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Characterization of Structural Determinants of Granzyme B Reveals Potent Mediators of Extended Substrate Specificity*

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Granzymes are trypsin-like serine proteases mediating apoptotic cell death that are composed of two genetically distinct subfamilies: granzyme A-like proteases resemble trypsin in their active site architecture, while granzyme B-like proteases are quite distinct. Granzyme B prefers substrates containing P4 to P1 amino acids Ile/Val, Glu/Met/Gln, Pro/Xaa, and aspartic acid N-terminal to the proteolytic cleavage. By investigating the narrow extended specificity of the granzyme B-like proteases the mediators of their unique specificity are being defined. The foci of this study were the structural determinants Ile^{99}, Tyr^{174}, Arg^{192}, and Asn^{218}. Even modest mutations of these residues resulted in unique extended specificity profiles as determined using combinatorial substrate libraries and individual fluorogenic substrates. As with other serine proteases, Ile^{99} completely defines and predicts P2 specificity, primarily through the binding constant K_m. Asn^{218} variants have minor effects alone but in combination with mutations at Arg^{192} and Ile^{99} alter P2 through P4 extended specificity. For each variant, the activity on its cognate substrate was equal to that of granzyme B for the same substrate. Thus, mutations at these determinants change extended selectivity preferentially over catalytic power. Additionally Asn^{218} variants result in increased activity on the wild type substrate, while the N218A/I99A variant disrupts the additivity between P2 and P4 specificity. This defines Asn^{218} not only as a determinant of specificity but also as a structural component required for P2 and P4 independence. This study confirms four determinants of granzyme B extended substrate specificity that constitute a canon applicable to the study of the remaining family members.

Cytotoxic lymphocytes serve as the major immune system defense against virally and tumor-infected cells (1). They efficiently initiate apoptosis in the target cell by the directional release of cytotoxic granules containing the granzyme family of serine proteases, the pore-forming protein perforin, and proteoglycans (2). The granzymes are trypsin-like serine proteases expressed as preproproteases. The pre region targets them to the granules where activation occurs through the proteolytic processing of the pro region by dipeptidyl protease 1 (3). Of particular interest is granzyme B because of its unique specificity for aspartic acid-containing substrates (4). Granzyme B initiates the apoptotic cascade by activating caspases 3 and 7 (5) and proteolyzing a specific set of intercellular substrates such a DNA fragmentation factor 45 (6, 7). In addition to the strict requirement by granzyme B for an aspartic acid residue at the primary, or P1,[1] position the substrate preferentially contains an Ile or Val at the P4 position; Glu, Gln, or Met at P3; any amino acid at P2; non-charged amino acids at P1′; and Ser, Ala, or Gly at P2′ for efficient hydrolysis (9, 10). Granzyme B homologues have been located on rat, mouse, and human chromosomes 14 in a tightly linked gene cluster with chymase, cathepsin G, rat mast cell protease, and granzymes C through H (11, 12). Proteases in this gene cluster share >46% identity and >56% sequence similarity with granzyme B. Despite the homology, these proteases have five identified or predicted primary specificities and unique substrates (13). The specific interactions that determine these narrow extended substrate specificities may occur through a constellation of amino acids located on the highly variable loops surrounding the active site (14–17).

The amino acids 189, 191, 216, and 226 [2] and the loops containing them have been investigated for their roles in P1 specificity in trypsin-like proteases (19–21), and their optimization for specific recognition of one to two amino acids has been demonstrated. Compared with trypsin, the granzyme B-like serine proteases are missing the disulfide-linked cysteines at 191 and 220, have a truncated loop containing a cis-Pro at amino acid 224, and have the primary S1 specificity determinant translocated to amino acid 226. This amino acid is Arg in granzyme B, and it makes a salt bridge interaction with the P1-Asp of the substrate (22). Mutating 226 to Asp results in a protease with P1 basic specificity against ester substrates (23). However, the successful efforts to alter trypsin to chymotrypsin specificity required grafting entire active site loops and second shell mutagenesis (19, 24). Thus the primary binding site in the granzyme B proteases exhibits remarkable accommodation, and engineering the P1 specificity is perhaps less difficult. In this subfamily, S1 has evolved to the chymase preference for aromatic amino acids Phe and Tyr (16, 25), the cathepsin G

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[1] Serine protease substrate recognition sites are labeled according to the method of Schecter and Berger (8). Labels increase in number from P_1, P_2, . . . P_n for the substrate amino acids N-terminal to the scissile bond and P_1', P_2', . . . P_n' for the substrate amino acids C-terminal to the scissile bond. The corresponding substrate recognition pockets on the enzyme are labeled S_n . . . S_2, S_1, S_1', S_2' . . . S_n'.

[2] Amino acids are numbered according to their alignment with the serine protease chymotrypsin (18).

dual specificity for basic and aromatic amino acids (14), and the granzyme B preference for aspartic acid.

The high sequence identity of the granzyme B-like subfamily narrows the number of possible structural determinants and suggests that mutagenic changes will introduce radical alterations in substrate specificity. The coincidence of crystal structures of granzyme B (22, 26, 27), chymase (16), rat mast cell protease (28), and cathepsin G (14) and combinatorial assays for determining substrate specificity (29) provide a powerful basis for understanding extended substrate specificity using site-directed mutagenesis. This study seeks to demonstrate, through site-directed mutagenesis, the role of amino acids identified by the crystal structure of rat granzyme B in the selective modification of its specificity. The contributions of subfamily-specific amino acids were probed with a series of variants constructed along the active site binding cleft. Eight single and three combination variants were designed with substitutions at Tyr^{174}, Ile^{99}, Arg^{192}, and Asn^{218}. We used P1 to P4 positional scanning synthetic combinatorial libraries (PSSCLs)[3] of substrates to screen each variant for changes in specificity and designed individual substrates to probe kinetic activity through Michaelis-Menten kinetics. The steady state kinetic constants reveal the extent of the specificity changes and are used to dissect the effect of each mutation on the selectivity of granzyme B.

EXPERIMENTAL PROCEDURES

Materials—Ac-IEPD-AMC, Ac-IKPD-AMC, Ac-IETD-AMC, and Ac-AAD-*p*NA were purchased from Sigma. Ac-IEFD-AMC, Ac-LEFD-AMC, and Ac-LEPD-AMC were purchased from SynPep (Dublin, CA). The QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA) and used according to the manufacturer's instructions. All oligonucleotide primers and the *Pichia pastoris* expression system were purchased from Invitrogen. The Asp-ACC-conjugated resin was a gift from the laboratory of Jon Ellman (University of California, Berkeley). The P1-Asp-AMC library was a gift from Nancy Thornberry (Merck Laboratories, Rahway, NJ). The complete diverse ACC tetrapeptide substrate library was a gift of Youngchool Choe (Craik Laboratory).

Alignment of Granzyme B Subfamily Serine Proteases—Rat granzyme B[4] was used as a query to find related granzyme and granule proteases from the National Center for Biotechnology Information (NCBI) data base (National Library of Medicine, National Institutes of Health, Bethesda, MD) using PSI-BLAST (30) and HSSP (31). The sequences were aligned using ClustalW 8.1. The three-dimensional structure of rat granzyme B in a complex with ecotin (^{81}IEPD^{84}) was used to determine amino acids with side chain atoms within 4 Å of the substrate amino acid side chains. Amino acids were also evaluated that contact small molecule inhibitors in the human granzyme B, chymase, cathepsin G, and rat mast cell protease II structures.[5]

Variants of Rat Granzyme B—The wild type, R192A, and R192E rat granzyme B constructs were prepared as described previously (10). Additional variants of wild type rat granzyme B were constructed using the QuikChange protocol (Stratagene). The following 5′ to 3′ primers were made to introduce point mutations into the pPICZαA plasmid (mutations are underlined): *I99A*, CCA GCG TAT AAT TCT AAG ACA GCC TCC AAT GAC ATC ATG CTG; *I99F*, CCA GCG TAT AAT TCT AAG ACA TTC TCC AAT GAC ATC ATG CTG; *I99R*, CCA GCG TAT AAT TCT AAG ACA AGA TCC AAT GAC ATC ATG CTG; *Y174A*, GT GAG TCC TAC TTA AAA AAT GCT TTC GAC AAA GCC AAT GAG ATA; *N218A*, GGC ATC GTC TCC TAT GGA CAA GCT GAT GGT TCA ACT CCA CGG GCA; *N218T*, GGC ATC GTC TCC TAT GGA CAA ACT GAT GGT TCA ACT CCA CGG GCA. Each mutation was confirmed by sequencing with primers to the 5′ alcohol oxidase and 3′ alcohol oxidase regions. The resulting plasmid was transformed into X33 cells and selected with Zeocin (Invitrogen). All granzyme B variants were expressed and purified to homogeneity by the wild type protocol (10) with yields between 0.5 and 3 mg/liter. Activity was monitored in the supernatant using 1 μM Ac-AAD-*p*NA during expression. Following purification each variant was exchanged into a buffer containing 50 mM MES, pH 6.0, and 100 mM NaCl, quantified by absorbance at 280 nm (A_{280} = 22,900 M^{-1} cm^{-1}), titrated with wild type or M84D ecotin (10), and stored at 4 °C.

Positional Scanning Synthetic Combinatorial Libraries—Both the completely diversified and P1-Asp PSSCLs were measured into Microfluor black 96-well plates (DYNEX Technologies, Chantilly, VA) and diluted to a final concentration of 250 μM. Thus the tetrapeptide substrates in each pool are at a concentration far below the expected K_m of the variant protease, and the initial rates are proportional to the specificity constant k_{cat}/K_m. The granzyme B variants were assayed for activity against Ac-IEPD-AMC and diluted in granzyme activity buffer (50 mM Na-HEPES, pH 8.0, 100 mM NaCl, 0.01% Tween 20) to concentrations between 50 nM and 1 μM that yielded PSSCL activity levels approximately equal to 50 nM wild type granzyme B. Initially each variant was assayed in the P1 sublibrary of a tetrapeptide complete diverse PSSCL.[6] It consists of 20 spatial arrayed pools of 8000 fluorogenic tetrapeptide ACC substrates where the P1 amino acid is fixed (cysteine was deleted and norleucine (2-aminohexanoic acid) was included), while the P2, P3, and P4 amino acids consist of equimolar amino acid mixtures. To increase the signal to noise ratio and reduce the number of assayed peptide substrates, the P1-Asp 7-amino-4-methylcoumarin (AMC)-based PSSCL used to profile caspases and human (9) and rat granzyme B (10) was used to measure extended (P2 through P4) selectivity. Each sublibrary consists of 19 pools containing 361 peptides, one each for the spatially addressed amino acids (cysteine and methionine are deleted, and D-alanine and norleucine (2-aminohexanoic acid) are included). Enzymatic activity in the PSSCL was assayed for 1 h at 30 °C on a SpectraMAX Gemini fluorometric plate reader (Amersham Biosciences), and the rate of substrate hydrolysis was analyzed with the SOFTmax PRO software (Version 3.1.1, Amersham Biosciences). Excitation and emission were measured at 380 and 450 nm, respectively.

Synthesis of ACC Substrates—Rink resin-conjugated ACC-Asp(OtBu)-Fmoc was subjected to standard solid phase peptide synthesis, acylated, and cleaved by trifluoroacetic acid (32). The resulting substrates were purified to homogeneity by reverse phase high pressure liquid chromatography on a C_{18} column (Vydac, 5 μm, 4.6 × 250 mm) with a 20–60% gradient of 0.1% aqueous trifluoroacetic acid and 0.08% trifluoroacetic acid, 95% acetonitrile. The molecular weight of each substrate was confirmed by matrix-assisted laser desorption ionization mass spectrometry. The concentration of each substrate stock solution was verified by total hydrolysis of the ACC or AMC leaving group using saturating amounts of wild type granzyme B. Briefly a measured amount of each substrate was exposed to saturating amounts of enzyme, and the total fluorescence was measured over multiple days until all of the substrate was hydrolyzed.

Individual Kinetic Measurements—Individual kinetic measurements were performed using a SpectraMAX Gemini fluorometric plate reader. Each protease was diluted to between 50 nM and 1 μM in assay buffer. All ACC substrates were diluted in Me_2SO to between 5 and 500 μM, while AMC substrates were diluted to between 20 and 2000 μM. Each assay contained less than 5% (v/v) Me_2SO. Enzymatic activity was monitored every 15 s at excitation and emission wavelengths of 380 and 460 nm, respectively, for a total of 10 min. Rates of substrate hydrolysis were determined using the SOFTmax PRO data analysis software (Version 3.1.1, Amersham Biosciences) and fit to the Michaelis-Menten equation using Kaleidagraph (Version 3.5, Synergy Software, Reading, PA). When the apparent K_m values for a substrate were greater than 2.2 mM, only k_{cat}/K_m was reported. The substrates were assayed for hydrolysis by granzyme B and variants at least twice in duplicate.

RESULTS

General Considerations for Substrate Specificity Studies—From the three-dimensional structure of rat granzyme B with the pseudosubstrate ecotin (^{81}IEPD^{84}) (22, 26, 27), amino acid

[3] The abbreviations used are: PSSCL, positional scanning synthetic combinatorial library; AMC, 7-amino-4-methylcoumarin; *p*NA, *p*-nitroanilide; ACC, 7-amino-4-carbamoylmethylcoumarin; MES, 4-morpholineethanesulfonic acid; OtBu, *tert*-butoxy; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

[4] The amino acid sequence of rat granzyme B is found under the NCBI accession number NP_612526 or Swiss-Prot accession number 18291.

[5] The atomic coordinates of the analyzed structures are available from the Research Collaboratory for Structural Bioinformatics Protein Data Bank under the following identification numbers: rat granzyme B, 1FI8; human granzyme B, 1FQ3 and 1IAU; chymase, 1PJP and 1KLT; cathepsin G, 1AU8 and 1CGH; and rat mast cell protease II, 3RP2.

[6] Y. Choe, in preparation.

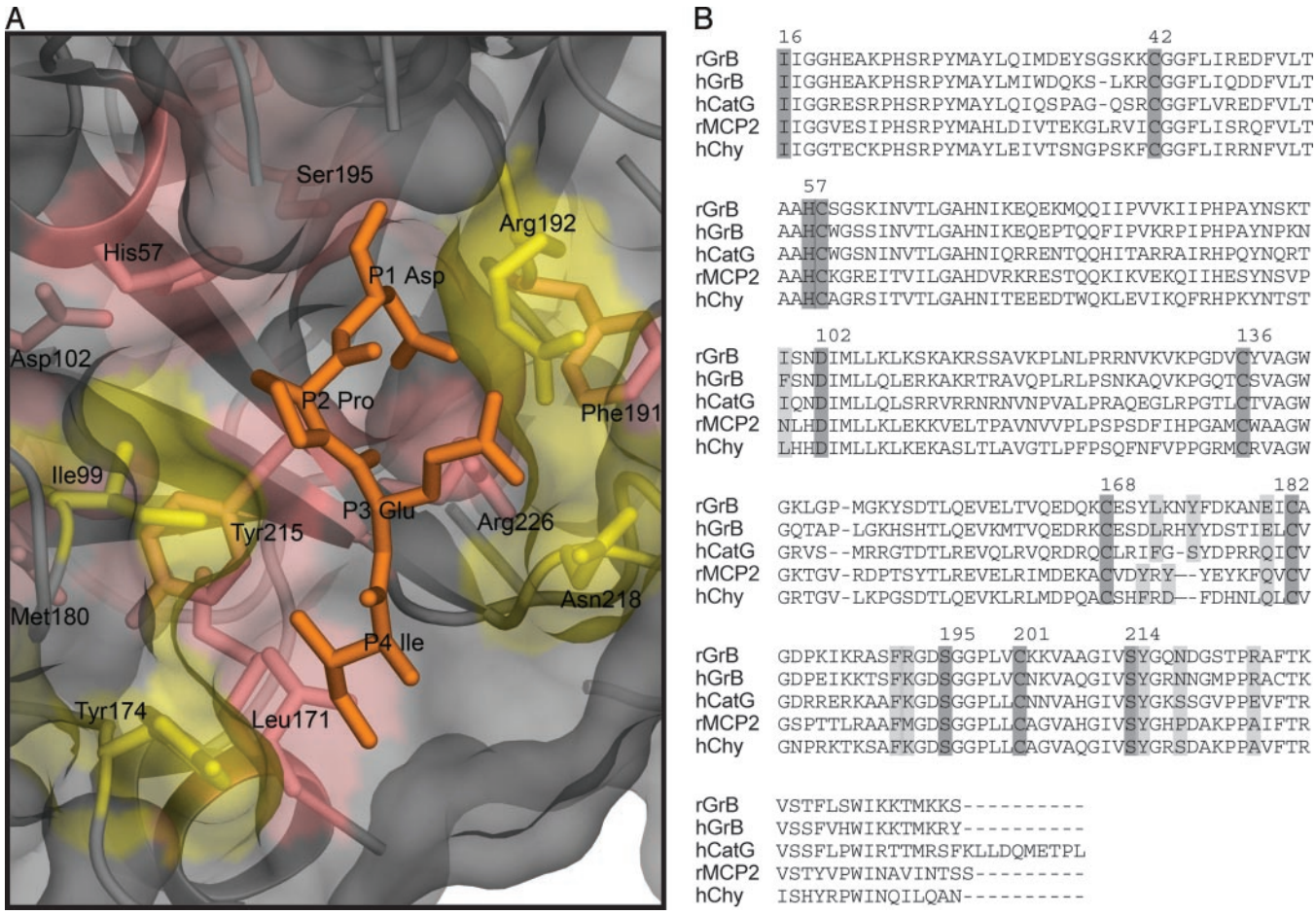

FIG. 1. **Structural determinants of granzyme B specificity.** *A*, a three-dimensional representation is used to highlight the determinants on the structure of rat granzyme B (N66Q) in complex with the P1 through P4 substrate-like amino acids of ecotin (^{81}IEPD^{84}) (*orange*). The catalytic triad and unmodified structural determinants are shown in *red* over a *gray* ribbon representation of the protein backbone. The amino acids mutated in this study are shown in *yellow*. The structure was visualized in three dimensions with PyMol (DeLano Scientific, San Carlos, CA). *B*, multiple sequence alignment of the granzyme B-related serine proteases. Serine proteases related to rat granzyme B (with the GenBank™ accession number followed by the Protein Data Bank code in parentheses) (NKP1_Rat, 1FI8) and characterized by x-ray crystallography include human cathepsin G (CATG_Human, 1AU8), human granzyme B (GRAB_Human, 1FQ3), rat mast cell protease II (MCP2_Rat, 3RP2), and human chymase (MCT1_Human, 1KLT). The S1 pocket of these enzymes has a distinct active site architecture: no disulfide-linked cysteines at amino acids 191 and 220, a truncated loop at 220 compared with trypsin, and a cis-Pro at amino acid 224. The disulfide-linked cysteines, catalytic triad (including Ser^{214}), and N terminus are labeled and highlighted with *dark gray bars*. Highlighted with *light gray bars* are the amino acids or their structural equivalents with side chains in close contact (<4 Å) of the modified ecotin inhibitor (from Protein Data Bank file 1FI8). The amino acids Ile^{99}, Tyr^{174}, Arg^{192}, and Asn^{218} were mutated in this study. *rGrB*, rat granzyme B; *hGrB*, human granzyme B; *hCatG*, human cathepsin G; *rMCP2*, rat mast cell protease II; *hChy*, human chymase.

side chains within 4 Å of side chains of the ecotin binding site loop were determined (Fig. 1*A*). They include Phe^{191} and Arg^{226} in the S1 pocket; Ile^{99} and His^{57} in the S2 pocket; Arg^{192} and Asn^{218} in the S3 pocket; and Gln^{180}, Phe^{174}, Tyr^{215}, and Leu^{172} in the S4 pocket. Amino acid 226 has been identified as the primary determinant of P1 specificity, and the determinants His^{57}, Leu^{172}, Phe^{191}, Gln^{180}, and Tyr^{215} vary between two or fewer amino acids in the subfamily or are absolutely required for catalytic activity (24, 33). Thus many potential determinants of specificity from crystallographic studies can be excluded from mutagenic studies because of known structural characteristics independent of extended specificity.

Initial activity in the complete diverse PSSCL indicated that all granzyme B variants specifically and uniquely hydrolyzed substrates containing P1 aspartic acid (data not shown). This result is consistent with the strict specificity of granzyme B for aspartic acid (29). The complete diverse library is made up of 16,000 sublibrary member substrates per well where P1 is aspartic acid in just 1/20 of them. By fixing the P1 amino acid and profiling with a P1-Asp-AMC PSSCL, the number of sublibrary member substrates was decreased to 400 per well increasing the signal to noise ratio. Since the focus of the PSSCL was to report alterations in substrate specificity, the reported activity in each well of the library is shown as a fraction of the highest rate at the substrate position. This format highlights the specificity changes upon mutation but masks the relative activity of each subsite.

PSSCL Reveals Extended Specificity Changes upon Mutagenesis—The PSSCL was used to profile the specificity changes for each variant. Initially alanine substitution was used to detect the importance of each amino acid to the specificity profile of granzyme B. The I99A variant had a large effect on the PSSCL specificity profiles at the P2 position. The mutation increased the hydrolysis of P2-Phe or -Tyr substrates and reduced the hydrolysis of non-hydrophobic side chains without altering the P3 or P4 profiles (Fig. 2). Activity for the P2 amino acid Phe was 9 times the activity for Pro in the PSSCL and extended to tetrapeptide substrates where the specificity constants, k_{cat}/K_m, for Ac-IEPD-AMC and Ac-IEFD-AMC were 5-fold different, 57 and 268 M^{-1} s^{-1}, respectively (Table I).

Two amino acids restrict specificity at P3. Asn^{218} was re-

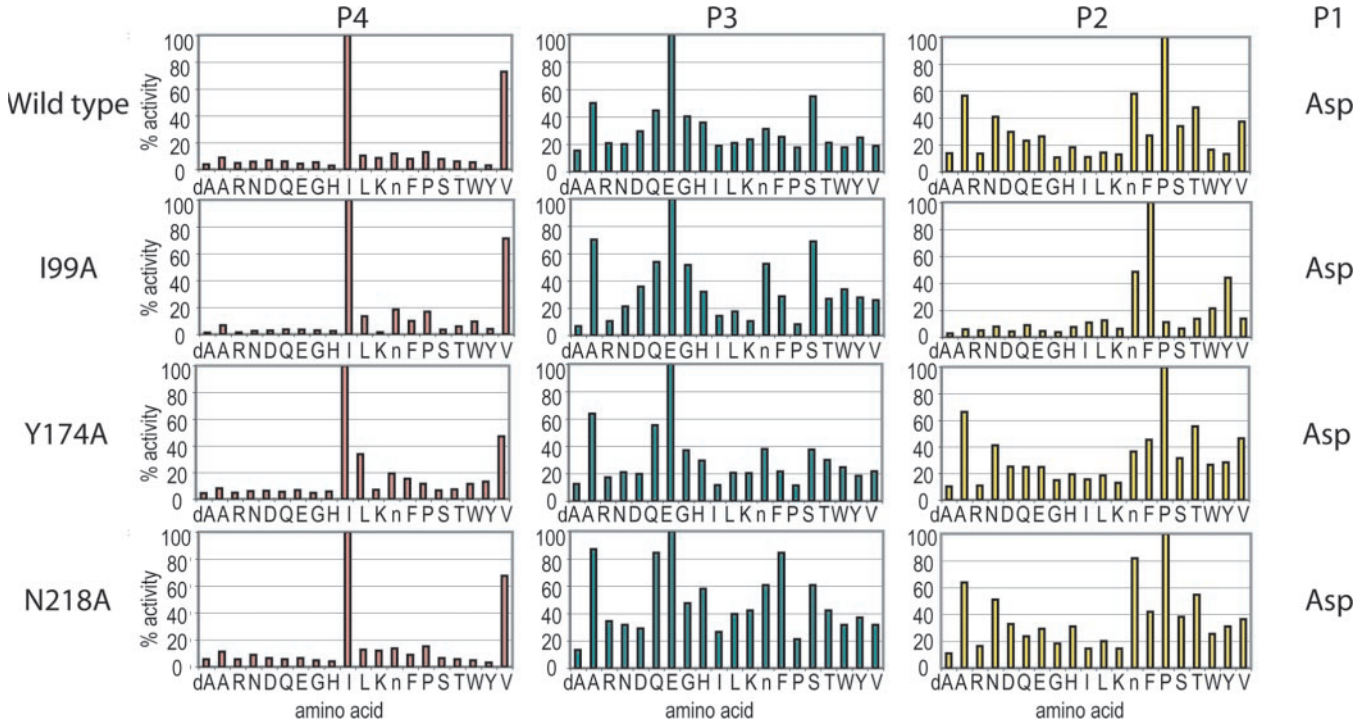

FIG. 2. **PSSCL for the I99A, N218A, and Y174A variants compared with wild type activity.** The PSSCL activity of each amino acid at the P2 (*yellow bars*), P3 (*blue bars*), and P4 (*pink bars*) positions is displayed along the *y axis* as a percentage of the activity of the best amino acid activity at that position. Note that this representation of the library showcases the specificity at each position independently and masks the activity of each extended substrate position in relation to each other. The concentration of protease was adjusted to match that of 50 nM wild type granzyme B in the library, and the activity was measured over 1 h. The one-letter code for the amino acid held fixed is listed alphabetically along the *x axis*. A *lowercase n* is used to represent norleucine (2-aminohexanoic acid), and *dA* is used to represent D-alanine.

TABLE I
Specificity constants for granzyme B variants

Steady state kinetic specificity constants (k_{cat}/K_m) for Ac-IEPD-AMC and one contrasting substrate illustrate the magnitude of specificity alteration for the variants. Each ratio consists of the wild type preference (P) and the variant preference (X) shown in bold for activity in the PSSCL libraries (PSSCL ratio) and between individual tetrapeptide substrates (activity ratio).

Protease	k_{cat}/K_m	PSSCL ratio (P:**X**)	Activity ratio (P:**X**)
	$M^{-1}\ s^{-1}$		
Wild type			
Ac-IEPD-AMC	3300 ± 500		
I99A			
Ac-IEPD-AMC	57 ± 2.6		
Ac-IE**F**D-AMC	270 ± 18	1:10	1:5
I99F			
Ac-IEPD-AMC	510 ± 41		
Ac-**L**EPD-AMC	200 ± 22	2.3:1	2.6:1
I99R			
Ac-IEPD-AMC	330 ± 26		
Ac-IE**F**D-AMC	505 ± 45	1:1.3	1:1.5
Y174A			
Ac-IEPD-AMC	470 ± 36		
N218A			
Ac-IEPD-AMC	2200 ± 190		
Ac-I**K**PD-AMC	220 ± 19	2.2:1	10:1
N218T			
Ac-IEPD-AMC	6200 ± 600		
Ac-I**K**PD-AMC	600 ± 4	2.5:1	10:1
N218A/R192A			
Ac-IEPD-AMC	92 ± 11		
Ac-I**K**PD-AMC	55 ± 2.8	1:1.5	1.7:1
N218A/R192E			
Ac-IEPD-AMC	10 ± 0.35		
Ac-I**K**PD-AMC	73 ± 3.4	1:14	1:7

placed with Ala to probe the role of the hydrogen bond observed between the amino group of Asn^{218} and the carboxyl group of the ecotin Glu^{83} in the three-dimensional structure (22). N218A granzyme B did not change the P2 or P4 specificity profiles but decreased the preference for PSSCL substrates with Glu at the P3 position, broadened the number of accepted P3 amino acids to include Ser and Ala, and increased the preference for Met and Gln (Fig. 2). Hydrolysis of Ac-I**K**PD-AMC (where boldface denotes the amino acid that is different from the wild type sequence) by N218A granzyme B was increased >2-fold compared with wild type activity from 99 to 220 $M^{-1}\ s^{-1}$, whereas hydrolysis of Ac-IEPD-AMC was reduced from 3300 to 2200 $M^{-1}\ s^{-1}$ (Table I). Finally the Y174A variant exhibited no alteration in the PSSCL profile preferring the canonical P4-Ile, P3-Glu, and P2-Pro sequence (Fig. 2). Instead the specificity constant for Y174A granzyme B was ~10-fold less than the wild type (470 *versus* 3300 $M^{-1}\ s^{-1}$). Because the specificity did not change despite the enlargement of the S4 pocket, the Y174A mutation probably does not play a direct role in the selection of the P4 amino acid.

The alanine mutations revealed extensive alterations in substrate specificity except at the P4 position where Ile and Val continued to dominate, so a series of variations based on subfamily homology was designed. The I99R and I99F variants test amino acids found in other members of the granzyme subfamily. Phe^{99} occurs in the human homolog of rat granzyme B, while Arg^{99} is found in granzymes A and C (Fig. 1*B*). The I99F variant retains the wild type granzyme B preference for Ile-Glu-Pro by the PSSCL, but there is an increase in the specificity of P4-Leu substrates (Fig. 3). The preference for P4-Ile *versus* P4-Leu tetrapeptide substrates is reduced to 2.6-fold for I99F granzyme B where the specificity constants are 510 and 200 $M^{-1}\ s^{-1}$, respectively (Table I). In the variant I99R, a hydrophobic preference at P2 appears, but it is less apparent than that in the I99A variant. Here Pro and Phe tetrapeptide

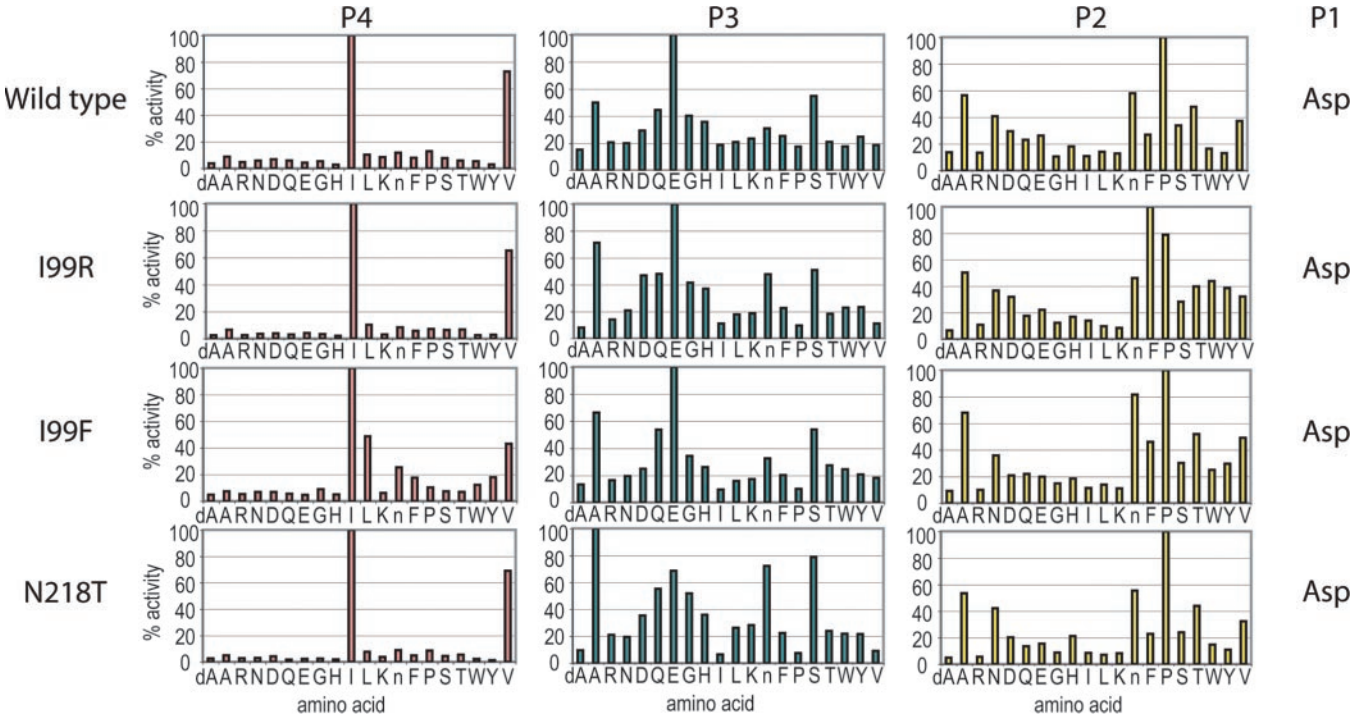

FIG. 3. **Mutations mimicking homologous protease amino acids at position 99 alter P2 and P4 specificity.** The P1-Asp-AMC PSSCL highlights the narrow P2 specificity of the I99R variant and the increase in P4-Leu preference for I99F granzyme B. A lowercase n is used to represent norleucine (2-aminohexanoic acid), and *dA* is used to represent D-alanine. *Pink bars*, P4; *blue bars*, P3; *yellow bars*, P2.

substrates are hydrolyzed with similar specificity constants (330 and 505 M^{-1} s^{-1}, respectively).

Asn^{218} was replaced to probe the role of the hydrogen bond between it and the carboxylate group of Glu^{82} in ecotin. Mutations were designed to test this interaction individually and in combination with mutations of Arg^{192}. The N218T variant had decreased preference for the PSSCL substrates containing Glu at the P3 position, a broader number of accepted P3 amino acids including Ser and Ala, and increased preference for Met and Gln (Fig. 3). The mutation did not change the P2 or P4 profiles. However, the hydrolysis of Ac-IEPD-AMC was faster by the N218T variant than by the wild type protease. Ala was the most preferred P3 amino acid, while the specificity constant for basic amino acids such as Lys remained less than 10-fold the value for Glu (600 *versus* 6200 M^{-1} s^{-1}) (Table II). The R192A/N218A variant had no change at P2 and P4, an increased preference for Ala and Ser at P3 (Fig. 4), and a slight preference for Glu over Lys. This variant also had low activity similar to the single R192A mutation (k_{cat}/K_m (Ac-IEPD-AMC) = 92 M^{-1} s^{-1} (10)). When combined with the R192E variant, the N218A mutation completely reversed the P3 specificity from acidic to basic. From PSSCL results, the activity of P3-Glu *versus* -Lys was reversed to favor Lys by 9-fold. The kinetic constants demonstrate a significant preference as well. The specificity constants for N218A/R192E for Ac-I**E**PD-AMC and Ac-I**K**PD-AMC are 10 and 73 M^{-1} s^{-1}, respectively (Table II). This represents a 7-fold preference for the basic amino acid.

The Role of Structural Determinants of Specificity in the Transition State—According to transition state theory, an optimal substrate is one where the enzyme binds preferentially to the transition state of the reaction, reducing the activation barrier between the enzyme substrate-associated Michaelis complex and the final cleaved product and regenerated enzyme. Serine proteases follow a multistep catalytic mechanism in which the substrate and enzyme associate to form the Michaelis complex. The hydroxyl of Ser^{195} attacks the carbonyl carbon of the reactive or scissile bond generating an acyl-enzyme intermediate and the first product followed by hydrolysis of the acyl-enzyme intermediate to regenerate the active protease, the second product, and a water molecule. This is shown in Equation 1. When the substrate is hydrolyzed at an amide bond as in peptide and AMC synthetic substrates, the deacylation rate is much faster than the acylation rate ($k_3 \gg k_2$), and the rate constants reduce to Equations 2 and 3.

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} E*S \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + R\text{-}COOH \quad (H_2N\text{-}R') \qquad \text{(Eq. 1)}$$

$$k_{cat} = k_2 \qquad \text{(Eq. 2)}$$

$$K_m = K_s = k_{-1}/k_1 \qquad \text{(Eq. 3)}$$

The catalytic rate constants for granzyme B on a series of tetrapeptide substrates shown in Table III illustrate its selectivity toward the optimal substrate sequence Ile-Glu-Pro-Asp. Variation at the P2 position of the substrate results in lower k_{cat} values (1.20 *versus* 0.15 and 0.39 s^{-1}) but not higher K_m values (370 *versus* 150 and 250 μM). A similar observation was made for the activity of the protease chymotrypsin upon substrates of increasing length. For chymotrypsin, it was hypothesized that extended substrates increase k_{cat} by improving transition state stabilization and conversion of binding energy to catalysis (34). The substrates with non-optimal P3 and P4 amino acids did not exhibit this effect. For Ac-**L**EPD-AMC and Ac-I**K**PD-AMC both the k_{cat} and K_m measurements were worse than wild type. The K_m values increased at least 3-fold from 370 to 1100 and 1600 μM, while the k_{cat} values decreased greater than 7-fold from 1.2 to 0.15 and 0.096 s^{-1}, respectively. Non-optimal substrate sequences at P3 and P4 have less affinity for the protease and are not able to efficiently convert the ground state binding into catalytic activity.

TABLE II

Individual kinetic constants for wild type granzyme B against a panel of tetrapeptide substrates

Steady state kinetic rate constants for individual tetrapeptide substrates illustrate the magnitude of altered specificity where bold amino acids deviate from the wild type sequence. Each ratio consists of the wild type preference (P) and the variant preference (X) shown in bold for activity in the PSSCL libraries (PSSCL ratio) and between individual tetrapeptide substrates (activity ratio). ND, not determined.

Protease	k_{cat}/K_m	k_{cat}	K_m	PSSCL ratio (P:**X**)	Activity ratio (P:**X**)
	$M^{-1}\ s^{-1}$	s^{-1}	μM		
Wild type					
Ac-IEPD-AMC	3300 ± 500	1.2 ± 0.033	370 ± 70		
Ac-IE**T**D-AMC	1600 ± 260	0.39 ± 0.02	250 ± 53	2.1:1	2.1:1
Ac-IE**F**D-AMC	1000 ± 99	0.15 ± 0.003	150 ± 34	3.8:1	3.3:1
Ac-I**A**PD-ACC	110 ± 5	ND	>1000	2:1	30:1
Ac-I**K**PD-AMC	99 ± 5	0.15 ± 0.01	1600 ± 190	4.4:1	33:1
Ac-**L**EPD-AMC	87 ± 6.8	0.096 ± 0.0055	1100 ± 200	9.8:1	38:1

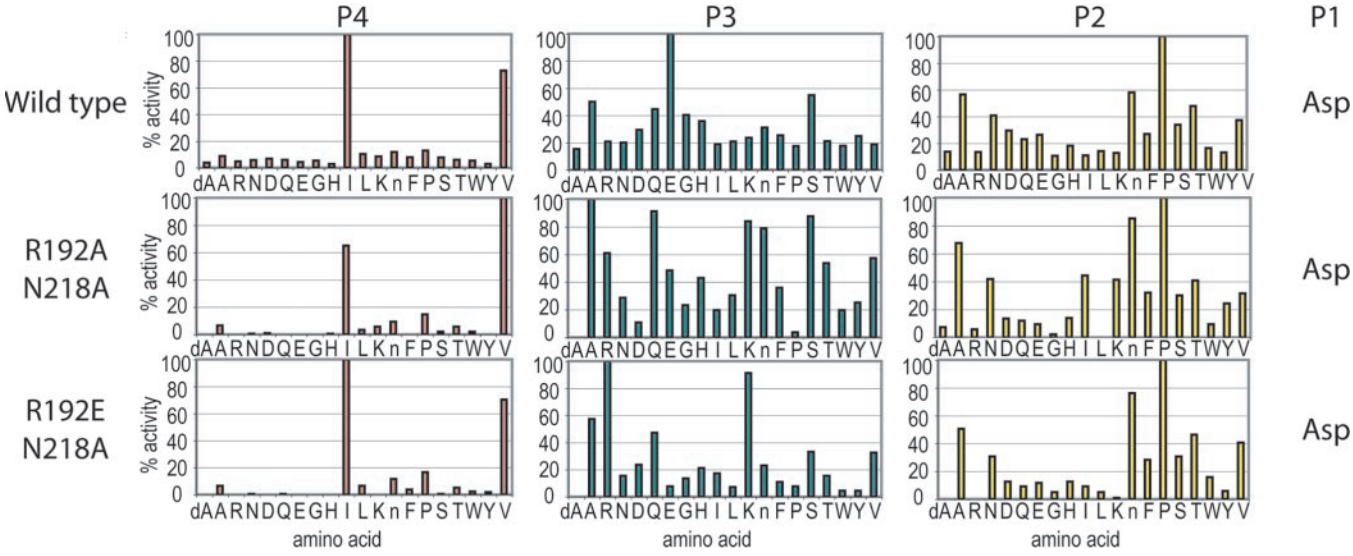

FIG. 4. **Arg^{192} and Asn^{218} mutations in combination reduce the preference for acidic P3 amino acids.** P1-Asp-AMC PSSCL profiles for the variants N218A/R192A and N218A/R192E highlight the reversal of P3 specificity from acidic to alanine and basic residues. A *lowercase n* is used to represent norleucine (2-aminohexanoic acid), and *dA* is used to represent D-alanine. *Pink bars*, P4; *blue bars*, P3; *yellow bars*, P2.

TABLE III

Kinetic constants for single variants compared to wild type

The preference for hydrophobic residues at P2 for the I99A, I99F, I99R, and N218A/I99A variants highlights the increase in K_m for the Ac-IEFD-AMC substrate. At P3, the preference for acidic amino acids is totally reversed with the R192E/N218A variant, but the activity is not better than that of wild type. Rather the N218A and N218T variants prefer basic P3 residues with improvedk_{cat} and no decrease in K_m. ND, not determined.

Substrate	k_{cat}/K_m	k_{cat}	K_m
	$M^{-1}\ s^{-1}$	s^{-1}	μM
Ac-IEFD-AMC			
Wild type	1000 ± 99	0.15 ± 0.003	150 ± 34
I99A	270 ± 18	0.24 ± .010	880 ± 96
I99F	410 ± 77	ND	
I99R	505 ± 45	0.47 ± 0.01	930 ± 190
I99A/N218A	290 ± 24	0.26 ± .002	890 ± 350
Ac-IKPD-AMC			
Wild type	99 ± 5	0.15 ± 0.01	1600 ± 190
N218A	220 ± 19	0.38 ± 0.09	1800 ± 550
N218T	600 ± 4	1.1 ± 0.01	1800 ± 30
N218A/R192A	55 ± 2.8	ND	>2000
N218A/R192E	73 ± 3.4	ND	>2000

The variant proteases act in a similar yet subtly different manner. First the Ile^{99} variant proteases have activities that are less than the wild type activity on the same substrate, Ac-IEFD-AMC, despite its place as a highly preferred substrate. The kinetic constants for Ac-IE**F**D-AMC exhibit K_m values up to 8-fold higher than the wild type protease providing most of the overall rate reduction (100 *versus* 880 and 930 μM) (Table III). The increase in K_m values suggests that while the S2 pocket has been enlarged in the I99A and I99R variants, the energy increase provided by the additional van der Waals contacts of a Phe residue is not enough to return the binding constant to the original value. For the Asn^{218} variants, the opposite result is observed. The Ac-I**K**PD-AMC substrate is poorly turned over by granzyme B but has a higher affinity to the variants N218A and N218T. The binding constant K_m remains fairly constant for all of the variants (1600 through >1800 μM), while the k_{cat} values result in the activity increases (Table III). Despite the completely reversed preference for P3-Lys by the N218A/R192E variant, the activity is no better than granzyme B on Ac-I**K**PD-AMC due primarily to a low kinetic constant, k_{cat}. The importance of differences in k_{cat} toward the overall activity of the variant arises from the ability of the variant to convert the binding energy associated with Michaelis complex formation into transition state stabilization.

Asn^{218} Is a Structural Determinant Required for Efficient Transition State Stabilization—The N218T and N218A variants have a wider range of preferred P3 amino acids and increased acylation rate compared with wild type granzyme B. This indicates that Asn^{218} is present to select substrates with acidic P3 residues rather than to maximize the rate constants. The double mutation N218A/I99A had a dramatic effect on extended specificity by PSSCL, altering the P2 through P4 specificity to the sequence Pro-Ala-Phe-Asp (Fig. 5). Like the I99A and I99R variants, the P2 specificity is narrowed to prefer large hydrophobic amino acids, but in addition the P3 and P4 specificity has been broadened to include most aliphatic and β

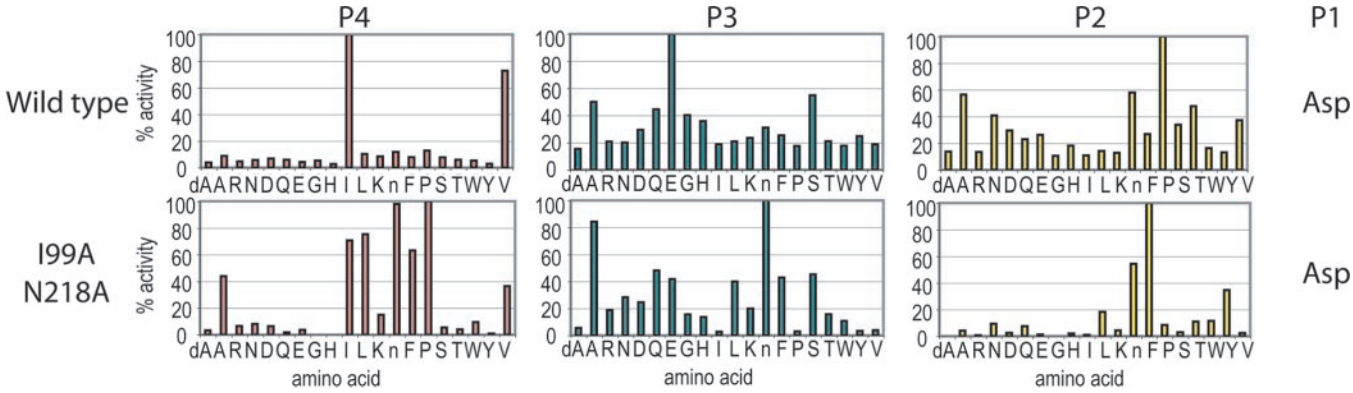

FIG. 5. **I99A/N218A granzyme B has dramatically altered extended specificity.** Results of the P1-Asp-AMC PSSCL compared with wild type granzyme B illustrate a broad profile at P4 and P3 positions and a narrow preference for P2-Phe, -Tyr and -2-aminohexanoic acid . A *lowercase n* is used to represent norleucine (2-aminohexanoic acid), and *dA* is used to represent D-alanine. *Pink bars*, P4; *blue bars*, P3; *yellow bars*, P2.

TABLE IV

Kinetic constants and change in free energy of wild type and the N218A/I99A variant on a series of substituted tetrapeptide substrates

Steady state kinetic constants illustrate the overall decrease in activity as the substrates become less preferred and the detailed changes in K_m and k_{cat}. The calculated change in free energy ($\Delta\Delta G_T^{\ddagger} = -RT \ln((k_{cat}/K_m)_{IEPD}/(k_{cat}/K_m)_{modified})$) of the N218A/I99A variant is also calculated in comparison to wild type granzyme B. ND, not determined.

Protease	k_{cat}/K_m	k_{cat}	K_m	$\Delta\Delta G_T^{\ddagger}$
	$M^{-1}\ s^{-1}$	s^{-1}	μM	*kcal mol*$^{-1}$
Wild type				
Ac-IEPD-AMC	3300 ± 500	1.2 ± 0.033	370 ± 70	0
Ac-IE**F**D-AMC	1000 ± 99	0.154 ± 0.003	150 ± 34	+0.71
Ac-**L**EPD-AMC	88 ± 6.8	0.096 ± 0.0006	1100 ± 200	+2.18
Ac-**LEF**D-AMC	14 ± .86	0.0087 ± 0.0003	600 ± 260	+3.26
I99A/N218A				
Ac-IEPD-AMC	32 ± 1.9	0.087 ± 0.0008	2700 ± 590	0
Ac-IE**F**D-AMC	290 ± 24	0.26 ± .002	890 ± 350	−1.33
Ac-**L**EPD-AMC	1.3 ± 0.11	ND	>2000	+1.92
Ac-**LEF**D-AMC	37 ± 9.0	0.047 ± 0.0005	1300 ± 168	−0.09

branched amino acids. The specificity constant of the N218A/I99A variant is comparable to that of the I99A variant against the Ac-IEFD-AMC substrate (270 *versus* 290 $M^{-1}\ s^{-1}$, respectively) (Table IV). The tetrapeptide substrate with the highest specificity constant is Ac-IEFD-AMC (290 $M^{-1}\ s^{-1}$) rather than the PSSCL preferred Ac-LEFD-AMC (37 $M^{-1}\ s^{-1}$). This effect indicates that some cooperativity in substrate preference is masked by the combinatorial nature of the libraries.

To demonstrate the interaction between the P2 and P4 substrate amino acids and its contribution to transition state stabilization, a series of substituted substrates (Ac-IEPD-AMC, Ac-LEPD-AMC, Ac-IEFD-AMC, and Ac-LEFD-AMC) were kinetically characterized for wild type and N218A/I99A granzyme B (Table IV). The extent of nonadditivity, or cooperativity, between the extended subsites is determined by the difference between the transition state stabilization energy (35) calculated using the difference in specificity constant, k_{cat}/K_m, between a substituted substrate and the wild type (Ac-IEPD-AMC) substrate.

$$\Delta\Delta G_T^{\ddagger} = -RT \ln((k_{cat}/K_m)_{IEPD}/(k_{cat}/K_m)_{modified}) \qquad \text{(Eq. 4)}$$

The difference in free energy is positive if the wild type substrate, IEPD, is a better substrate than the modified substrate. There is nonadditivity between extended positions when the $\Delta\Delta G_T^{\ddagger}$ for a single amino acid modification does not equal the $\Delta\Delta G_T^{\ddagger}$ for the same modification made in the context of an additional modification.

In wild type granzyme B, the $\Delta\Delta G_T^{\ddagger}$ for P2-Pro *versus* P2-Phe is +0.71 and +1.1 kcal mol^{-1} when P4 is Ile or Leu, respectively (Table IV). The same result is observed for the P4 amino acid change for Ile to Leu ($\Delta\Delta G_T^{\ddagger}$, +2.18 and 2.55 kcal mol^{-1}). This highlights the wild type granzyme B requirement of an extended substrate for efficient hydrolysis. The identity of the P4 amino acid contributes 1.5 kcal mol^{-1} to the transition state stabilization and is directly additive to the identity of the P2 amino acid. When a non-preferred amino acid or no amino acid at all is present in the substrate, the activity is reduced significantly regardless of the identity of the P2 amino acid (Fig. 6*A*). The N218A/I99A variant introduces cooperativity into the specificity profile. The $\Delta\Delta G_T^{\ddagger}$ for P2-Pro *versus* P2-Phe is −1.33 kcal mol^{-1} when the P4 amino acid is Ile and −2 kcal/mol when the P4 amino acid is Leu. Cooperativity is also observed when the P4 amino acid is changed from Ile to Leu ($\Delta\Delta G_T^{\ddagger}$, +1.92 and +1.24 kcal mol^{-1} for P2-Pro and P2-Phe, respectively.) The amount of binding energy contributed to the transition state stabilization from an ideal or non-ideal P2 amino acid is dependent on the identity of the P4 amino acid in the N218A/I99A variant.

DISCUSSION

The granzyme B-like subfamily of serine proteases plays an important role in granule-mediated immune responses. They are clustered on chromosome 14 of the human, rat, and mouse species and have been proposed to have processing rather than digestive proteolytic properties. It is hypothesized that processing enzymes like the granzymes trade catalytic potency for selectivity, accepting reductions in catalytic rates in return for focused activity on a distinct extended substrate. The granzyme B subfamily has an active site architecture that is distinct among trypsin-like proteases. This active site, including the S1 through S4 loop regions, the catalytic triad, and the calcium and the sodium binding sites, represents sufficient diversity to differentiate the granzyme B subfamily in a phylogenic tree of full-length serine proteases (36). From this anal-

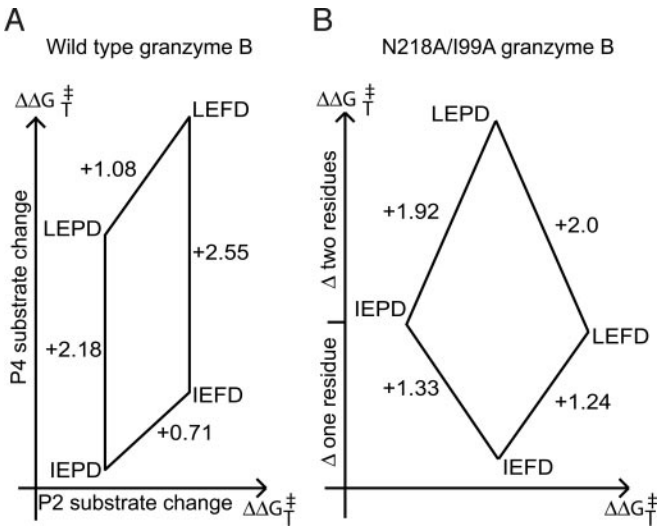

FIG. 6. **Free energy ($\Delta\Delta G_T^{\ddagger} = -RT\ln((k_{cat}/K_m)_{IEPD}/(k_{cat}/K_m)_{modified})$) analysis of wild type granzyme B compared with the N218A/I99A variant.** *A*, the change in free energy in wild type granzyme B of changing the P4 amino acid from Ile to Leu is ~2 kcal mol^{-1} and more important to overall catalytic activity than the identity of the P2 amino acid ($\Delta\Delta G_T^{\ddagger}$ of changing P2 from Pro to Phe is <1 kcal mol^{-1}). The *rectangular* shape of the free energy diagram illustrates that the $\Delta\Delta G_T^{\ddagger}$ for P4 is independent of the identity of the P2 amino acid. *b*, the N218A/I99A variant exhibits a cooperative effect between the P2 and P4 amino acids because the $\Delta\Delta G_T^{\ddagger}$ is dependent on the number rather than the position that was changed. This is illustrated best along the *y axis* of the diagram where a single amino acid change is ~1.3 kcal mol^{-1} and a second amino acid change is an additional 1.9 kcal/mol regardless of the order of the changes to the substrate. The P4 amino acid is no longer the most important extended site amino acid for activity because the change in $\Delta\Delta G_T^{\ddagger}$ is equivalent for both P2 and P4 varied substrates.

ysis, the amino acids that vary between subfamily members are likely to have important differentiating roles in specificity and catalysis. For granzyme B, four of these structural determinants have been investigated: positions 99, 174, 192, and 218.

The variants profiled in this study represent a detailed analysis of mutations to extended specificity, and the PSSCLs provide a powerful method for evaluating the changes in their specificity. Even slight mutations result in differences in the PSSCL profiles of the variants. Within a 10-fold dynamic range, the specificity profiles generated by PSSCL are mirrored in the individual kinetic constants of substrates. The profiles reveal specificity changes masked by traditional individual substrate analysis and immediately identify optimal individual substrates.

The dependence of P2 specificity on the identity of the amino acid at position 99 has been investigated by multiple groups, but the I99A, I99R, and I99F variants highlight the universal role of the size and hydrophobicity of position 99 as a determinant of P2 specificity. A dramatic change in P2 specificity occurred first when Ile^{99} was mutated to Arg and Ala and then in combination with N218A. These substitutions enlarged the S2 pocket so that bulky hydrophobic residues like Tyr and Phe preferentially fill the pocket by increasing the number of favorable van der Waals contacts. For P2, all of the additional binding energy of P2-Phe appears to be converted to transition state stabilization because the binding constants of non-optimal substrates at P2 are similar to each other. In a case that mirrors the profiling result for the I99R variant, profiling data for the human and mouse homologs of granzyme A indicate P2 hydrophobic specificity (37) where position 99 is Arg. Previous experiments with coagulation proteases suggest the converse specificity prediction also holds true. Tyr^{99} directly contacts the pyrrolidine ring of the bound inhibitor in the crystal structure of Factor Xa and blocks access to the P2 pocket by large side chains (38). Similarly Tyr^{99} blocks P2 substrate binding in Factor IXa (39), and when Thr^{99} of activated protein C is mutated to Tyr, it has an increased activity against substrates with a P2-Gly residue (40). The correlation between favorable van der Waals interactions in the S2 pocket and substrate specificity indicates that small amino acids at equivalent structural positions to Ile^{99} of granzyme B in other trypsin-like serine proteases may result in selectivity for bulky hydrophobic amino acids.

The other determinant with dramatic effects on specificity was Asn^{218}. It is structurally unique to the granzyme B subfamily of serine proteases and, in granzyme B, serves as a hydrogen bond donor selecting for the P3-Glu/Gln/Met preference. Single amino acid substitutions at Asn^{218} resulted in variants with increased acylation rates and broadened P3 specificity. This indicated a dual role in granzyme B specificity, the exclusion of substrates containing small or basic P3 amino acids and the maintenance of the P2 subsite additivity. Usually the amino acid at Arg^{192} is implicated as the primary P3 determinant as seen in studies of granzyme B (10), activated protein C (41), and Factor Xa (42). Therefore in granzyme B, P3 specificity is determined by both amino acids 218 and 192. Arg^{192} provides electrostatic repulsion against Arg and Lys substrate side chains, while the polar Asn^{218} acts as a selector to exclude large hydrophobic, β branched amino acids, and small amino acids. As with Ile^{99}, the reduction in activity for less preferred substrates is primarily in k_{cat}, suggesting that the selection is due to differences in transition state stabilization rather than formation of the Michaelis complex.

The results of the systematic mutagenesis described here demonstrate that amino acids in direct contact with the extended substrate amino acids by side chain to side chain interactions contribute to the extended specificity of the enzyme. Mutation of the structural determinants changed the specificity profiles and both the binding constant K_m and the catalytic rate k_{cat}. Importantly the hydrolysis of individual substrates by the variant proteases demonstrates that the alterations are primarily in selectivity rather than catalytic power. Thus, the alterations to specificity arise from the exclusion of non-ideal substrates rather than increased affinity of ideal substrates or an improvement of the transition state stabilization.

Cooperativity and interdependence between the extended substrate sites was also measured for granzyme B and its variants. In granzyme B, an Ile or Val at the P4 position is required for efficient hydrolysis. Concurrently the free energy analysis revealed that the catalytic rate changes dependent on the identity of the P2 and P4 amino acids are completely independent. Substrates with non-optimal P2 amino acids have lower k_{cat} values reminiscent of the increase in activity of chymotrypsin and porcine pancreatic elastase on increasingly extended substrates. It is hypothesized that extended substrates increase k_{cat} by improving transition state stabilization and conversion of binding energy to catalysis (34). Thus, for granzyme B an effective catalytic ceiling exists where hydrophobic, steric, and structural effects all contribute to efficient catalysis of substrates by modulating the formation of the Michaelis complex.

Asn^{218} plays a pivotal role in substrate specificity not limited to P3 selectivity. Two results point to an additional role in orienting the entire length of a tetrapeptide substrate for efficient hydrolysis. First, the Y174A mutation did not alter the P4 specificity, yet the addition of N218A to I99A had a dramatic effect on it. Second, the N218A/I99A double mutation introduced cooperative effects in substrate recognition by decoupling the independence between the P2 and P4 positions. This mutation also changed the relative importance of the identity

of the P2 and P4 amino acids compared with wild type. Structurally the truncation of 218 may increase the number of degrees of freedom of the substrate backbone. This would allow additional hydrophobic amino acids with reduced affinities (higher K_m) to be accommodated by the S4 pocket while still retaining efficient hydrolytic activity. Conversely the cooperativity between P2 and P4 amino acids suggests that the loop at 220 may have become more flexible requiring the expenditure of binding energy for loop stabilization. The role of Asn^{218} in granzyme B then is both to select the P3 substrate amino acid and to influence the catalytic activity of the protease.

By examining the amino acids that line the active site cleft of a granzyme B-like protease, logical predictions of extended specificity can be made. Position 99 has been well defined as a determinant of P2 specificity, and the combination of 192 and 218 defines P3 specificity. Position 174 stabilizes the S4 pocket, while positions 99 and 218 alter the P4 specificity. The remaining uncharacterized members of the granzyme B subfamily have a variety of amino acids at the structural determinants shown in this study to alter specificity: $Arg^{99}/Glu^{192}/Thr^{218}$ (granzyme C), $Asn^{99}/Ser^{192}/Asn^{218}$ (granzyme F), $Gly^{99}/Glu^{192}/Asn^{218}$ (granzyme G), and $Phe^{99}/Lys^{192}/Lys^{218}$ (granzyme H). Applying the results described here to these remaining granzymes suggests that granzymes C and G will have a hydrophobic P2 preference and polar or acidic P3 preference, while granzyme H will prefer small P2 amino acids. These extended specificity predictions define the specificities of the uncharacterized granzymes as unique rather than redundant. Each member has distinct characteristics at extended positions that suggest disparate roles in the apoptotic cascade.

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