depicted in Scheme 1. These results reaffirm the assertion of Krantz and his colleagues that peptide (acyloxy)methyl ketones represent a new class of cysteine protease inactivators that are highly potent, and yet chemically inert (Smith et al., 1988a; Krantz et al., 1991).

[3] There is a possibility that some of the labeled protein is p22, since p22 and p20 are not well resolved using this gel system.

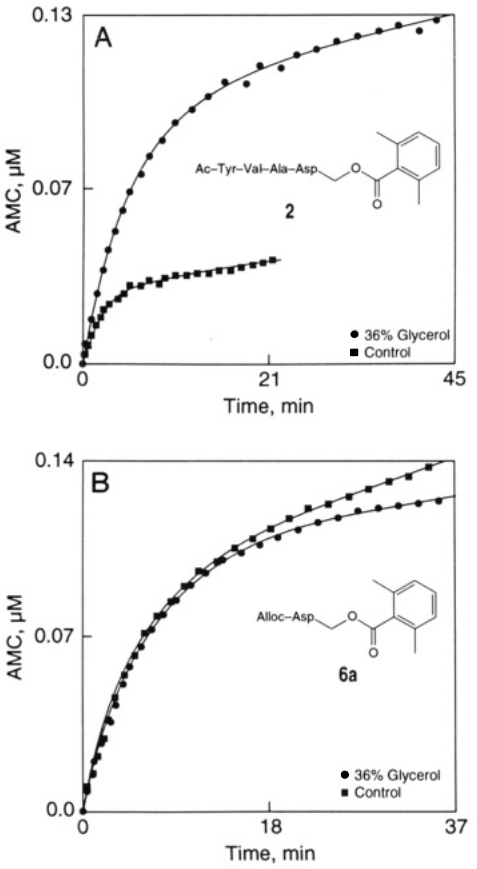


FIGURE 2: Diffusion-controlled inactivation by Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**2**). The second-order rate constant for inactivation was determined in the presence and absence of 36% glycerol for **2** (panel A) and **6a** (panel B). Reactions were initiated by adding enzyme (0.6 nM) to reactions containing inhibitor (**2**, 5 nM; **6a**, 10 μM), substrate (14 μM), DTT (10 mM), and 100 mM Hepes, pH 7.5, ± 36% glycerol. The solid line is theoretical for a fit of the data to eq 3. The influence of glycerol on the second-order inactivation rate for the two inhibitors, summarized in Table 2, indicates that the rate of inactivation by the tetrapeptide inhibitor **2** is directly proportional to macroviscosity while the rate constant for the less potent single amino acid (acyloxy)methyl ketone **6a** is virtually identical in the presence and absence of glycerol.

Table 3: Effect of 36% Glycerol on Inactivation Rates by Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**2**) and Alloc-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**6a**)

		k (M^{-1} s^{-1})	
additive	η/η_0	compd **2**	compd **6a**
none	1.0 ± 0.03	(2.75 ± 0.07) × 10^6	788 ± 27
36% glycerol	2.5 ± 0.1	(1.1 ± 0.1) × 10^6	745 ± 21

The peptide requirements for potent inactivation of ICE by these compounds are consistent with the reported substrate specificity of the enzyme (Sleath et al., 1990; Howard et al., 1991; Thornberry et al., 1992). Tetrapeptide (acyloxy)methyl ketones inactivate the enzyme with second-order inactivation rates that are at least 100-fold faster than their single amino acid counterparts, consistent with the enzyme's requirement for four amino acids to the left of the cleavage site in peptide substrates. In addition, the enzyme's reported ability to tolerate liberal substitutions in the P_2 position have been exploited here to develop a potent and highly selective affinity label containing a biotin tag.

The potency and specificity of these inhibitors is probably also determined by the ability of ICE to accommodate the relatively bulky (arylacyl)oxy leaving group. Previous studies have shown that, while peptide (acyloxy)methyl ketones rapidly inactivate cathepsin B, with rate constants in certain cases

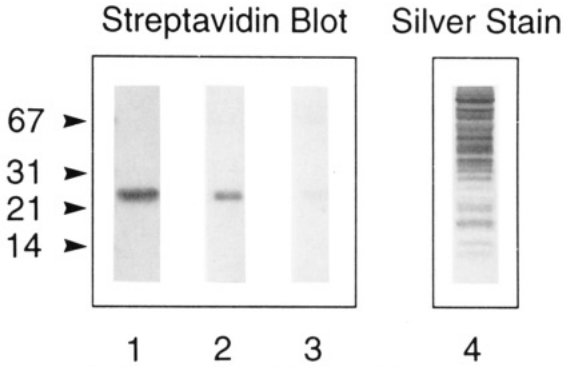


FIGURE 3: Affinity labeling of THP.1 cell lysates with Ac-Tyr-Val-(biotin)Lys-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph (**3**). Selectivity was evaluated by treating the cytosol from THP.1 cells with the affinity label **3** for 28 half-lives. In a typical experiment, samples were incubated with affinity label at 200 nM for 10 min at 25 °C, followed by SDS–PAGE, transfer to nitrocellulose, and detection with ^{125}I-streptavidin as described in the Materials and Methods. Samples include affinity-purified ICE (lane 1), THP.1 cell cytosol (lane 2), and THP.1 cell cytosol treated with 10 μM Ac-Tyr-Val-Ala-Asp-CHO (lane 3). The distribution of proteins in a THP.1 cell cytosol is shown in lane 4. The results indicate selective labeling of only one protein, p20.

exceeding 1 × 10^6 M^{-1} s^{-1}, they are relatively weak inhibitors of smooth muscle calpain, a Ca^{2+}-dependent cysteine protease (Pliura et al., 1992). Pliura et al. have suggested that the active site of calpain may not tolerate the steric bulk of the (arylacyl)oxy leaving group. In contrast, ICE is clearly able to accommodate bulky residues in P_1', as exemplified by the fluorogenic substrate used in these studies (Ac-Tyr-Val-Ala-Asp-AMC), which has a K_m of 14 μM.

The most notable feature of inhibition of ICE by tetrapeptide (acyloxy)methyl ketones and (aryloxy)methyl ketones is the extraordinarily rapid rate of inactivation observed, even for compounds with leaving groups of relatively high pK_a. For all of the tetrapeptide inhibitors investigated, the second-order rate constant is independent of leaving group pK_a with an approximate value of 1 × 10^6 M^{-1} s^{-1}, leading to the proposal that the rate-determining step in inactivation is binding. This was confirmed by demonstrating that the rate of inactivation by these inhibitors is directly proportional to the macroviscosity of the reaction milieu. In contrast, a much less potent single amino acid (acyloxy)methyl ketone has a second-order inactivation rate that is independent of reaction viscocity, providing an important control for this experiment. Using the technique of viscosity variation to identify diffusion-controlled reactions has precedence in elegant work in the laboratories of Kirsch, Knowles, and others (Hardy & Kirsch, 1984; Kurz et al., 1987; Pocker & Janjie, 1987; Blacklow et al., 1988).

It is interesting to note that the second-order rate constant for binding of the tetrapeptide aldehyde, Ac-Tyr-Val-Ala-Asp-CHO, to ICE is also approximately 1 × 10^6 M^{-1} s^{-1} (Thornberry et al., 1992).[4] Recent studies in this laboratory have established that this interaction is also proportional to macroviscosity (data not shown). The value for this rate constant is at the low end of the range of 10^6–10^8 M^{-1} s^{-1} typically observed for enzyme–substrate and protein–ligand interactions (Hammes & Schimmel, 1970; Fersht, 1985). It is tempting to speculate that this slow rate of association will be a characteristic of all tetrapeptide-based ICE inhibitors.

[4] The second-order rate constant for binding of the peptide aldehyde is 2.7 × 10^6 M^{-1} s^{-1}. This differs from that previously reported (3.8 × 10^5 M^{-1} s^{-1}), as it is corrected for hydration of the peptide aldehyde (14%).

The observation that binding, rather than chemistry, is the rate-limiting step in inactivation of ICE by tetrapeptide (acyloxy)methyl ketones contrasts sharply with the behavior of the cathepsin B inactivators. In this case there is a strong dependence of second-order inactivation rate on the leaving group pK_a (Smith et al., 1988a; Krantz et al., 1991). Thus, cathepsin B inhibitors containing a carboxylate leaving group of relatively high pK_a (2,6-dimethylbenzoate, pK_a 3.35) are slow inactivators ($k_{inact}/K_i = 1.4 \times 10^4$ M^{-1} s^{-1}) when contrasted with the analogous ICE tetrapeptide (acyloxy)-methyl ketone **2** ($k_{inact}/K_i = 1 \times 10^6$ M^{-1} s^{-1}). A comparison of the binding constants of the corresponding aldehydes for ICE (0.1 nM)[5] and cathepsin B (21 nM) (Smith et al., 1988b), a difference of 200-fold, suggests a potential rationale for this difference in potency. Krantz has proposed that inactivation of cysteine proteases by (acyloxy)methyl ketones proceeds through formation of a thiohemiketal, analogous to the thiohemiacetal formed with peptide aldehydes (Krantz et al., 1991). Consequently, the increased potency of these inhibitors for ICE vs cathepsin B could be entirely accounted for by an increase in binding affinity, rather than a difference in the catalysis of inactivation.

Four lines of evidence suggested that tetrapeptide (acyloxy)-methyl ketones would be highly selective ICE inhibitors. First, the enzyme has a stringent and unusual requirement for aspartic acid in the P_1 position. Second, tetrapeptide aldehydes are potent and highly selective reversible inhibitors (Thornberry et al., 1992). Third, as noted above, rapid rates of inactivation are achieved with inhibitors containing leaving groups that are relatively difficult to displace by other thiol proteases. Finally, the second-order rate constant for inactivation of the enzyme by a representative member of this class, **2**, is at least 10^9-fold larger than the rate constant for the reaction of glutathione with this inhibitor measured under the same conditions (data not shown). Selectivity was demonstrated for the biotin conjugate of this inhibitor, **3**, through affinity labeling of crude THP.1 cell cytosol, where ICE constitutes <0.001% of the total protein. Under conditions where greater than 99.99% of the ICE is inactivated ($28t_{1/2}$), there is no detectable labeling of any other proteins.

It is not yet clear that ICE is an appropriate target for therapeutic intervention in inflammatory diseases. Although studies with IL-1 receptor antagonist indicate that IL-1 is a major mediator of inflammation (Dinarello & Thompson, 1991), the relative contributions of IL-1α and IL-1β *in vivo* have yet to be determined. While the recent discovery of the cowpox virus ICE inhibitor (crmA) establishes the importance of ICE, and thus IL-1β, in the host response to infection, it also raises serious questions as to the safe administration of ICE inhibitors. Tetrapeptide (acyloxy)methyl ketones, because of their selectivity and potency, are excellent candidates for *in vivo* studies aimed at addressing these issues.

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[5] The value of 0.1 nM differs from the previously reported value (0.76 nM) as it is corrected for hydration (14%) of the peptide aldehyde.

REFERENCES

Black, R. A., Kronheim, S. R., & Sleath, P. R. (1989) *FEBS Lett. 247* (2), 386–390.

Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D., & Knowles, J. R. (1988) *Biochemistry 27*, 1158–1167.

Chapman, K. T. (1992) *Bioorg. Med. Chem. Lett. 2* (6), 613–618.

Dinarello, C. A., & Thompson, R. C. (1991) *Immunol. Today 12* (11), 404–410.

Fersht, A. (1985) in *Enzyme Structure and Mechanism, 2nd ed.*, W. H. Freeman and Co., New York.

Griffin, P. R., Coffman, J. A., Hood, L. E., & Yates, J. R. (1991) *Int. J. Mass Spectrom. Ion Phys. 111*, 131–149.

Hammes, G. G., & Schimmel, P. R. (1970) *The Enzymes (3rd Ed.)* 2, 67–114.

Hardy, L. W., & Kirsch, J. F. (1984) *Biochemistry 23*, 1275–1282.

Howard, A. D., Kostura, M. J., Thornberry, N., Ding, G. J. F., Limjuco, G., Weidner, J., Salley, J. P., Hogquist, K. A., Chaplin, D. D., Mumford, R. A., Schmidt, J. A., & Tocci, M. J. (1991) *J. Immunol. 147* (19), 2964–2969.

Kortüm, G., Vogel, W., & Adrussow, K. (1961) *Dissociation Constants of Organic Acids in Aqueous Solution*, Butterworths, London.

Kostura, M. J., Tocci, M. J., Limjuco, G., Chin, J., Cameron, P., Hillman, A. G., Chartrain, N. A., & Schmidt, J. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5227–5231.

Krantz, A., Copp, L. J., Coles, P. J., Smith, R. A., & Heard, S. B. (1991) *Biochemistry 30*, 4678–4687.

Kurz, L. C., Weitkamp, E., & Frieden, C. (1987) *Biochemistry 26*, 3027–3032.

Mahler, H. R., & Cordes, E. H. (1971) in *Biological Chemistry, 2nd ed.*, pp 267–273, Harper & Row, New York.

Miller, D. K., Ayala, J. M., Egger, L. A., Raju, S. M., Yamin, T. T., Ding, G. J.-F., 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. (1993) *J. Biol. Chem. 268* (24), 18062–18069.

Molineaux, S. M., Casano, F. J., Rolando, A. M., Peterson, E. P., Limjuco, G., Chin, J., Griffin, P. R., Calaycay, J. R., Ding, G. J.-F., Yamin, T.-T., Palyha, O. C., Luell, S., Fletcher, D., Miller, D. K., Howard, A. D., Thornberry, N. A., & Kostura, M. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1809–1813.

Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.

Pliura, D. H., Bonaventura, B. J., Smith, R. A., Coles, P. J., & Krantz, A. (1992) *Biochem. J. 288*, 759–762.

Pocker, Y., & Janjie, N. (1987) *Biochemistry 26*, 2597–2606.

Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., & Pickup, D. J. (1992) *Cell 69*, 597–604.

Sleath, P. R., Hendrickson, R. C., Kronheim, S. R., March, C. J., & Black, R. A. (1990) *J. Biol. Chem. 265* (24), 14526–14528.

Smith, R. A., Copp, L. J., Coles, P. J., Pauls, H. W., Robinson, V. J., Spencer, R. W., Heard, S. B., & Krantz, A. (1988a) *J. Am. Chem. Soc. 110*, 4429–4431.

Smith, R. A., Copp, L. J., Donnelly, S. L., Spencer, R. W., & Krantz, A. (1988b) *Biochemistry 27*, 6568–6573.

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., MacCoss, M., Mumford, R. A., Schmidt, J. A., & Tocci, M. J. (1992) *Nature 356*, 768–774.