Human and Murine Cytotoxic T Lymphocyte Serine Proteases: Subsite Mapping with Peptide Thioester Substrates and Inhibition of Enzyme Activity and Cytolysis by Isocoumarins†

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ABSTRACT: The active site structures of human Q31 granzyme A, murine granzymes (A, B, C, D, E, and F), and human granzymes (A, B, and 3) isolated from cytotoxic T lymphocytes (CTL) were studied with peptide thioester substrates, peptide chloromethyl ketone, and isocoumarin inhibitors. Human Q31, murine, and human granzyme A hydrolyzed Arg- or Lys-containing thioesters very efficiently with k_{cat}/K_M of 10^4–10^5 M^{-1} s^{-1}. Murine granzyme B was found to have Asp-ase activity and hydrolyzed Boc-Ala-Ala-Asp-SBzl with a k_{cat}/K_M value of 2.3×10^5 M^{-1} s^{-1}. The rate was accelerated 1.4-fold when the 0.05 M NaCl in the assay was replaced with $CaCl_2$. The preparation of granzyme B also had significant activity toward Boc-Ala-Ala-AA-SBzl substrates, where AA was Asn, Met, or Ser [$k_{cat}/K_M = (4–5) \times 10^4$ M^{-1} s^{-1}]. Murine granzymes C, D, and E did not hydrolyze any thioester substrate but contained minor contaminating activity toward Arg- or Lys-containing thioesters. Murine granzyme F had small activity toward Suc-Phe-Leu-Phe-SBzl, along with some contaminating trypsin-like activity. Human Q31 granzyme A, murine, and human granzyme A were inhibited quite efficiently by mechanism-based isocoumarin inhibitors substituted with basic groups (guanidino or isothiureidopropoxy). Although the general serine protease inhibitor 3,4-dichloroisocoumarin (DCI) inactivated these tryptases poorly, it was the best isocoumarin inhibitor for murine granzyme B ($k_{obs}/[I] = 3700–4200$ M^{-1} s^{-1}). Murine and human granzyme B were also inhibited by Boc-Ala-Ala-Asp-CH_2Cl; however, the inhibition was less potent than that with DCI. DCI, 3-(3-aminopropoxy)-4-chloroisocoumarin, 4-chloro-3-(3-isothiureidopropoxy)isocoumarin, and 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin inhibited Q31 cytotoxic T lymphocyte mediated lysis of human JY lymphoblasts ($ED_{50} = 0.5–5.0$ μM).

Cell-mediated killing by cytotoxic T lymphocytes (CTL)[1] and natural killer cells (NK) is important in defense against tumor cell proliferation and viral infection. Cytolytic T lymphocytes contain dense cytoplasmic granules, which are able to lyse red blood cells and tumor cells (Henkart et al., 1984; Podack, 1986; Masson et al., 1985). Considerable evidence implicates cellular proteases in the cell-killing process. Plasma protease inhibitors such as α_1-antichymotrypsin suppress natural killer cell mediated lysis (Hudig et al., 1981, 1984). Synthetic inhibitors of serine proteases, such as DFP, PMSF, TLCK, and H-D-Pro-Phe-Arg-chloromethyl ketone block CTL-mediated killing (Henkart et al., 1987; Kramer & Simon, 1987; Hudig et al., 1984; Chang & Eisen, 1980). Mechanism-based isocoumarins also block RNK-16 lymphocyte granule mediated cytolysis (Hudig et al., 1987, 1989).

Perforin and a family of highly homologous serine proteases have been isolated from mouse granules (Masson & Tschopp, 1987). Perforin is a lytic pore-forming protein (Masson & Tschopp, 1985; Podack et al., 1985; Young et al., 1986a,b) which exhibits structural and functional homology to the complement component C9 (Young et al., 1986c,d; Tschopp et al., 1986; Tschopp & Nabholz, 1990). Several serine protease genes have been sequenced from CTL and NK cells from a variety of species, and a number of enzymes have been isolated and partially characterized (Table I). Seven serine proteases, referred to as granzymes A, B, C, D, E, F, and G, have been found in the mouse granules. Granzyme A is related to a protease (HF/CTLA3) predicted from a CTL-specific clone (Gershenfeld & Weissman, 1986; Brunet et al., 1986), and granzyme B is related to a protein (CTLA1/CCPI) encoded by the CTL-specific cDNA clone (Brunet et al., 1986; Lobe et al., 1986; Jenne et al., 1988a). Granzymes C (CCPII), D, E, F, and G are highly homologous to each other and are similar to rat mast cell protease II (Lobe et al., 1986; Jenne et al., 1988b,c, 1989). Granzyme C has 67% sequence identity with granzyme B, 40% sequence identity with granzyme A, and 46% sequence identity with RMCP II, while granzyme D shows 43% sequence identity with RMCP II and only 39% homology to granzyme A. Granzymes D, E, F, and G are

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[1] Abbreviations: AA, amino acid residue; α_1-PI, α_1-protease inhibitor; AMC, 7-amino-4-methylcoumarin; Apc, *S*-(3-aminopropyl)cysteine; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; Boc, *tert*-butyloxycarbonyl; CCPI, cytotoxic cell protease I; CTL, cytotoxic T lymphocyte; DCI, 3,4-dichloroisocoumarin; DFP, diisopropyl fluorophosphate; DMF, dimethylformamide; ED_{50}, effective dose for 50% inhibition; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HF, Hannuka factor; NK, natural killer; PMSF, phenylmethanesulfonyl fluoride; Mes, 2-(*N*-morpholino)ethanesulfonic acid; RMCP II, rat mast cell protease II; RNK, rat natural killer cells; SBzl, thiobenzyl ester; SBu-i, thioisobutyl ester; THF, tetrahydrofuran; TLCK, *N*-tosyllysine chloromethyl ketone; TPCK, *N*-tosylphenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl.

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Table I: Cytotoxic T Lymphocyte Serine Proteases

enzyme	source	representative substrates	inhibitors	ref
granzyme A	mouse	Z-Lys-SBzl Pro-Phe-Arg-AMC	DFP PMSF, benzamidine, aprotinin, leupeptin	Masson & Tschopp, 1987 Young et al., 1986e Simon et al., 1986
granzyme A	human	Gly-Pro-Arg-AMC Val-Pro-Arg-AMC		Krahenbuhl et al., 1988
Q31 granzyme A (Q31 tryptase)	human	Z-Lys-SBzl, Z-Arg-SBzl D-(ε-Z)Lys-Pro-Arg-AMC	APMSF, leupeptin *p*-aminobenzamidine	Poe et al., 1988
granzyme B	mouse	NF[a]	DFP (marginal)[b]	Masson & Tschopp, 1987
granzyme B	human	NF		Krahenbuhl et al., 1988
granzyme C	mouse	NF	NF	Masson & Tschopp, 1987
granzyme D	mouse	Suc-Ala-Phe-Lys-AMC	DFP TLCK, TPCK	Masson & Tschopp, 1987 Jenne & Tschopp, 1988
granzyme E	mouse	NF	DFP (marginal)[b]	Masson & Tschopp, 1987
granzyme F	mouse	NF	NF	Masson & Tschopp, 1987
granzyme G	mouse	NF	NF	Jenne et al., 1989

[a] NF = no substrate or inhibitor was found. [b] Longer reaction time was needed.

much more similar to each other (80–90% sequence identity; Jenne et al., 1989).

Two serine proteases, granzymes A and B, have been isolated from human CTL granules and are homologous to the murine enzymes (Krahenbuhl et al., 1988). Human granzyme A has a higher reactivity for Pro-Arg-AMC substrates and a lesser activity toward other Arg- or Lys-containing substrates. Human granzyme A is also identical with the HuTPS recently isolated from a human $CD8^+$ CTL line (Fruth et al., 1987). Human Q31 granzymes A and B have been purified from the granules of human cytolytic T lymphocyte line Q31 (Poe et al., 1988, 1991a), and Q31 granzyme A appears to be identical with human granzyme A.[2] Several Arg- and Lys-containing peptide nitroanilides and thioesters are substrates, and several classes of serine protease inhibitors have also been found to inhibit this tryptase. A third human granzyme with trypsin-like specificity has been isolated from lymphokine-activated killer (LAK) cell granules (Hameed et al., 1988).

A number of commercially available synthetic substrates and inhibitors have been tested with murine and human CTL serine proteases (Table I). Murine granzymes A and D have trypsin-like activity with granzyme A having much higher activity. No substrates have been found for the other granzymes. Granzymes A and D react strongly with DFP; granzymes B and E react with DFP to a lesser extent, while granzymes C and F do not react with DFP at all. Other serine protease inhibitors including PMSF, aprotinin, leupeptin, and benzamidine are good inhibitors for the esterolytic activity of granzyme A. TPCK and TLCK inhibited granzyme D effectively but had no effect on granzyme A. Here we report studies on the substrate specificity of murine and human granzymes and Q31 granzyme A with a series of peptide thioester substrates. We have discovered that granzyme B has Asp-ase activity and the granules contain significant Met-ase activity. In addition, we report a number of specific mechanism-based isocoumarin and peptide chloromethyl ketone inhibitors which should be useful for defining the functional role of these granule enzymes in cell-mediated killing.

Materials and Methods

Q31 granzyme A and Q31 granzyme B were purified from granules of a human cytotoxic lymphocyte line by a three-step procedure (Poe et al., 1988, 1991a). The active site concentration was standardized by titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Coleman et al., 1976). Murine granzymes A–F were purified from cytoplasmic granules of cytotoxic T lymphocytes by a previously described method (Masson & Tschopp, 1987). Human granzymes A and B were isolated from granules of cloned human cytotoxic T lymphocytes as previously described (Krahenbuhl et al., 1988). Human granzyme 3 was isolated as described previously (Hameed et al., 1988) except that granules of human CTL clone KV 10 were used (Krahenbuhl et al., 1988). Z-Lys-SBzl was obtained from Sigma Chemical Co., St. Louis, MO. Suc-Phe-Leu-Phe-SBzl and all Boc-amino acids used for the synthesis of new peptide thiobenzyl esters were purchased from Bachem Bioscience Inc., Philadelphia, PA. All the Arg-containing peptide thioesters (McRae et al., 1981; Kam et al., 1987), Boc-Ala-Ala-AA-SBzl (AA = various amino acid residues; Harper et al., 1984), MeO-Suc-Ala-Ala-Pro-Lys-SBzl, and MeO-Suc-Ala-Ala-Pro-Apc-SBzl (T. Ueda, unpublished results) were synthesized as previously described. DCI (Harper et al., 1985), 3-(benzyloxy)-4-chloroisocoumarin, 4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin, 4-chloro-3-ethoxy-7-nitroisocoumarin (Harper & Powers, 1985), isocoumarins substituted with basic groups (guanidino, aminopropoxy, or isothiureidopropoxy; Kam et al., 1988), 1-naphthoyl-Arg-CF_3 (Ueda et al., 1990), and MeO-Suc-Ala-Ala-Pro-$NHCH(CH_2Ph)P(O)(OPh)_2$ (Oleksyszyn & Powers, 1989) have also been synthesized previously.

Synthesis. All new Boc and Suc peptide thiobenzyl esters were prepared with methods described previously (Harper et al., 1984) except for Asp analogues. The syntheses of Boc-Ala-Ala-Asp-SBzl and Boc-Ala-Ala-Asp-CH_2Cl are described briefly below; more complete procedures along with the syntheses of all other new compounds are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

Boc-Ala-Ala-Asp-SBzl. Boc-Asp(O-*t*-Bu)-SBzl was prepared from Boc-Asp(O-*t*-Bu)-OH by reaction with benzylmercaptan using 1-hydroxybenzotriazole hydrate and dicyclohexylcarbodiimide in THF at −5 °C. The crude product

[2] Human Q31 granzyme A has previously been called human Q31 tryptase (Poe et al., 1988). In this paper, we will use the term granzyme for any lymphocyte enzyme whose gene sequence has been determined. The terms chymase and tryptases have been frequently used in the literature to describe intracellular chymotrypsin-like and trypsin-like enzymes, primarily those found in granules in phagocytic cells such as neutrophils, mast cells, and lymphocytes. Some investigators suggest that the terms chymase and tryptase should be reserved only for the well-characterized serine proteases from mast cells such as RMCP II. We do not subscribe to such a narrow view and suggest that cathepsin G (neutrophils), RMCP II (mast cells), and the chymotrypsin-like enzyme(s) in lymphocytes are all chymases. In this paper, we use the term chymase (or tryptase) to describe a lymphocyte chymotrypsin-like (or trypsin-like) activity which has not yet been assigned to one of the granzymes, as a term describing the specificity of one or more of the granzymes, and as a term describing the family of enzymes from phagocytic cells.

was purified by chromatography (silica gel; ethyl acetate/*n*-hexane, 1:10) to give the final product (85% yield) as a pale yellow oil. H-Asp-SBzl·HCl was prepared by deblocking Boc-Asp(O-*t*-Bu)-SBzl by adding a saturated solution of HCl in ethyl acetate solution at 0 °C and then stirring for 2.5 h at 25 °C. Removal of the solvent and addition of fresh ethyl acetate give the product as a white precipitate which was dried in vacuo (87% yield). Boc-Ala-Ala-OH was converted into the corresponding mixed anhydride with *N*-methylmorpholine and isobutyl chloroformate at −15 °C in THF for 2 min, followed by the addition of triethylamine (2 equiv) in cold THF. The reaction mixture was then added to a DMF solution of H-Asp-SBzl·HCl at −15 °C, followed by stirring for 1 h. After quenching with 1 N HCl and workup, the crude product was purified by chromatography (silica gel; $CHCl_3/CH_3OH$, 50:1) and solidified with *n*-hexane to give the final product (70% yield) as a white powder: mp 72–79 °C; R_f = 0.52 ($CHCl_3/CH_3OH/CH_3CO_2H$, 80:10:5). Analysis is given in the supplementary material.

Boc-Ala-Ala-Asp-CH_2Cl. Boc-Asp(OBzl)-CHN_2 (oil) was prepared from Boc-Asp(OBzl)-OH and converted to H-Asp(OBzl)-CH_2Cl·HCl (a white powder) according to the procedure of Kettner and Shaw (1981). Without further purification, this was coupled with Boc-Ala-Ala-OH to give Boc-Ala-Ala-Asp(OBzl)-CH_2Cl by a mixed anhydride procedure. The crude Boc-Ala-Ala-Asp(OBzl)-CH_2Cl was purified by chromatography (silica gel; $CHCl_3/CH_3OH$, 100:1) and recrystallized ($CHCl_3$–CCl_4) to give pure product (64%) as white crystals: R_f = 0.23 ($CHCl_3/CH_3OH$, 20:1). The benzyl blocking group was removed by vigorously stirring with 5% Pd–C in THF for 1 h at room temperature under an atmosphere of hydrogen. The crude product was purified by chromatography (silica gel; $CHCl_3/CH_3OH$, 20:1) and solidified with *n*-hexane to give the final product (0.19 g, 64% yield) as a white powder: mp 60–75 °C; R_f = 0.40 ($CHCl_3/CH_3OH/CH_3CO_2H$, 80:10:5); mass spectrum *m/e* 408 ($M^+ + 1$). Analysis is given in the supplementary material.

Stability of the Chloromethyl Ketones. The stability of Boc-Ala-Ala-Asp-CH_2Cl and Boc-Ala-Gly-Glu-CH_2Cl was followed by TLC after incubation in water, 0.1 M Hepes and 0.05 M $CaCl_2$ at pH 7.5, and 0.1 M phosphate and 0.5 M NaCl at pH 7.5 for varing lengths of time. The Asp chloromethyl ketone was stable for several hours but exhibited ca. 30% decomposition after 6 h and 50% decomposition after 22 h in the two buffers. Little decomposition was observed in water. The Glu chloromethyl ketone was much less stable and decomposed 30% after 1 h and 60% after 3 h in the buffers. In water it was fairly stable for 6 h.

Determination of the Concentration of Murine and Human Granzyme A. Murine and human granzyme A were titrated with increasing amounts of equine antithrombin III in the presence of a small amount of heparin according to the procedure described previously for thrombin (Danielsson & Bjork, 1982). The concentration of antithrombin III solution was standardized by reaction with titrated bovine trypsin. Trypsin solution was titrated with *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1970), and the stoichiometry of inhibition of trypsin by antithrombin III was determined by incubating trypsin (0.15 μM) with increasing amounts of inhibitor (0.1–0.48 μM) and a small amount of heparin (0.19 mg/mL, 28 units/mL) for 2 min at room temperature in 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5. The residual activity of trypsin was measured by transfering 25 μL of the enzyme–inhibitor solution into 2 mL of buffer containing 150 μL of 4,4'-dithiopyridine (0.34 mM) and 25 μL of Z-Arg-SBzl (0.061 mM). The plot of residual activity of trypsin vs inhibitor concentration gave the exact amount of inhibitor required to form a one to one complex with enzyme. Murine granzyme A (0.09 μM) and human granzyme A (0.39 μM) were titrated with various amounts of antithrombin III (0.02–0.28 and 0.1–0.48 μM, respectively) according to the same procedure with the exception that a longer incubation time was used (15 and 20 min).

Substrate Kinetics. The enzymatic hydrolysis of peptide thioester substrates was measured in 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, buffer containing 8% Me_2SO and at 25 °C in the presence of 4,4'-dithiodipyridine (Grassetti & Murray, 1967). Stock solutions of substrate were prepared in Me_2SO and stored at −20 °C. The initial rates were measured at 324 nm (ϵ_{324} = 19800 M^{-1} cm^{-1}) on a Beckman 35 or Varian DMS-90 spectrophotometer after 10–25 μL of an enzyme stock solution was added to a cuvette containing 2.0 mL of buffer, 150 μL of 4,4'-dithiodipyridine (5 mM), and 25 μL of substrate. The same volumes of substrate and 4,4'-dithiodipyridine were added to the reference cell in order to compensate for the background hydrolysis rate of the substrates. Initial rates were measured in duplicate for each substrate concentration and were averaged in each case. The kinetic constants k_{cat}, k_M, and k_{cat}/k_M were obtained from Lineweaver–Burk plots which had correlation coefficients greater than 0.99.

Inhibition Kinetics: Incubation Method. The inactivation reaction was initiated at 25 °C by adding a 25-μL aliquot of inhibitor in Me_2SO to 0.3 mL of buffered enzyme solution (30–600 nM) such that the final Me_2SO concentration was 8–9%. Aliquots were removed at various time intervals and diluted into substrate solution (90-fold dilution), and the residual activity was measured spectrophotometrically. Q31 granzyme A, murine granzyme A, and human granzyme A were assayed with Z-Arg-SBzl, and murine granzyme B was assayed with Boc-Ala-Ala-Ser-SBzl, by the procedure described above. First-order inactivation rate constants (k_{obs}) were obtained from plots of $\ln v_i/v_o$ vs time and had correlation coefficients greater than 0.98. Inactivation rate constants were the average of duplicate experiments.

Several isocoumarin inhibitors of Q31 granzyme A had both time-dependent and time-independent components of inhibition. To measure the time-independent components, which reflected the binding affinity of the enzyme for the isocoumarin, a plot of enzyme activity at time t [$v(t)$] was made versus time for five to nine inhibitor concentrations at 0.3 mM substrate; the plots were extrapolated to the time of inhibitor–enzyme mixing to get v at zero time [$v(t=0)$]. A nonlinear least-squares fit to a plot of $v(t=0)$ versus inhibitor concentration was used to calculate IC_{50}, the concentration of inhibitor required to inhibit Q31 granzyme A by 50% before the time-dependent component of inhibition made any significant contribution to inhibition.

Cytotoxicity Inhibition Assays. Cytotoxicity assays [see Engers et al. (1986) for a detailed protocol] were done with JY target cells (a lymphoblastoid cell line) maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (Hyclone Labs, Logan, UT), glutamine, and penicillin–streptomycin. Q31 cells (a human cytotoxic T lymphocyte cell line) were maintained in the same media with 30 units/mL recombinant IL-2 (Amgen Biologicals, Thousand Oaks, CA). All other media components were from GIBCO (Bethpage, NY). Q31 and JY (Biddison et al., 1984) were a gift from Dr. Biddison at the Neuroimmunology Branch of

NINCDS (Bethesda, MD), grown by M. Gammon at the Department of Immunology of Merck. Cells were grown in 5% CO_2 at 37 °C. Cell viability was determined by the trypan blue exclusion method.

For the cytotoxicity assays, JY target cells in logarithmic growth were labeled with $Na_2{}^{51}CrO_4$ by use of 200 μCi with (1–6) × 10^6 cells/mL for 60 min at 37 °C in growth media. Isocoumarins were dissolved in Me_2SO at a concentration such that 0.5% Me_2SO would be present in the cytotoxicity assays. An initial titration was done to establish the ratio of Q31 to JY, i.e., effector to target, so that 50–70% of the JY targets were lysed by the Q31. For inhibitor studies, Q31 cells in microtiter plates were mixed with various concentrations of inhibitor in 0.1 mL of growth media and allowed to incubate for 15 min at 37 °C prior to the addition of 0.1 mL of labeled JY target cells. Following a 4-h incubation at 37 °C, the plates were centrifuged for 3 min (20*g*), and 0.1 mL of the supernate was removed for counting. The released radioactivity was corrected for release by 0.1 mL of 1 N HCl (total lysis). A plot of specific lysis [(actual lysis − spontaneous lysis)/(total lysis − spontaneous lysis)] versus isocoumarin concentration was visually interpolated to the concentration that inhibited Q31-mediated lysis of JY by 50%. Repeated determination of ED_{50} for a particular isocoumarin was always reproducible to better than ±30%. For a further discussion of the cytotoxicity assays, see Poe et al. (1991b).

Results

Substrate Specificity. Unfractionated murine CTL granules were tested for serine protease activity by use of thioester substrates containing various amino acid residues at the P_1 site [subsite nomenclature of Schecter and Berger (1967)] such as Ala, Apc, Arg, Asn, Glu, Ile, Leu, Lys, Met, Nle, Nva, Phe, Pro, Ser, and Val (Table 1 of the supplementary materials). The granules effectively hydrolyzed substrates which contain Arg or Lys at P_1 (herein referred to as tryptase activity) and Suc-Phe-Leu-Phe-SBzl (chymase activity), even though the latter hydrolysis rate was 30-fold lower than the rate with Z-Arg-SBzl. The granules also hydrolyzed Boc-Ala-Ala-AA-SBzl, where AA was Met, Asn, and Ser, but had no activity with typical elastase substrates or with substrates where AA was Pro and Glu. Granules treated with 1.5 M NaCl and 10 mM benzamidine showed similar activity toward thioester substrates; however, the rates were 3–10-fold lower when compared to granules in buffer (100 mM KCl, 3.5 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1.25 mM EGTA, 10 mM Pipes, pH 6.8) and Percoll (1.11 g/cm^3).

Murine granule proteins of CTL line B6.1, which had been depleted of perforin, were loaded onto a Mono S cation exchange column and were subsequently eluted by increasing the NaCl concentration [elution pattern shown in Jenne and Tschopp (1988)]. Activities of the protein fractions were monitored with five thioester substrates: Z-Arg-SBzl, Suc-Phe-Leu-Phe-SBzl, Boc-Ala-Ala-Asp-SBzl, Boc-Ala-Ala-Met-SBzl, and Boc-Ala-Ala-Ser-SBzl. Two peaks were found to have Z-Arg-SBzl activity. The large peak which was eluted with 650–830 mM NaCl corresponds to the fractions containing granzymes A, F, and C, while the small peak which was eluted at 500 mM NaCl corresponds to the fractions eluting after the granzyme D and granzyme E fractions. Suc-Phe-Leu-Phe-SBzl hydrolyzing activity was also found in the granzyme A and F fractions. The peak with Boc-Ala-Ala-Asp-SBzl activity corresponds to the fractions containing granzyme B (elution at 500 mM NaCl). These fractions also contain Boc-Ala-Ala-Met-SBzl and Boc-Ala-Ala-Ser-SBzl hydrolyzing activities.

Purified murine granzymes A, B, C, D, E, and F were tested with the same set of substrates used in Table 1 of the supplementary material; the data are shown in Table 2 of the supplementary material. Granzyme A hydrolyzed Arg- or Lys-containing substrates most effectively but also hydrolyzed substrates such as Suc-Phe-Leu-Phe-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl at much slower rates. This enzyme did not hydrolyze substrates containing Val or any other amino acid residues at the P_1 site. Granzyme B hydrolyzed Boc-Ala-Ala-AA-SBzl (AA = Asp, Asn, Met, Ser, Thr, and Nval) effectively and had lesser activity toward some P_1 Glu, Arg, and Phe substrates. Granzyme C did not hydrolyze any thioester substrate, while granzymes D, E, and F showed a little activity toward Arg- or Lys-containing substrates. Granzyme F also had activity toward Suc-Phe-Leu-Phe-SBzl (Table 2, supplementary material).

Once the most reactive substrates for various granzymes had been determined, Michaelis–Menten parameters were measured with these substrates. Murine granzyme A hydrolyzed all P_1 Arg and Lys thioesters quite efficiently, and the kinetic constants of these substrates are shown in Table II. Surprisingly, this enzyme also hydrolyzed Suc-Phe-Leu-Phe-SBzl, a chymase substrate, with a k_{cat}/K_M value ca. 30-fold lower than that of the P_1 Arg thioesters. The exact concentration of murine and human granzyme A stock solutions was determined by titration with antithrombin III in the presence of a small amount of heparin (0.19 mg/mL, 28 units/mL). It is assumed that granzyme A forms a one to one complex with antithrombin III in the presence of heparin. Without heparin, the inhibition of granzyme A by antithrombin III was very slow; however, the addition of heparin to the mixture of granzyme A and inhibitor resulted in complete inhibition after 20 min. Attempts to titrate granzyme A with methylumbelliferyl *p*-guanidinobenzoate (Coleman et al., 1976) or *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1970) were unsuccessful.

Murine granzyme B hydrolyzed Boc-Ala-Ala-AA-SBzl, where AA was Asp, Asn, Met, and Ser, with k_{cat}/K_M values of (4–23) × 10^4 M^{-1} s^{-1}. Granzyme B had similar activity with thioester substrates when compared to granzyme A. The best granzyme B substrate is Boc-Ala-Ala-Asp-SBzl with a k_{cat}/K_M value of 230000 M^{-1} s^{-1}, which is 1.6-fold lower than the value of Z-Gly-Arg-SBzl for granzyme A. Mouse granzyme B also hydrolyzed the tripeptide substrates Boc-Ala-Phe-Asp-SBzl and Boc-Phe-Ala-Asp-SBzl with almost the same rate as for Boc-Ala-Ala-Asp-SBzl, but the hydrolysis rate of the amino acid analogue Z-Asp-SBzl was very low (Table II). Granzymes D, E, and F hydrolyzed Z-Arg-SBzl with k_{cat}/K_M values of 11 000, 13 000, and 37 000 M^{-1} s^{-1}, respectively, and these values were 1 order of magnitude lower than the k_{cat}/K_M of the same substrate with granzyme A. This low thioesterase activity is probably due to the presence of contaminating granzyme A or another tryptase activity in the granzyme D, E, and F samples.

The effect of pH on the activity of murine granzyme B was also measured in the pH range of 6.0–8.0 with two substrates, Boc-Ala-Ala-Asp-SBzl and Boc-Ala-Ala-Met-SBzl (Figure 1). The pH optimum for both Boc-Ala-Ala-Asp-SBzl and Boc-Ala-Ala-Met-SBzl was pH 7.5. The effect of salt on activities of granzyme B was also tested in 0.1 M Hepes, pH 7.5, buffer (Table IV). The enzymatic hydrolysis rate of Boc-Ala-Ala-Met-SBzl was 70–80% higher in the presence of 0.1–1.0 M NaCl than in its absence. The maximum rate of Boc-Ala-Ala-Asp-SBzl hydrolysis was obtained in 0.05 M $CaCl_2$, and this rate was ca. three times the rate measured in

Table II: Kinetic Constants for the Hydrolysis of Peptide Thioester Substrates by Murine Granzyme A, Murine Granzyme B, Human Granzyme A, and Human Q31 Granzyme A[a]

substrates	kinetic constants[b]	enzymes: mouse granzyme A	mouse granzyme B	human granzyme A	Q31 granzyme A
Z-Lys-SBzl	k_{cat}	22	NH[c]	3.1	
	K_M	130		190	
	k_{cat}/K_M	170 000		16 000	
Z-Arg-SBzl	k_{cat}	83		8.8	6.6
	K_M	315		120	120
	k_{cat}/K_M	260 000		71 000	56 000
Z-Gly-Arg-SBzl	k_{cat}	59		6.1	6.2
	K_M	160		105	200
	k_{cat}/K_M	370 000		58 000	31 000
Bz-Lys-Arg-SBzl	k_{cat}				24
	K_M				110
	k_{cat}/K_M				220 000
Bz-Glu-Lys-Arg-SBzl	k_{cat}				14
	K_M				280
	k_{cat}/K_M				48 000
Boc-Ala-Ala-Arg-SBzl	k_{cat}	45			
	K_M	140			
	k_{cat}/K_M	320 000			
MeO-Suc-Ala-Ala-Pro-Lys-SBzl	k_{cat}	21			
	K_M	410			
	k_{cat}/K_M	52 000			
Z-Ile-Ala-Gly-Arg-SBzl	k_{cat}				
	K_M				
	k_{cat}/K_M				22 000
Suc-Phe-Leu-Phe-SBzl	k_{cat}	1.2			
	K_M	130			
	k_{cat}/K_M	9 100			
Boc-Ala-Ala-Met-SBzl	k_{cat}	NH	24		
	K_M		550		
	k_{cat}/K_M		43 000		
Boc-Ala-Ala-Asp-SBzl	k_{cat}	NH	116		
	K_M		500		
	k_{cat}/K_M		230 000		
Boc-Ala-Ala-Asn-SBzl	k_{cat}	NH			
	K_M				
	k_{cat}/K_M		49 000		
Boc-Ala-Ala-Ser-SBzl	k_{cat}	NH			
	K_M				
	k_{cat}/K_M		43 000		

[a] All kinetic constants were measured in 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, at 25 °C in the presence of 4,4′-dithiodipyridine. Enzyme concentrations: mouse granzyme A, 8.0–77 nM; mouse granzyme B, 3.9–7.8 nM; human granzyme A, 23.3 nM; human Q31 granzyme A, 7.1 nM. [b] The units for k_{cat}, K_M, and k_{cat}/K_M are s^{-1}, μM, and M^{-1} s^{-1}. [c] NH = no hydrolysis.

Table III: Enzymatic Hydrolysis Rates of Peptide Thioesters by Mouse Granzyme B and Human Q31 Granzyme B[a]

substrates	[S] (μM)	rates (nM s^{-1}): mouse	human Q31
Z-Asp-SBzl	100	4.2	
Boc-Ala-Ala-Asp-SBzl	118	52.0	18.7
Boc-Ala-Phe-Asp-SBzl	104	62.8	19.4
Boc-Phe-Ala-Asp-SBzl	117	42.9	13.5
Boc-Ala-Ala-Met-SBzl	117		1.7
Boc-Ala-Ala-Glu-SBzl	227		2.9

[a] Hydrolysis rates were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 8% Me_2SO at 25 °C. 10 μL of mouse granzyme B stock solution (0.2 mg/mL) or 25 μL of human Q31 granzyme B stock solution (1.29 μg/mL) was added to assay mixtures containing 2 mL of buffer, 0.15 mL of 4,4′-dithiodipyridine (0.34 mM), and 25 μL of substrate.

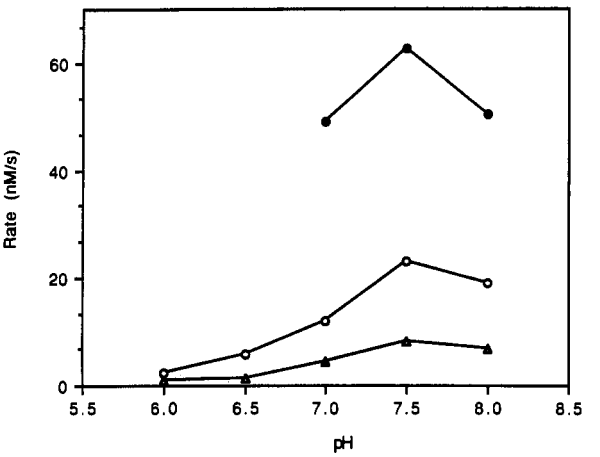


FIGURE 1: pH profile of mouse granzyme B. Hydrolysis rates of Boc-Ala-Ala-Asp-SBzl (●, ○) and Boc-Ala-Ala-Met-SBzl (▲) were measured in buffer solutions containing 0.1 M Mes (pH 6.0 and 6.5), Hepes (pH 7.0 and 7.5), and Tris (pH 8.0) with 0.5 M NaCl (○, ▲) or 0.05 M $CaCl_2$ (●) at 25 °C. Substrate concentrations were 100 μM for Boc-Ala-Ala-Asp-SBzl and 150 μM for Boc-Ala-Ala-Met-SBzl. Mouse granzyme B concentration was 1.3 nM.

0.05 M NaCl. Other salts such as KCl or $MgCl_2$ had approximately the same effect as NaCl, when the salt concentration was 0.1 M.

Human granzymes A, B, and 3 were also tested with the same set of thioester substrates (Table 3 of the supplementary material). The human enzyme, like mu.ine granzyme A, also hydrolyzed P_1 Arg or Lys thioester substrates with comparable k_{cat}/K_M values (Table II); however, it did not hydrolyze thioesters with P_1 Phe or any other amino acid residue. Human Q31 granzyme A also hydrolyzed most P_1 Arg or Lys thioesters with k_{cat}/K_M values of 10^4–10^5 M^{-1} s^{-1} (Table II).

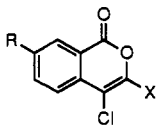
Table IV: Effect of Salt on Activity of Mouse Granzyme B with Two Substrates[a]

salt	concn (M)	rate (nM s^{-1}) Boc-Ala-Ala-Asp-SBzl	rate (nM s^{-1}) Boc-Ala-Ala-Met-SBzl
NaCl	0	33.7[b]	3.5
	0.01	8.8[b]	
	0.05	21.0[b]	
	0.1	34.3	5.9
	0.25	29.9	6.3
	0.5	23.6	5.9
	1.0	17.7	6.3
KCl	0.1	34.3	5.9
$CaCl_2$	0.01	43.8[b]	
	0.05	62.6[b]	
	0.1	38.0[b]	
	0.1	35.8	6.7
$MgCl_2$	0.1	38.7	6.3

[a] Hydrolysis rates were measured in 0.1 M Hepes buffer solutions at 25 °C. Substrate concentrations were 83 μM for Boc-Ala-Ala-Asp-SBzl and 97 μM for Boc-Ala-Ala-Met-SBzl. Mouse granzyme B concentration was 1.3 nM. [b] The concentration of Boc-Ala-Ala-Asp-SBzl was 100 μM.

Although human granzyme A and Q31 granzyme A were purified from different CTL cell lines, these two enzymes have similar activity toward Arg-containing thioester substrates. Human granzyme B and 3 had very low activities toward P_1 Arg or Lys substrates. Human CTL Q31 granzyme B had Met-ase and Glu-ase activities, but compared to the Asp-ase activity these activities were very low (Table III).

Inhibition Kinetics. Dichloroisocoumarin and other substituted isocoumarins (mechanism-based inhibitor structures shown in Figure 2) were tested as inhibitors for murine granzymes A and B and human granzyme A, and the second-order inhibition constants ($k_{obs}/[I]$) are shown in Table V. The inhibition of Q31 granzyme A by various substituted isocoumarins, the trifluoromethyl ketone 1-naphthoyl-Arg-CF_3 (a transition-state analogue), and the peptidyl phosphonate MeO-Suc-Ala-Ala-Pro-NHCH(CH_2Ph)P(O)($OPh)_2$ (phosphonylates Ser-195) was measured at pH 7.5 or 7.25 (Table VI). DCI, a general serine protease inhibitor, inhibited all three tryptases (murine granzyme A, human granzyme A, and Q31 granzyme A) poorly with $k_{obs}/[I]$ values of 30–50 M^{-1} s^{-1}. Substituted isocoumarins with basic functional groups (aminopropoxy, guanidino, or isothiureidopropoxy), first synthesized as inhibitors for trypsin and blood coagulation enzymes (Kam et al., 1988), inhibited the tryptase very efficiently. The most potent inhibitor for murine granzyme A was 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**) with a $k_{obs}/[I]$ value of 26 000 M^{-1} s^{-1}, which is similar to the values observed with some coagulation enzymes. 4-Chloro-3-(3-isothiureidopropoxy)isocoumarin (**6**) also inhibited this enzyme quite well but with a slightly lower inhibition constant. For human granzyme A and Q31 granzyme A, the isothiureido compound (**6**) was the most potent inhibitor with a $k_{obs}/[I]$ value of 13 000–18 000 M^{-1} s^{-1} at pH 7.5, while the guanidino isocoumarin (**9**) inhibited these two enzymes less effectively ($k_{obs}/[I]$ = 6000–7000 M^{-1} s^{-1}). 7-Amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**7**) was a reasonably good inhibitor for both murine and human granzyme A, but had $k_{obs}/[I]$ values ca. 3–6-fold less than the value for the isothiureido compound (**6**).



R = H, X = Cl (1, DCI)
R = H, X = O-$(CH_2)_3$-SC(=NH_2^+)NH_2 (**6**)
R = NH_2, X = O-$(CH_2)_3$-SC(=NH_2^+)NH_2 (**7**)
R = NHC(=NH_2^+)NH_2, X = OCH_2CH_3 (**9**)

FIGURE 2: Structures of representative substituted isocoumarins. The R group is located at the 7-position and the X group at the 3-position of the isocoumarin ring.

The best isocoumarin inhibitor for murine granzyme B was DCI with a $k_{obs}/[I]$ value of 4180, 3700, and 3930 M^{-1} s^{-1}, respectively, when Boc-Ala-Ala-Asp-SBzl, Boc-Ala-Ala-Ser-SBzl, and Boc-Ala-Ala-Met-SBzl were used to monitor the residual enzyme activity. All other compounds with various substituents showed only slight inhibitory potency. The peptide chloromethyl ketone Boc-Ala-Ala-Asp-CH_2Cl, which has the same sequence as the best substrate for murine granzyme B, was a slow inhibitor ($k_{obs}/[I]$ = 2.0 M^{-1} s^{-1}). The inhibition rates of granzyme B by Boc-Ala-Gly-Glu-CH_2Cl and Boc-Ala-Ala-Asp(OBzl)-CH_2Cl were even slower than the rate with Boc-Ala-Ala-Asp-CH_2Cl. Murine granzymes D, E, and F were also tested with DCI and the guanidinoisocoumarin (**9**) using Z-Arg-SBzl as the substrate. With DCI, granzymes D, E, and F had $k_{obs}/[I]$ values of 110, 240, and 140 M^{-1} s^{-1}, respectively. With guanidinoisocoumarin (**9**), granzymes D, E, and F had $k_{obs}/[I]$ values of 31 000, 36 000, and 51 000 M^{-1} s^{-1}, respectively. Murine granzymes A and B were also tested with α_1-protease inhibitor. No inhibition was observed when either granzyme A or B was incubated with α_1-PI ([I]/[E] = 300–5600) at room temperature in the pH 7.5 buffer for 20 min.

Table V: Inactivation Rates of Murine Granzyme A, Murine Granzyme B, and Human Granzyme A by Substituted Isocoumarins[a]

inhibitors	[I] (μM)	$k_{obs}/[I]$ (M^{-1} s^{-1}) mouse granzyme A	mouse granzyme B	human granzyme A
3,4-dichloroisocoumarin (**1**)	8.2		3 700	
	45–500	50	(4 200)[b] (3 900)[c]	50
7-amino-4-chloro-3-methoxyisocoumarin (**2**)	420		8.1	
4-chloro-3-methoxyisocoumarin (**3**)	39		21	
4-chloro-3-methoxy-7-[(phenylcarbamoyl)amino]isocoumarin (**4**)	44		18	
3-(3-aminopropoxy)-4-chloroisocoumarin (**5**)	1.1–15	770		2 000
4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**6**)	1.1–3.9	18 000		18 000
7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**7**)	3.7–4.3	3 000		6 800
4-chloro-7-guanidino-3-methoxyisocoumarin (**8**)	0.41–2.6	15 000		
4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**)	0.42–3.8	26 000		6 900
	420		17	
4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**10**)	0.43–4.9	6 400		

[a] Inactivation rates were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 8% Me_2SO at 25 °C by the incubation method. Z-Arg-SBzl (74–85 μM) was used as the substrate to monitor the residual enzymatic activity of murine and human granzyme A. Boc-Ala-Ala-Ser-SBzl (121 μM) was used as the substrate for murine granzyme B. [b] Boc-Ala-Ala-Asp-SBzl (130 μM) was used as the substrate for murine granzyme B. [c] Boc-Ala-Ala-Asp-SBzl (123 μM) was used as the substrate for murine granzyme B.

Table VI: Inhibition of Human Q31 CTL Granzyme A by Substituted Isocoumarins and Other Classes of Serine Protease Inhibitors[a]

compounds	IC_{50} (μM)[b]	$k_{obs}/[I]$ (M^{-1} s^{-1})
3,4-dichloroisocoumarin (**1**)	130	12
		29[c]
3-(benzyloxy)-4-chloroisocoumarin	3000	7.1
4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin	≫50	NI[d]
4-chloro-3-ethoxy-7-nitroisocoumarin	26	7200
3-(3-aminopropoxy)isocoumarin	240	NI[d]
3-(3-aminopropoxy)-4-chloroisocoumarin (**5**)	≫10	480
4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**6**)		13000[c]
7-amino-4-chloro-3-(3-isothiureidopropoxy)-isocoumarin (**7**)		2000[c]
7-guanidino-3-methoxyisocoumarin	630	48
4-chloro-7-guanidino-3-methoxyisocoumarin (**8**)	6.7	3300
		6600[c]
3-ethoxy-7-guanidinoisocoumarin	2500	62
4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**)	≫10	1500
		6200[c]
7-guanidino-3-(2-phenylethoxy)isocoumarin	210	30
4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**10**)		1900[c]
1-naphthoyl-Arg-CF_3	160	11[e]
MeO-Suc-Ala-Ala-Pro-NHCH(CH_2Ph)-P(O)($OPh)_2$	420	9.0

[a] Inhibition constants were measured in 50 mM Hepes, 0.3 M NaCl, and 10 mM $CaCl_2$, pH 7.25, at 25 °C, which are the same conditions used previously with the Q31 CTL granzyme A (Poe et al., 1988). [b] IC_{50} was the concentration of inhibitor that gave 50% inhibition of Q31 granzyme A at the time of mixing, before any significant contribution was made by the time-dependent component of inhibition (see Materials and Methods). [c] Inhibition constants were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 8% Me_2SO at 25 °C by the incubation method. Z-Arg-SBzl (74 μM) was used as a substrate. [d] No time dependence in inhibition. [e] Slow tight-binding inhibitor.

The inhibitors of human Q31 granzyme A with the largest $k_{obs}/[I]$ values at pH 7.25 are the isothiureidoisocoumarin (**6**) and 4-chloro-3-ethoxy-7-nitrosoisocoumarin. The isocoumarins with bulky hydrophobic groups such as benzyloxy at the 3-position had much smaller $k_{obs}/[I]$ values with this lymtryptase. The guanidinoisocoumarins without the chlorine at the 4-position had 25–70-fold smaller $k_{obs}/[I]$ values than those with the chlorine (**8**, **9**, or **10**). The arginine trifluoromethyl ketone 1-naphthoyl-Arg-CF_3, which inhibits trypsin and several coagulation enzymes (Ueda et al., 1990), had a small inhibition constant for Q31 granzyme A. MeO-Suc-Ala-Ala-Pro-NHCH(CH_2Ph)P(O)($OPh)_2$, which is a potent inhibitor for chymotrypsin (Oleksyszyn & Powers, 1989), was also a poor inhibitor of the Q31 granzyme A.

The lysis of human JY lymphoblasts by Q31 cytotoxic T lymphocytes was measured at six to eight concentrations of various inhibitors (Table VII). The concentration of inhibitor required to reduce lysis by 50% (ED_{50}) was calculated from plots of lysis versus inhibitor concentration. The inhibitors with the smallest ED_{50} values were the two isothiureidoisocoumarins **6** and **7**. Three compounds, DCI, 4-chloro-3-ethoxy-7-nitroisocoumarin, and 3-(3-aminopropoxy)-4-chloroisocoumarin, had ED_{50} values significantly smaller than the IC_{50} (the concentration required for 50% inhibition of Q31 granzyme A). This may indicate the involvement of other enzymes in cytolysis or processes which affect the concentrations or stability of the various inhibitors.

Table VII: Inhibition of Human Q31 Cytotoxic T Lymphocyte Mediated Lysis of Human JY Lymphoblasts by Substituted Isocoumarins and Other Serine Protease Inhibitors

compounds	ED_{50} (μM)[a]
3,4-dichloroisocoumarin (**1**)	5.0
3-(benzyloxy)-4-chloroisocoumarin	32
4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin	53
4-chloro-3-ethoxy-7-nitroisocoumarin	6.0
3-(3-aminopropoxy)isocoumarin	>20
3-(3-aminopropoxy)-4-chloroisocoumarin (**5**)	5.0
4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**6**)	0.46
7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**7**)	3.2
7-guanidino-3-methoxyisocoumarin	>250
4-chloro-7-guanidino-3-methoxyisocoumarin (**8**)	>20
3-ethoxy-7-guanidinoisocoumarin	>250
4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**)	>20
7-guanidino-3-(2-phenylethoxy)isocoumarin	20
4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**10**)	10
1-naphthoyl-Arg-CF_3	>250
MeO-Suc-Ala-Ala-Pro-NHCH(CH_2Ph)P(O)($OPh)_2$	>250

[a] ED_{50} is the concentration of inhibitor that inhibits Q31/JY cytolysis by 50%.

Discussion

Cytolytic T and natural killer lymphocytes kill virally infected cells and tumor cells. Synthetic inhibitors of serine proteases, such as DFP, PMSF, TLCK, and H-D-Pro-Phe-Arg-CH_2Cl, blocked the CTL-mediated killing (Redelman & Hudig, 1980; Hudig et al., 1984; Henkart et al., 1987; Kramer & Simon, 1987; Chang & Eisen, 1980). Plasma protease inhibitors such as α_1-antichymotrypsin also suppress the lysis when present in the natural killer (NK) cell assays (Hudig et al., 1981, 1984). These effects suggest that granule proteases from cytotoxic T and natural killer lymphocytes are involved in the cell-mediated killing.

In an attempt to understand the mechanism of the target cell lysis and the functional role of these granule proteases in the cell-mediated killing, murine granzymes A, B, C, D, E, F, and G (Masson & Tschopp, 1987), human granzymes A, B, and 3 (Krahenbuhl et al., 1988; Hameed et al., 1988), and human Q31 granzymes A and B have been isolated and purified. Granzyme A (HF), granzyme B (CCPI), granzyme C (CCPII), and granzymes D, E, F, and G have been cloned, sequenced, and structurally characterized as serine proteases (Gershenfeld & Weissman, 1986; Brunet et al., 1986; Lobe et al., 1986; Jenne et al., 1988a,b, 1989). Granzymes A and D have trypsin-like activity with synthetic substrates, but no substrates have been found for the other granzymes (Masson & Tschopp, 1987; Jenne & Tschopp, 1988).

When we tested unfractionated mouse granules with a series of very sensitive peptide thioester substrates, high trypsin-like activity was found, but the granules also hydrolyzed peptide thioester substrates containing Phe, Met, Asn, or Ser at P_1, although at slower rates than with the Lys and Arg substrates. Thus it appeared that the granules contained a tryptase activity, a chymase activity, an activity directed toward small polar amino acid residues, and an activity which we referred to as a Met-ase activity. At this point it was not clear if all of these activities were distinct enzymes since thioester substrates are very reactive, but also fairly unspecific.

Granzyme A. Murine granzyme A is clearly a potent tryptase as evidenced by its high thioesterase activity toward substrates containing P_1 Arg or Lys residues. This enzyme prefers Arg substrates over Lys substrates and is more reactive toward extended peptide substrates than simple amino acid derivatives. Mouse granzyme A is also an excellent tryptase toward thioester substrates, with k_{cat}/K_M values near 10^5 M^{-1} s^{-1}, which is 1 order of magnitude smaller than the values obtained with trypsin (McRae et al., 1981). The Arg- and Lys-specific peptide thioesterase activity is consistent with the earlier observations that granzyme A effectively hydrolyzed

Table VIII: Important Amino Acid Residues in the Substrate Binding Site of Murine Granzymes[a]

enzymes	key residues					predicted specificity at P_1 site
				S_1		
	S_4 (192)	S_3 (218)	S_2 (99)	189	226	
granzyme A	Asn	Glu	Arg	Asp	Gly	Arg/Lys
granzyme B	Arg	Lys	Phe	Ala	Arg	Asp
granzyme C	Glu	Thr	Arg	Ala	Gln	Asn/Ser
granzyme D	Lys	Asn	Phe	Thr	Gly	Phe/Leu
granzyme E	Lys	Asn	Phe	Thr	Gly	Phe/Leu
granzyme F	Ser	Asn	Asn	Ser	Gly	Phe/Leu
granzyme G	Glu	Asn	Gly	Ala	Gly	Phe/Leu

[a] The numbering of the sequences is based on chymotrypsinogen.

Z-Lys-SBzl and peptidyl 7-amino-4-methylcoumarinamide derivatives with Arg or Lys at P_1.

Human granzyme A from two cell lines was 6–10-fold less reactive than the murine enzyme but also seemed to prefer Arg substrates. The catalytic efficiency (k_{cat}/K_M) of the Q31 granzyme A for Z-Arg-SBzl of 56 000 M^{-1} s^{-1} is similar to the catalytic efficiency for Z-Arg-SBzl of human granzyme A (71 000 M^{-1} s^{-1}). Thus it appears that the granzymes A expressed by two different lines of human CTLs are catalytically similar but less effective than murine granzymes A.

Murine granzyme A also had slight activity toward the cathepsin G/chymase substrate Suc-Phe-Leu-Phe-SBzl. This chymase activity in granzyme A could be due to a contaminating enzyme (possibly granzyme F) in the granzyme A preparation or could represent an intrinsic activity of granzyme A. Thioester substrates are very reactive but not highly specific. For example, both trypsin and chymotrypsin will cleave Z-Gly-Arg-SBu-i and Suc-Phe-Leu-Phe-SBzl. Bovine trypsin hydrolyzes Z-Gly-Arg-SBu-i ca. 90-fold faster than Suc-Phe-Leu-Phe-SBzl, while the hydrolysis rate of Suc-Phe-Leu-Phe-SBzl by chymotrypsin is 70-fold higher than the rate of Z-Gly-Arg-SBu-i hydrolysis. In the case of murine granzyme A, Z-Gly-Arg-SBzl was hydrolyzed ca. 40-fold faster than Suc-Phe-Leu-Phe-SBzl.

A molecular model of mouse granzyme A (HF) from cytotoxic T lymphocytes has recently been constructed on the basis of the crystal structures of bovine trypsin and RMCP II (Murphy et al., 1988). Key amino acid residues in the substrate binding site of the murine granzymes and the predicted specificity at the P_1 residue of substrates are shown in Table VIII. The S_1 pocket of serine proteases is formed from a number of amino acid residues with 189 and 226 usually have a significant role in determining the primary substrate specificity. Granzyme A, like bovine trypsin, has an aspartate residue at position 189 in the S_1 specificity pocket, and therefore a preference for lysine or arginine as the P_1 residue of substrates is predicted. The high tryptase activity of granzyme A provides support for the granzyme A model and points to the importance of Asp-189 in determining the specificity of trypsin-like enzymes. Granzyme A also has an arginine at residue 99, which suggests a preference for Asp or Glu as the P_2 residue. The residue at position 99 usually is in a position to interact with the P_2 residue in a substrate while 192 and 218 can interact respectively with the P_3 and P_4 residues. The presence of Glu at 218 and Arg at 99 suggests that the natural substrate of granzyme A may contain a very charged sequence such as (Arg or Lys)-(Asp or Glu)-Arg*AA-, where the asterisk indicates the point of cleavage.

Granzyme B. A molecular model of murine granzyme B has also been constructed by Murphy et al. (1988) using RMCP II as a template. Granzyme B has an arginine residue at position 226 and thus is predicted to prefer Asp or Glu at the P_1 position in a substrate. We had previously synthesized Boc-Ala-Ala-Glu-SBzl but found no significant activity upon assaying the substrate with granzyme B. Since it was possible that the long Arg side chain in the S_1 pocket of the enzyme might result in an unfavorable spacing with P_1 Glu substrates, we decided to synthesize Boc-Ala-Ala-Asp-SBzl, which has one less methylene group in the P_1 side chain. Indeed, we found Boc-Ala-Ala-Asp-SBzl to be one of the most potent murine granzyme B substrates with k_{cat}/K_M = 230 000 M^{-1} s^{-1}. This substrate is also an effective substrate of human Q31 granzyme B and has a k_{cat}/K_M = 76 000 M^{-1} s^{-1} in 0.1 M Hepes buffer at pH 7.0 (Poe et al., 1991a). The low activity of murine granzyme B toward Boc-Ala-Ala-Glu-SBzl indicates that there is an optimum spacing between the carboxylate of the S_1 residue and the guanidino group of Arg-226, which is achieved only with Asp substrates. Very few other serine proteases are specific for substrates with P_1 acidic residues; one example is the *Staphylococcus aureus* V-8 protease which cleaves Glu substrates (Drapeau, 1976).

Both Boc-Ala-Phe-Asp-SBzl and Boc-Phe-Ala-Asp-SBzl are also as effective as Boc-Ala-Ala-Asp-SBzl for murine and human Q31 granzyme B (Table III). However, Z-Asp-SBzl is a less reactive substrate, indicating the prescence of an extended substrate binding site in the enzyme. The molecular model of murine granzyme B predicts favorable binding for substrates with small hydrophobic residues at the P_2 position due to the presence of Phe-99. The substrate with a P_2 Phe was hydrolyzed slightly faster than the one with a P_2 Ala, confirming the importance of the S_2–P_2 interaction. We are planning to synthesize additional substrates for granzyme B which will further define the nature of granzyme B's extended substrate binding site.

In addition to the Asp-ase activity, murine granzyme B has moderate activity toward Boc-Ala-Ala-Met-SBzl, Boc-Ala-Ala-Asn-SBzl, and Boc-Ala-Ala-Ser-SBzl and lesser activity toward Boc-Ala-Ala-Nva-SBzl and Boc-Ala-Ala-Glu-SBzl. The activity toward the Asn and Ser substrates is not unreasonable since both substrates have hydrogen-bonding groups and are of appropriate length to interact with Arg-226. However, it is less clear whether the Met-ase activity belongs to granzyme B. Evidence suggesting that the Met-ase activity belongs to granzyme B includes the observation that the Met-ase and Asp-ase activities coelute from the Mono S column and the nearly identical inhibition rates of granzyme B with DCI when three different substrates are used: Boc-Ala-Ala-Asp-SBzl, Boc-Ala-Ala-Ser-SBzl, and Boc-Ala-Ala-Met-SBzl. Evidence supporting the presence of two different enzymes is the observation by Poe et al. (1991a) that the Met-ase activity is separated from the Asp-ase during the purification of human granzyme B. Clearly, future purification of murine granzyme B will be required before we are certain whether the Met-ase activity is due to granzyme B or another unidentified murine granzyme.

Granzyme C. Granzyme C has Gln at 226 and Arg at 99;

therefore, it is predicted to have a preference for polar residues at P_1 and acidic residues at P_2. Our initial survey of thioesters yielded no substrates. We then synthesized Suc-Ser-SBzl and Suc-Asn-SBzl as possible substrates on the basis of the predicted S_1 and S_2 structure of granzyme C but found no enzymatic activity with granzyme C.

Granzymes D, E, and F. The granules also have significant chymotrypsin-like activity and effectively cleave the substrate Suc-Phe-Leu-Phe-SBzl (Harper et al., 1981). Granzymes D, E, and F are predicted to have activity toward Phe/Leu substrates since the S_1 pocket is formed from amino acid residues with small side chains (Table VIII). For example, granzyme F has Gly at 226 and Asn at 99 and is therefore predicted to prefer a hydrophobic residue at the P_1 site and a polar residue at the P_2 residue in a substrate. Thioesterase activity toward Suc-Phe-Leu-Phe-SBzl appeared in the Mono S column fractions containing granzyme A and F, and purified granzyme F also showed a chymotrypsin-like activity when higher concentrations of enzyme were used (Table 2, supplementary material). Murine granzyme F thus appears to be a chymase, although it is quite poor with the substrates tested at present. Neither granzyme D nor granzyme E had significant chymotrypsin-like activity. It is possible that the appropriate substrates have not yet been tested for these three predicted chymases. Development of a good substrate for human neutrophil cathepsin G required extensive subsite mapping studies (Tanaka, 1985), and we plan to synthesize addition peptide substrates for testing as possible substrates for granzymes D, E, and F.

It should also be noted that granzymes D and F have an Ala replacing Ser 214, while granzyme F has a Thr instead of Ala. Meyer et al. (1988) suggest that Ser 214 is a part of the catalytic tetrad in serine proteases, and its absence in granzymes D, E, and F could result either is loss of enzyme activity or diminished catalytic efficiency.

The preparations of granzymes D, E, and F also showed some activity toward Z-Lys-SBzl and Z-Arg-SBzl. Since the Mono S column fractions following the granzyme D and E fractions contained a small amount of trypsin-like activity, we suspect that traces of tryptases are responsible for this thioesterase activity in granzymes D and E. Granzyme D has previously been observed to have activity toward basic substrates and exhibited a pH optimum at 5.0 (Jenne & Tschopp, 1988). The chloro ketones TPCK and TLCK inhibited granzyme D efficiently. However, granzymes D and E have Gly at 226 in the S_1 pocket and Phe at 99 in the S_2 pocket, which predicts that both enzymes will prefer a hydrophobic residue such as Phe or Leu as the P_1 residue in substrates and a small hydrophobic residue such as Ala at P_2. We suspect that the trypsin-like activity in these column fractions is due to another tryptase, which has not yet been identified.

Synthetic Inhibitors. Previous studies have shown that granzymes A and D react strongly with DFP, granzymes B and E react with DFP to a lesser extent, and granzymes C and F do not react with DFP at all (Table I; Masson & Tschopp, 1987). Other serine protease inhibitors like PMSF, aprotinin, leupeptin, and benzamidine are good inhibitors for the esterolytic activity of granzyme A (Masson et al., 1986b; Young et al., 1986f; Simon et al., 1986a). PMSF, *p*-aminobenzamidine, human α_1-protease inhibitor, and human antithrombin III have been found to inhibit human Q31 granzyme A.

Since mouse and human granzyme A and human Q31 granzyme A showed trypsin-like activity toward peptide thioesters, it was expected that these enzymes would be inhibited by isocoumarins substituted with basic groups such as isothiureidoalkoxy and guanidino groups. These mechanism-based inhibitors have previously been shown to be effective inhibitors for trypsin and coagulation serine proteases (Kam et al., 1988) and selectively block RNK-16 lymphocyte granule mediated cytolysis (Hudig et al., 1989). The inhibitor with the largest $k_{obs}/[I]$ for mouse granzyme A was the 7-guanidino inhibitor **9**, and the inhibitor with the largest $k_{obs}/[I]$ for human granzyme A and Q31 granzyme A was the isothiureidoisocoumarin **6**. The $k_{obs}/[I]$ values of these inhibitors for these trypsin-like enzymes were in the range of 10^4 M^{-1} s^{-1}, which is comparable to the values observed with some coagulation serine proteases (Powers et al., 1989). Murine and human granzyme A like several coagulation proteases were inhibited more potently by the isothiureidopropoxy compound (**6**) than by the aminopropoxy compound (**5**), which showed that both enzymes prefer guanidino or guanidino-like side chains relative to a simple aminoalkyl group. This effect is also reflected in the substrate studies of murine granzyme A, where Z-Arg-SBzl (guanidino side chain) was a better substrate than Z-Lys-SBzl (aminoalkyl side chain). The rates of inhibition of the trypsin-like activity of granzymes A, D, E, and F by DCI and the 7-guanidino isocoumarin (**9**) were all quite similar, which is additional evidence that the trypsin-like activity in granzymes D, E, and F is due to small amounts of another unidentified tryptase or contaminating granzyme A.

Granzyme B was inhibited poorly by all the substituted isocoumarins except DCI, which is expected since none of the isocoumarins have acidic substituents. Therefore, we decided to synthesize a peptide chloromethyl ketone inhibitor for granzyme B on the basis of the structure of the substrate Boc-Ala-Ala-Asp-SBzl. Peptide chloromethyl ketones are well-known serine protease inhibitors, and the sequences found in good substrates are usually effective as chloromethyl ketone inhibitors (Powers, 1977; Powers & Harper, 1986). Boc-Ala-Ala-Asp-CH_2Cl inhibited mouse granzyme B slowly ($k_{obs}/[I]$ = 2.0 M^{-1} s^{-1}) and also inhibited human Q31 granzyme B with the same rate (data not shown). The inhibition rates of granzyme B by Boc-Ala-Gly-Glu-CH_2Cl and Boc-Ala-Ala-Asp(OBzl)-CH_2Cl were even smaller than the value of Boc-Ala-Ala-Asp-CH_2Cl, confirming a preference for the Asp residue at the P_1 site. Although DCI is a more effective inhibitor, the Asp chloromethyl ketone is more specific and should be useful for studying the biological role of granzyme B.

Two of the isocoumarins (**6** and **7**) which had high $k_{obs}/[I]$ values against human Q31 granzyme A (Table VI) were also effective inhibitors of the lysis of JY lymphoblastoid cells mediated by Q31 cytotoxic T lymphocytes (Table VII). Three other guanidinoisocoumarins (**8**, **9**, **10**) which also had high $k_{obs}/[I]$ were not effective inhibitors of lysis, perhaps due to cell-membrane transport problems. Three compounds in Table VI (**1**, **5**, and 4-chloro-3-ethoxy-7-nitroisocoumarin) had ED_{50} values significantly smaller than the IC_{50} values versus Q31 granzyme A. It is possible that only a small percentage of the Q31 granzyme A activity must be inhibited to block lysis. Alternately, another protease, or proteases such as granzyme B, may be required for lysis and is inhibited by the isocoumarins. It is also possible that these compounds are concentrated by the whole cells or metabolized to more potent structures. Thus, it is clear that mechanism-based inhibitors of serine proteases block CTL-mediated cytolysis, although additional experiments will be required to elucidate their mechanism of action.

Summary. Several Arg- or Lys-containing thiobenzyl esters have been found to be good substrates for mouse granzyme A, human granzyme A, and human Q31 granzyme A. Mouse granzyme B was shown to have enzymatic activity and found to effectively hydrolyze Boc-Ala-Ala-Asp-SBzl and slowly hydrolyze Boc-Ala-Ala-AA-SBzl, where AA was Asn, Met, or Ser. Granzyme F was found to have some chymotrypsin-like activity with the substrate Suc-Phe-Leu-Phe-SBzl. No substrates have been found for murine granzymes C, D, and E, although granzymes D and E are predicted to be chymotrypsin-like enzymes from their sequences. Isocoumarins with basic substituents effectively inhibited human Q31 granzyme A, murine granzyme A, and human granzyme A in a time-dependent manner. Boc-Ala-Ala-Asp-CH_2Cl inhibited murine and human granzyme B but was less potent than 3,4-dichloroisocoumarin, a general serine protease inhibitor. DCI and several isocoumarins with basic substituents were effective in blocking Q31 CTL-mediated lysis.

Acknowledgments

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Supplementary Material Available

Syntheses of several new peptide thiobenzyl esters and peptide chloromethyl ketones and Tables 1, 2, and 3 describing the enzymatic hydrolysis rates of peptide thioesters by mouse CTL granules, enzymatic hydrolysis rates of peptide thioesters by murine granzymes A, B, C, D, E, and F, and enzymatic hydrolysis rates of peptide thioesters by human granzymes (11 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 51050-59-0; **2**, 62252-26-0; **3**, 24672-89-7; **4**, 126062-30-4; **5**, 113251-24-4; **6**, 113273-56-6; **7**, 113251-07-3; **8**, 113251-08-4; **9**, 113251-10-8; **10**, 112901-86-7; Z-Lys-SBzl, 69861-90-1; Z-Arg-SBzl, 88253-86-5; Z-Gly-Arg-SBzl, 130698-88-3; Bz-Lys-Arg-SBzl, 108113-41-3; Bz-Glu-Lys-Arg-SBzl, 108113-45-7; Boc-Ala-Ala-Arg-SBzl, 102838-96-0; MeO-Suc-Ala-Ala-Pro-Lys-SBzl, 120253-71-6; Z-Ile-Ala-Gly-Arg-SBzl, 79864-39-4; Suc-Phe-Leu-Phe-SBzl, 80651-94-1; Boc-Ala-Ala-Met-SBzl, 90171-58-7; Boc-Ala-Ala-Asp-SBzl, 102838-95-9; Boc-Ala-Ala-Asn-SBzl, 90171-61-2; Boc-Ala-Ala-Ser-SBzl, 90171-60-1; Z-Asp-SBzl, 130698-89-4; Boc-Ala-Phe-Asp-SBzl, 130698-90-7; Boc-Phe-Ala-Asp-SBzl, 130698-91-8; Boc-Ala-Ala-Glu-SBzl, 90171-62-3; Boc-Ala-Ala-Asp-CH_2Cl, 130698-92-9; Boc-Ala-Gly-Glu-CH_2Cl, 130698-93-0; Boc-Ala-Ala-Asp(OBzl)-CH_2Cl, 130698-94-1; MeO-Suc-Ala-Ala-Pro-NHCH(CH_2Ph)P(O)($OPh)_2$, 122299-44-9; 3-(benzyloxy)-4-chloroisocoumarin, 99033-23-5; 4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin, 99033-24-6; 3-(3-aminopropoxy)isocoumarin, 113251-23-3; 7-guanidino-3-methoxyisocoumarin, 113301-72-7; 3-ethoxy-7-guanidinoisocoumarin, 113251-09-5; 7-guanidino-3-(2-phenylethoxy)isocoumarin, 113251-11-9; 1-naphthoyl-Arg-CF_3, 125652-38-2; serine protease, 37259-58-8; 4-chloro-3-ethoxy-7-nitroisocoumarin, 62252-29-3.

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Crystal Structure of Unliganded *Escherichia coli* Dihydrofolate Reductase. Ligand-Induced Conformational Changes and Cooperativity in Binding†,‡

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ABSTRACT: The crystal structure of unliganded dihydrofolate reductase (DHFR) from *Escherichia coli* has been solved and refined to an *R* factor of 19% at 2.3-Å resolution in a crystal form that is nonisomorphous with each of the previously reported *E. coli* DHFR crystal structures [Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, B. C., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13650–13662; Bystroff, C., Oatley, S. J., & Kraut, J. (1990) *Biochemistry 29*, 3263–3277]. Significant conformational changes occur between the apoenzyme and each of the complexes: the $NADP^+$ holoenzyme, the folate·$NADP^+$ ternary complex, and the methotrexate (MTX) binary complex. The changes are small, with the largest about 3 Å and most of them less than 1 Å. For simplicity a two-domain description is adopted in which one domain contains the $NADP^+$ 2′-phosphate binding site and the binding sites for the rest of the coenzyme and for the substrate lie between the two domains. Binding of either $NADP^+$ or MTX induces a closing of the PABG-binding cleft and realignment of α-helices C and F which bind the pyrophosphate of the coenzyme. Formation of the ternary complex from the holoenzyme does not involve further relative domain shifts but does involve a shift of α-helix B and a floppy loop (the Met-20 loop) that precedes αB. These observations suggest a mechanism for cooperativity in binding between substrate and coenzyme wherein the greatest degree of cooperativity is expressed in the transition-state complex. We explore the idea that the MTX binary complex in some ways resembles the transition-state complex.

This is the second of two crystallographic papers describing substrate binding and induced conformational changes in *Escherichia coli* dihydrofolate reductase (DHFR).[1] The previous paper (Bystroff et al., 1990) reported the structures of the $NADP^+$ holoenzyme and the folate·$NADP^+$ ternary

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‡ Coordinates for the apo-dihydrofolate reductase described have been deposited in the Brookhaven Protein Data Bank under 5DFR.
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[1] Abbreviations: ABD, adenosine binding domain; DHFR, dihydrofolate reductase; MTX, methotrexate; $NADP^+$ and NADPH, nicotinamide adenine dinucleotide phosphate (oxidized and reduced, respectively); NMN, nicotinamide mononucleotide; PABG, (*p*-aminobenzoyl)glutamate.

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