

Cleavage Specificity of *Mycobacterium tuberculosis* ClpP1P2 Protease and Identification of Novel Peptide Substrates and Boronate Inhibitors with Anti-bacterial Activity*

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Background: ClpP1P2 is a novel protease complex essential for viability of *Mycobacterium tuberculosis*.

Results: Cleavage preferences of ClpP1P2 were defined, which allowed us to design potent substrate-based boronate inhibitors showing anti-mycobacterial activity.

Conclusion: Excellent new fluorogenic peptide substrates of ClpP1P2 were obtained, and novel enzyme properties were identified.

Significance: Selective inhibition of ClpP1P2 activity is a promising approach for drug development.

The ClpP1P2 protease complex is essential for viability in *Mycobacteria tuberculosis* and is an attractive drug target. Using a fluorogenic tripeptide library (Ac- $X_3X_2X_1$ -aminomethylcoumarin) and by determining specificity constants (k_{cat}/K_m), we show that ClpP1P2 prefers Met \gg Leu > Phe > Ala in the X_1 position, basic residues or Trp in the X_2 position, and Pro \gg Ala > Trp in the X_3 position. We identified peptide substrates that are hydrolyzed up to 1000 times faster than the standard ClpP substrate. These positional preferences were consistent with cleavage sites in the protein GFPssrA by ClpXP1P2. Studies of ClpP1P2 with inactive ClpP1 or ClpP2 indicated that ClpP1 was responsible for nearly all the peptidase activity, whereas both ClpP1 and ClpP2 contributed to protein degradation. Substrate-based peptide boronates were synthesized that inhibit ClpP1P2 peptidase activity in the submicromolar range. Some of them inhibited the growth of Mtb cells in the low micromolar range indicating that cleavage specificity of Mtb ClpP1P2 can be used to design novel anti-bacterial agents.

Tuberculosis is a devastating disease that causes nearly 2 million deaths annually. About a third of all humans are infected with latent *Mycobacterium tuberculosis* (Mtb),² which is becoming increasingly resistant to available antibiotics. Present treatments are not optimal; they require high doses of multiple agents for long periods and have numerous side effects. Consequently, it is important to identify and characterize new thera-

peutic targets and agents that selectively hit these targets. Recently, we characterized an enzyme that has the potential to be such a target: the protease complex ClpP1P2 (1, 2). ClpP1 and ClpP2 are both essential for viability and infectivity (3). Moreover, no enzyme homologous to ClpP1P2 is present in the cytosol of mammalian cells, where protein breakdown occurs by very different enzymes composing the ubiquitin proteasome pathway or autophagy-lysosome process. Also ClpP1P2 is quite different structurally from the ClpP complex in the mitochondrial matrix (4, 5).

The ClpP enzymes are a highly conserved family of multimeric serine proteases originally discovered and extensively characterized in *Escherichia coli* (6, 7). ClpP homologs exist in a wide range of bacteria as well as in chloroplasts and mitochondria in eukaryotes (4). The active enzyme is a tetradecamer composed of two heptameric rings that form a hollow cylinder with 14 proteolytic sites compartmentalized within its central chamber (8, 9). Most microorganisms possess a single *clpP* gene, whereas some, like Mtb, have two or more *clpPs* (4, 10, 11). By itself, *E. coli* ClpP is able to rapidly hydrolyze oligopeptides but not large globular protein without first forming a complex with an AAA ATPase, such as ClpA or ClpX in *E. coli* or ClpC in other species (12, 13). These hexameric ATPases associate with both ends of ClpP (12–14) and activate it but also selectively bind protein substrates, unfold them, and translocate them into the ClpP proteolytic chamber for degradation (14–18). Mtb contains two Clp ATPases, ClpC1 and ClpX, both of which are essential for viability (3). In fact, in collaborative studies, we recently showed that the cyclic peptide antibiotic lassomycin selectively kills mycobacteria by preventing ClpC1-dependent protein breakdown by ClpP1P2 protease (19).

We recently demonstrated that recombinant ClpP1 or ClpP2 by themselves form tetradecamers that lack proteolytic activity, but when mixed together, especially in the presence of low molecular weight peptide activators (N-terminal-blocked

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² The abbreviations used are: Mtb, *M. tuberculosis*; amc, aminomethylcoumarin; MIC, minimal inhibitory concentration; BoroMet, methionine boronic acid; Z, benzyloxy-carbonyl.

dipeptides or derivatives), they form a mixed tetradecameric complex that is very active in degrading peptides (1). The exact role of each ring in proteolysis is unclear and has been investigated here. The dramatic activation (up to >1000-fold) of the enzyme by the activator occurs in a unique fashion; they bind to the ClpP1 and ClpP2 inactive homo-tetradecamers promoting their dissociation into heptameric rings and re-associate to form the mixed functional ClpP1P2 complex containing one ring of ClpP1 and one ring of ClpP2. This activation by synthetic dipeptides is reversible and presumably mimics the action of some novel activating factor (e.g. chemical chaperone) that functions *in vivo*.

In designing or identifying selective inhibitors for an enzyme, it is important to have some knowledge of its specificity. It has been known for 25 years that the activity of ClpP from *E. coli* could be readily assayed with the fluorescent peptide, Suc-Leu-Tyr-amc (6, 7), which has also been widely used to assay ClpPs from other bacteria and mitochondria. However, this compound is a rather poor substrate for Mtb ClpP1P2. Here, by using an *N*-acetyl tripeptide-aminomethylcoumarin library (Ac- $X_3X_2X_1$ -amc), we elucidated the cleavage preferences of Mtb ClpP1P2 and identified a number of novel substrates that are up to 1000-fold better than the standard substrate.

On this basis we investigated the specific roles of ClpP1 and ClpP2 in degrading peptides and proteins. In addition, we synthesized a series of substrate-based peptide boronate derivatives that inhibit ClpP1P2 peptidase activity and protein degradation in the presence of ClpC1 and ClpX ATPases. Some of these inhibitors prevented growth of Mtb selectively. These observations provide a platform for further development of potent inhibitors of Mtb ClpP1P2 with anti-tuberculosis activity.

EXPERIMENTAL PROCEDURES

Materials

Human 20 S proteasomes purified from red blood cells was purchased from Boston Biochem, Cambridge, MA. Individual substrates for kinetic analysis were custom-synthesized by AnaSpec and Biomol (Plymouth Meeting, PA).

Bacterial Strains, Plasmids, Expression, and Growth of Cells

H37Rv strain was used in all experiments involving Mtb. All Mtb proteins were expressed in an *E. coli* BL21 strain lacking endogenous ClpP and ClpX. For expression of mature forms of wild type and active site mutants of ClpP1 (lacking 6 N-terminal amino acids) and ClpP2 (lacking 11 N-terminal amino acids), pTetOR plasmid, which has an inducible tetracycline promoter, was used. Induction with anhydrotetracycline (100 ng/ml) was carried out overnight. ClpC1 was expressed using pET28a plasmid and induced for 3 h at 37 °C by 1 mM isopropyl 1-thio- β -D-galactopyranoside. A truncated form of ClpX (residues 60–426) was used throughout this study. It was expressed from pTrc99 in a 3-h induction at 16 °C with 0.2 mM isopropyl 1-thio- β -D-galactopyranoside. All proteins had C-terminal His₆ tags except ClpX, which contained an N-terminal His₆ tag.

Characterization of Ac- $X_3X_2X_1$ -amc Library

The ChemRX Protease Profiler library of *N*-acetylated tripeptide-amc peptides was from Discovery Partners International (20). In addition to the 20 standard amino acids, L-citrulline and L-ornithine were present in the X_1 position. In the X_2 and X_3 positions, cysteine was replaced by L-ornithine in the 20-standard amino acid set. All peptides contained an N-terminal acetyl group. Of the possible 8800 tripeptides ($22 X_1 \times 20 X_2 \times 20 X_3$) 5920 were useable based on yield and purity. The average purity was 85–90% for any individual substrate.

Purification of Mtb Enzymes

Purification of recombinant Mtb ClpP1, ClpP2, and their inactive mutants was carried out as described previously (1). Purification of Mtb ClpX and ClpC1 was carried out at 4 °C using buffer B (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1 mM DTT, 1 mM Mg-ATP, and 10% glycerol). Cells were resuspended in two volumes of buffer and lysed with a French press at 1500 p.s.i. The extract was centrifuged at $100,000 \times g$ and mixed with nickel-nitrilotriacetic acid-agarose. After a 4-h incubation, nickel-nitrilotriacetic acid-agarose resin was transferred to a column, and proteins were eluted using step gradient (25, 50, 100, and 200 mM) of imidazole in buffer B. The active fractions containing nearly homogeneous proteins were combined and concentrated to 1–3 mg/ml by Millipore MWCO 10-kDa cut filter and fractionated further by gel filtration on a column (2.5 \times 22 cm) of Sephacryl S-300 equilibrated in the same buffer. The protein peak was collected, concentrated to ~ 3 mg/ml, and stored at -80 °C. All enzymes purified migrated as a single band in the SDS-PAGE.

Peptidase Assay

All assays of peptidase activity were performed at 37 °C in black 384-well plates using a Plate Reader SpectraMax M5 (Molecular Devices). 2–3 min were allowed for the reaction mixtures to reach 37 °C. Each well contained 10 μ M fluorogenic peptide, 20–50 nM ClpP1P2 in 80 μ l of buffer A (20 mM phosphate buffer, pH 7.6, with 100 mM KCl, 5% glycerol, and 5 mM Z-Leu-Leu). DMSO concentration never exceeded 2%. The reaction was initiated by the addition of the enzyme, and peptidase activity was followed in the linear range by monitoring the rate of production of fluorescent 7-amino-4-methylcoumarin-amc from peptide-amc substrates at 460 nm (excitation at 380 nm). The deviation of fluorescence value in three independent measurements was not >5%.

Determination of Kinetic Constants for Peptide Substrates and Boronate Inhibitors

Specificity constant (k_{cat}/K_m) for peptide substrates was calculated from the linear plot of enzyme activity at 10 μ M peptide concentrations, which is several hundred times less than the K_m of peptide substrates. K_i for peptide boronates were calculated using the formula $K_i = IC_{50}/(1 + [S]/K_m)$ (21). Under these conditions, K_i values equaled the IC_{50} . Inhibitors were assayed at eight different concentrations, and the IC_{50} (K_i) was calculated from four points for nonlinear curves using SpectroMax 5 program.

ClpP1P2 Cleavage Specificity and Boronate Inhibitors

Proteinase Assay

Mtb ClpP1P2 was also assayed continuously in 96-well plates using the fluorescent protein substrates, GFP_{ssrA}, and FITC-casein. To measure GFP_{ssrA} degradation by the ClpXP1P2 complex, each well contained 2 mM Mg-ATP, 500 nM GFP_{ssrA}, 75–100 nM ClpP1P2 tetradecamer, and 300–400 nM ClpX hexamer in 100 μ l of buffer A, and GFP_{ssrA} fluorescence was measured at 510 nm (excitation at 395 nm). To measure FITC-casein degradation by ClpC1P1P2, each well contained 2 mM Mg-ATP, 150–200 nM ClpP1P2, 500–700 nM ClpC1 hexamer, and 1–1.2 μ M FITC-casein in 100 μ l of buffer A. FITC-casein fluorescence was monitored at 518 nm (excitation at 492 nm), and the deviation of fluorescence value in three independent measurements was not >10%.

Products of GFP_{ssrA} Digestion by ClpXP1P2

To determine the cleavage preferences of ClpP1P2 in proteins, 1 μ M GFP_{ssrA} was incubated with 75–100 nM ClpP1P2, 400 nM ClpX hexamer, and 2 mM Mg-ATP in buffer A. After 50% degradation of protein, the products were extracted and analyzed by liquid chromatography and Tandem MS/MS according to Akopian *et al.* (1).

Determination of Peptidase and Proteinase Activity of ClpP1P2 Complexes in the Native Gel

WT or mutant forms of ClpP1 and ClpP2 (3 μ g of each) were mixed together in buffer A in the presence of the peptide activator Z-LL to form WT ClpP1P2 or mutant ClpP1P2(S-A) or ClpP1(S-A)P2 complexes, which were then isolated using the native PAGE. The formation of mixed ClpP1P2 complexes was confirmed by mass spectroscopy. To determine the peptidase activity, the gel was incubated for 25 min in buffer A containing 10 μ M Ac-PKM-amc and 5 mM Z-LL, and the appearance of amc fluorescence in the bands was detected in UV light using AlphaImager. To determine the proteinase activity, the gel was first incubated for 2 h with 0.1 mg/ml GFP-ssrA in buffer A to allow the GFP diffusion into the gel. Then the gel was incubated for 2 h with 0.02 mg/ml ClpX in buffer A with 5 mM Mg-ATP. The decrease of GFP fluorescence in proteolytically active ClpP1P2 bands was detected using AlphaImager.

ATPase Assay

ATP hydrolysis was measured with the enzyme-linked assay using pyruvate kinase and lactic dehydrogenase (PK/LDH). 2 μ g of pure ClpC1 or ClpX were mixed with 100 μ l of the assay buffer B containing 1 mM phosphoenolpyruvate, 1 mM NADH, 2 units of pyruvate kinase/lactic dehydrogenase, 4 mM MgCl₂, and 1 mM ATP, and the ATPase activity was followed by measuring the oxidization of NADH to NAD spectrometrically at 340 nm. Measurements were performed in triplicate, which agreed within 5%.

Determination of MIC₅₀ for Peptide Boronate Inhibitors against Mtb and Mycobacterium smegmatis

To determine the concentration of peptide boronates that inhibit cell growth, the colorimetric resazurin microtiter assay was used. 7H9 media (Difco) was dispensed in each well of a

sterile flat-bottom 96-well plate. Peptide boronates were serially diluted 2-fold across the plate. Bacteria were diluted to A₆₀₀ = 0.003, and equal volumes of culture were added to the serially diluted compounds. Plates were grown at 37 °C for 5 days for Mtb or overnight for *M. smegmatis*. At different times, 20 μ l of resazurin (0.05% w:v in water) were added to each well, and plates were incubated at 37 °C for 1 day (for Mtb) or 6 h for *M. smegmatis*. Growth and metabolism of bacteria is reflected by the reduction of resazurin, a blue non-fluorescent dye, to resorufin, a pink highly fluorescent dye. MICs for each boronate were defined as the highest concentration that resulted in no color change in resazurin.

Synthesis of the Peptide Boronate Inhibitors

Except for *N*-(picolinoyl)-Lys-boroMet and *N*-(benzo[b]thiophene-7-carbonyl)-Lys-boroMet (see below), all compounds were well characterized and reported (22). The target compounds were purified by reverse-phase (RP)-HPLC using a Varian semi-preparative system with a Discovery C18 569226-U RP-HPLC column. The mobile phase was typically made by mixing water (0.1% TFA) with acetonitrile (0.08% TFA) in gradient concentration. Purities determined by HPLC analysis were >95%.

N-(Picolinoyl)-Lys-boroMet—¹H NMR (D₂O) δ 8.73 (d, *J* = 4.3 Hz, 1H), 8.27–8.19 (m, 2H), 7.90–7.80 (m, 1H), 2.99 (t, *J* = 7.5 Hz, 2H), 2.80–2.70 (m, 1H), 2.60–2.50 (m, 2H), 2.06–1.90 (m, 5H), 1.80–1.65 (m, 4H), 1.55–1.45 (m, 2H). MS (ESI⁺) *m/z* (relative intensity): 365.3 ([M – H₂O + H]⁺, 100).

N-(Benzo[b]thiophene-7-carbonyl)-Lys-boroMet—¹H NMR (D₂O) δ 8.17 (d, *J* = 7.6 Hz, 1H), 7.94–7.85 (m, 1H), 7.75 (d, *J* = 5.6 Hz, 1H), 7.60–7.52 (m, 2H), 3.03 (t, *J* = 7.4 Hz, 2H), 2.85–2.75 (m, 1H), 2.54 (t, *J* = 7.4 Hz, 2H), 2.02–1.95 (m, 5H), 1.79–1.70 (m, 4H), 1.60–1.50 (m, 2H), 1.35 (d, *J* = 6.6 Hz, 1H). MS (ESI⁺) *m/z* (relative intensity): 420.1 ([M – H₂O + H]⁺, 100).

RESULTS

Residues Preferred by ClpP1P2 in the X₁ Position in the Tripeptide Library—During our initial characterization of ClpP1P2 protease using a small set of substrates (1), we observed that the complex had a preference for hydrophobic residues in the X₁ position. To define more precisely the positional preferences of ClpP1P2, we used an *N*-acetylated tripeptide-amc fluorogenic substrate library consisting of individual peptides covering the Ac-X₃X₂X₁-amc sequence space (20). First, the best substrate was identified for each of the 20 sublibraries with individual amino acid residues in the X₁ position (Fig. 1A). Substrates with the hydrophobic residues, Leu, Phe, Ala, and especially Met in the X₁ position were cleaved most efficiently by ClpP1P2 (Fig. 1A). Moreover, all of the 30 most rapidly hydrolyzed substrates (~0.5% of the library) and 49 of the 60 top substrates contained a Met in the X₁ position (data not shown). It is noteworthy that similar results were obtained when the average enzymatic activity for all substrates with specific residues in the X₁ position was calculated (Fig. 1B, Table 1). By contrast, little or no enzymatic activity was detected with other amino acids in the X₁ position, although some peptides with Lys, Arg, Glu, or Asp in this position could be cleaved much more slowly than

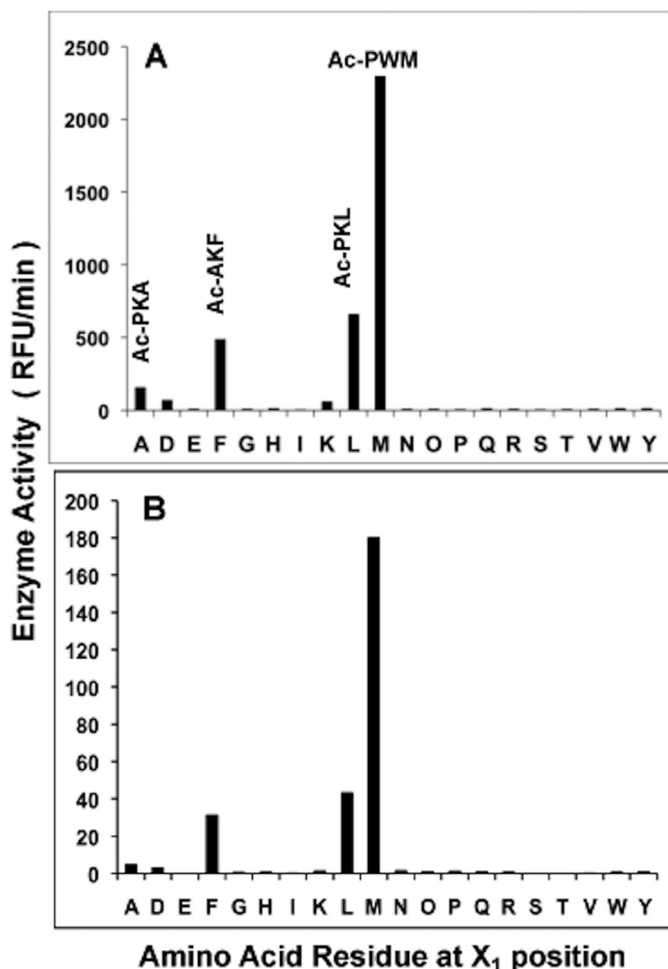


FIGURE 1. *Mtb* ClpP1P2 cleaves peptide-amc substrates mainly after Leu, Phe, Ala, and especially Met. *A*, to determine cleavage preferences for the X₁ position, ClpP1P2 activity was measured continuously using Ac-X₃X₂X₁-amc fluorogenic peptides library (at 10 μM) in buffer A containing 30 nM ClpP1P2. The ClpP1P2 activity against the best substrates with a given amino acid in X₁ position is reflected in the graph. *B*, average rate of hydrolysis of substrates with a given amino acid in the X₁ position. The activity was measured as in Fig. A. RFU, relative fluorescence units.

ones with Met or other hydrophobic residues in X₁ position (Table 2).

Residues Preferred by ClpP1P2 in the X₂ and X₃ Positions in the Tripeptide Library—The preferences at the X₂ and X₃ positions were calculated when the X₁ residue was fixed with one of the residues identified above (Met, Leu, Phe, or Ala). There was a clear preference for basic residues, particularly Lys and aromatic residues, particularly Trp, in the X₂ position, and Pro ≫ Ala > Trp residues in the X₃ position (Fig. 2). Table 1 presents the 10 best tripeptide substrates with Met, Leu, Phe, or Ala in the X₁ position, and most contained a basic residue in the X₂ and Pro in X₃ position. Of the 40 best substrates, 70% contain a positively charged amino acid in X₂ position, half of which were Lys. For the X₃ position, 58% contained Pro, and 20% contained Ala (Table 1).

The Tripeptides Most Efficiently Cleaved by ClpP1P2—To confirm the data obtained using the peptide library and to determine the catalytic efficiency (k_{cat}) for ClpP1P2 enzyme, a number of peptide substrates identified above were synthesized and retested. Due to limited solubility of many peptide-amc

TABLE 1

Cleavage preferences of *Mtb* ClpP1P2 for X₂ and X₃ positions with Met, Leu, Phe, or Ala at X₁ position

RFU, relative fluorescence units; Or = ornithine.

Ac-X ₃ X ₂ X ₁ -amc	X ₃	X ₂	X ₁	Peptidase activity (RFU)
PWM	Pro	Trp	Met	2299
HKM	His	Lys	Met	1957
PFM	Pro	Phe	Met	1754
PRM	Pro	Arg	Met	1712
PKM	Pro	Lys	Met	1497
POrM	Pro	Or	Met	1459
ARM	Ala	Arg	Met	1294
AKM	Ala	Lys	Met	1267
AWM	Ala	Lys	Met	1230
GKM	Gly	Lys	Met	1277
PKL	Pro	Lys	Leu	661
WKL	Trp	Lys	Leu	591
WYL	Trp	Tyr	Leu	478
PLL	Pro	Leu	Leu	385
POrL	Pro	Or	Leu	374
PWL	Pro	Trp	Leu	347
WRL	Trp	Arg	Leu	347
PRL	Pro	Arg	Leu	306
YKL	Tyr	Lys	Leu	276
PSL	Pro	Ser	Leu	272
AKF	Ala	Lys	Phe	489
PKF	Pro	Lys	Phe	454
GWF	Gly	Trp	Phe	440
PRF	Pro	Arg	Phe	423
AWF	Ala	Trp	Phe	320
AYF	Ala	Tyr	Phe	269
PYF	Pro	Tyr	Phe	265
WKF	Trp	Lys	Phe	238
ARF	Ala	Arg	Phe	235
WYF	Trp	Tyr	Phe	206
PKA	Pro	Lys	Ala	158
PRA	Pro	Arg	Ala	101
VKA	Val	Lys	Ala	87
ARA	Ala	Arg	Ala	52
POrA	Pro	Or	Ala	45
AKA	Ala	Lys	Ala	43
FKA	Phe	Lys	Ala	24
AOrA	Ala	Or	Ala	21
PWA	Pro	Trp	Ala	18
IRA	Ile	Arg	Ala	11

substrates, it was not possible to measure the V_{max} , which requires high concentrations of the substrates (e.g. for Ac-Pro-Lys-Met-amc $K_m > 3$ mM). To directly compare the enzyme against different substrates, we decided to determine the second order rate specificity constant k_{cat}/K_m (see “Experimental Procedures”). Two peptides, Ac-Pro-Lys-Met-amc and Ac-Pro-Trp-Met-amc, were determined to be the best substrates for ClpP1P2 (Table 2). Exchange of Met in the X₁ position for its close analog Nle (norleucine) decreased substrate cleavage 3-fold. The C-terminal dipeptides derived from the best two substrates, Ac-Pro-Lys-Met-amc and Ac-Pro-Trp-Met-amc, were also good substrates for ClpP1P2, especially Ac-Trp-Met-amc. It is noteworthy that all these four substrates are 100–1000× more efficiently cleaved than the standard fluorogenic substrate, Suc-Leu-Tyr-amc (7, 23). Based on its k_{cat}/K_m value, Ac-Pro-Lys-Met-amc is the best substrate among the peptides with Met at the X₁ position and was clearly better than Ac-Pro-Trp-Met-amc in contrast to our observations with the peptide library.

To be enzymatically active ClpP1P2 required the presence of the dipeptide activator Z-Leu-Leu, which induces dissociation of the inactive ClpP1 and ClpP2 complexes and reassociation

ClpP1P2 Cleavage Specificity and Boronate Inhibitors

TABLE 2
Specificity constant k_{cat}/K_m for Mtb ClpP1P2 and *E. coli*, *B. subtilis*, and *S. aureus* ClpPs against tripeptide-amc substrates
Nle, norleucine.

Substrate $X_3X_2X_1$ -amc	k_{cat}/K_m			
	<i>M. tuberculosis</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>
	$\text{M}^{-1}\text{S}^{-1}$			
Chymotryptic				
Ac-PKM	1327	331	176	2632
Ac-PKNle	411	69	239	2464
Ac-KM	144	56	36	630
Ac-PWM	1155	462	520	4745
Ac-WM	612	184	268	4095
Ac-ARM	410	236	526	1553
Ac-HKM	169	128	6	695
Ac-PKL	199	270	234	2021
Ac-PQL	63	183	247	2886
Ac-PYL	32	265	598	3302
Ac-PAL	42	127	282	1804
Ac-PKF	320	185	132	634
Ac-PAF	68	62	97	605
Ac-PAK	40	35	76	812
Z-LLL	0	14	0	46
Z-LL	0	8	2	58
Suc-LY	1	7	50	721
Suc-LLVY	2	2	1	26
Tryptic				
Ac-WEK	2	0	0	3
Ac-FAK	1	0	0	0
YMK	2	0	2	1
Z-LLR	5	0	1	1
Z-FVR	4	1	0	1
Acidic				
Z-nLPnLD	14	3	10	31
Z-APnLD	0	0	0	0
Ac-PnLD	0	4	0	0
Ac-LWD	2	1	0	14
Ac-WID	0	2	0	0

into the active ClpP1P2 complex (1). Our prior observation that the amc derivatives of the peptide activators are not good substrates (1) suggested that the activators bind either to sites distinct from the active site or in a non-productive mode to the active site. Accordingly, we found here that k_{cat}/K_m values of the amc derivatives of peptide activators Z-Leu-Leu-amc and Z-Leu-Leu-Leu-amc are very low (Table 2), confirming our prior observations and conclusions.

These Tripeptide Substrates Are Rapidly Cleaved by Other ClpP Enzymes but Not by the Proteasome—We also tested whether the cleavage specificity observed here for ClpP1P2 is unique or is shared by other members of ClpP family. To compare the specificity constant of ClpP1P2 with those of other members of the ClpP family, it is important to know how efficiently ClpP1 and ClpP2 associate into the active ClpP1P2 complex. Analysis by native gel electrophoresis indicates that in the presence of the activator nearly all of ClpP1 and ClpP2 are engaged in the mixed complex with a distinct mobility (Fig. 3) (the presence of both proteins in the band was confirmed by mass spectroscopy).

We found that the best substrates for ClpP1P2 are also very good substrates for *Bacillus subtilis*, *Staphylococcus aureus*, and *E. coli* ClpPs (Table 2) and that they are much better than the standard substrate, Suc-Leu-Tyr-amc. However, some individual differences were evident. For instance, the best substrates for Mtb ClpP1P2 were Ac-Pro-Lys-Met-amc and Ac-Pro-Trp-Met-amc, whereas *S. aureus* and *E. coli* ClpPs preferred Ac-Pro-Trp-Met-amc, and *B. subtilis* preferred Ac-

Pro-Tyr-Leu-amc. The standard ClpP substrate Suc-Leu-Tyr-amc is cleaved poorly by these enzymes except *E. coli* ClpP (Table 2).

Unlike most other bacteria, Mtb in addition to the ClpP1P2 expresses a 20 S proteasome that appears to be important for survival in the macrophage (24). Mtb proteasomes do not cleave tripeptides with Met in the X_1 position (25), the preferred substrates of ClpP1P2. We further tested whether the tripeptide substrates preferred by the Mtb proteasome were hydrolyzed by ClpP1P2. The seven best proteasome substrates (e.g. Ac-Arg-Ala-Trp-amc or Ac-Tyr-Gly-Trp-amc (25)) were poorly cleaved by ClpP1P2 (with k_{cat}/K_m from 0 to 14) (data not shown).

If a ClpP1P2 inhibitor were to be used in human patients, it is critical that it should not inhibit intracellular human proteases, the most important of which are proteasomes. We, therefore, tested if these ClpP substrates are degraded by human proteasomes and whether commonly used proteasome substrates are cleaved by ClpP1P2. The results show that the cleavage specificity of ClpP1P2 is quite different from that of the human proteasome (Fig. 4). For example, the standard substrate used for the mammalian proteasome chymotrypsin-like site, Suc-Leu-Leu-Tyr-amc, is a very poor substrate for Mtb ClpP1P2. Therefore, it should be possible to selectively inhibit ClpP1P2 without interfering with the proteasome function.

Cleavage Preferences in Proteins Resemble Those in the Peptide Substrates—We next tested whether ClpP1P2 shows similar cleavage preferences for the X_1 position in protein substrates as were observed with the tripeptide amc library. Rapid protein degradation requires protease association with a hexameric AAA ATPase complex, either ClpC1 or ClpX, which bind specific proteins and unfolds and translocates them into ClpP1P2 proteolytic chamber for processive degradation. We expressed His₆-tagged Mtb ClpX and ClpC1 ATPases in *E. coli*, purified them using affinity and size exclusion chromatography (to near-homogeneity by SDS-PAGE), and identified a fluorescent model protein substrate for each. Together with ClpX, the Mtb ClpP1P2 degrades GFPssrA-like ClpXP from *E. coli* (18) in a process requiring ATP. With ClpC1, ClpP1P2 degrades FITC-casein in an ATP-dependent manner (although some very slow breakdown of FITC-casein by ClpP1P2 alone was also observed) (1).

To determine the cleavage site preference of ClpP1P2 in protein substrates, GFPssrA was digested by ClpXP1P2, and the resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LS-MS/MS). Ninety-five unique peptides were identified covering >90% of the sequence of GFP. The sizes of the recovered peptides ranged from 4 to 26 residues in length, but most were 8–13 amino acids in length (Fig. 5A). Analysis of the C-terminal residues of these peptides indicate that ClpP1P2 cleaved GFPssrA most readily after Leu, Phe, and especially Met (Fig. 5B). Thus, in hydrolyzing proteins, ClpP1P2 showed similar preferences for the X_1 position as in digesting the peptide library. The main difference was that in digestion of GFPssrA, cleavages often occurred after Asn residues, which was not observed with the tripeptide library. Analysis of N-terminal amino acid residues of peptides generated during GFPssrA degradation allowed us also to determine if

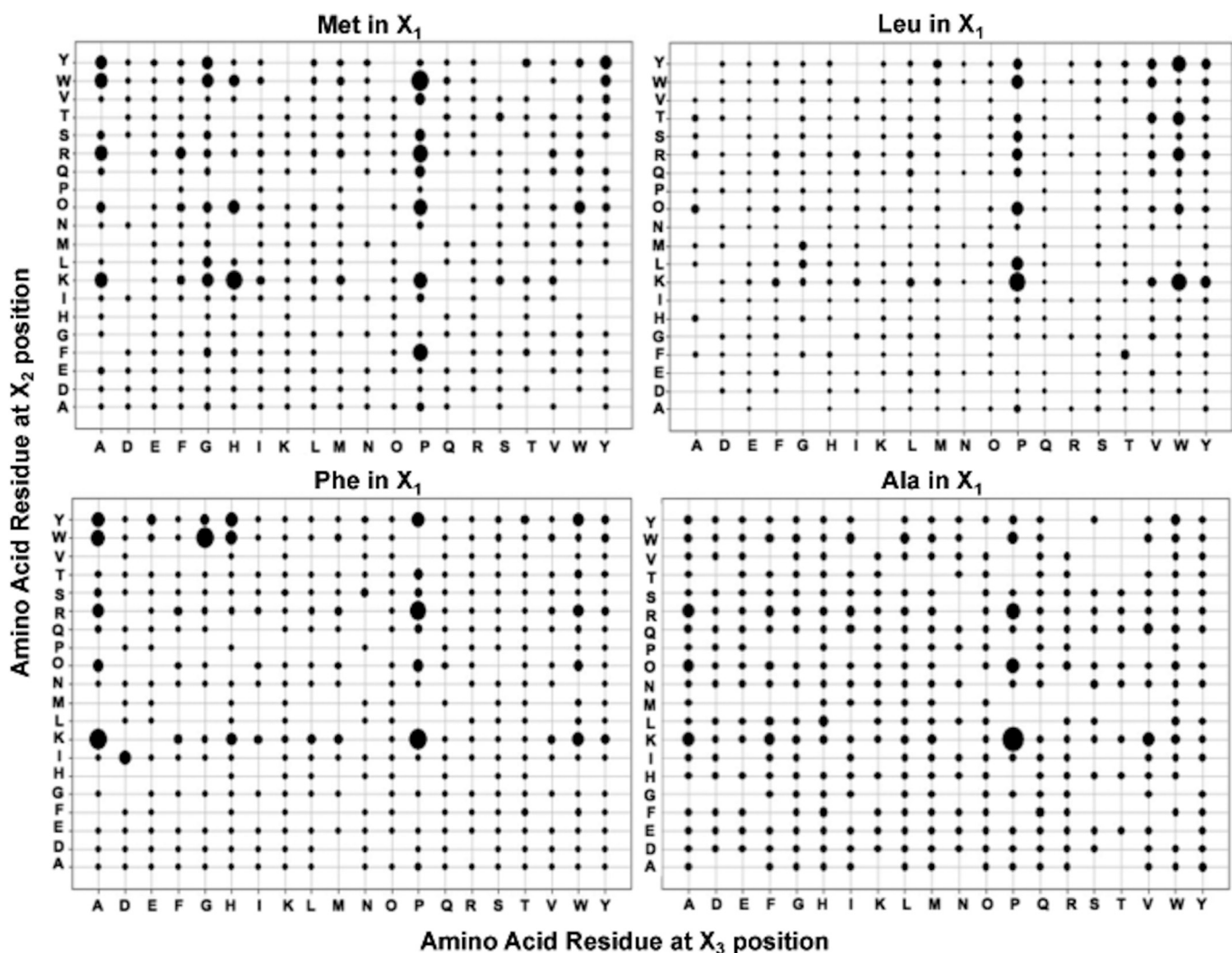


FIGURE 2. *Mtb* ClpP1P2 cleavage preferences for the X_2 and X_3 positions with fixed Met, Leu, Phe or Ala at the X_1 position. ClpP1P2 activity was measured using Ac- $X_3X_2X_1$ -amc fluorogenic peptides library as in Fig. 1A. The size of the dots represent the ClpP1P2 activity against the given peptide and is calculated as percent of the maximal value for each panel. Maximal activity (relative fluorescence units) for the libraries with fixed Met in X_1 position was 2300, with Leu 660, with Phe 490, or with Ala 160.

ClpP1P2 showed any preference for the nature of the residue(s) after the cleavage site (X_1'). As shown in Fig. 5C, ClpP1P2 seems to prefer positive amino acids (Arg, Lys, and His) and Ser in the X_1' position.

ClpP1 Accounts for Most of the Peptidase Activity Even though ClpP1 and ClpP2 Subunits Both Can Support Protein Breakdown—To understand the roles of ClpP1 and ClpP2 in the breakdown of proteins and peptides, we constructed ClpP1P2 complexes where only one of the subunits was enzymatically active. As we reported previously (1), both subunits must be present for enzymatic activity. Wild type and mutant ClpP1 and ClpP2 subunits were mixed together with the activator to form mixed complexes in which either of the subunits (or both) was inactive due to mutation of the active site serine to an alanine (S-A). When their abilities to degrade various tripeptide substrates were compared, surprisingly, the ClpP1P2(S-A) complex showed equal or even better activity than the WT enzyme (Fig. 6A). By contrast, ClpP1(S-A)P2 showed almost no activity against the best peptide substrates (even when tested at 1 mM concentration) and only limited

activity against the caspase substrate, Z-Nle-Pro-Nle-Asp-amc (Nle is norleucine; Fig. 6A). As expected, the double ClpP1(S-A)P2(S-A) mutant was inactive against all the substrates tested. Thus, ClpP1 is responsible for nearly all the activity against the tripeptide-amc substrates, as was suggested previously using other peptide substrates (1).

We then tested whether ClpP1 also played the predominant role in protein degradation. Surprisingly, in contrast to tripeptide degradation, ClpP1(S-A)P2 complexes degraded FITC-casein (with ClpC1) and GFPssrA (with ClpX) only slightly worse than ClpP1P2(S-A) or WT ClpP1P2 (Fig. 6B). Thus, even though ClpP1 and ClpP2 have very different activities against tripeptide substrates (Fig. 6A), the proteolytic activities of ClpP1 or ClpP2 do not determine the rates of degradation of protein substrates, which are determined by the ATPases, and in the presence of ATPases, either subunit has sufficient activity to digest these proteins almost as rapidly as the WT complex.

This conclusion was also confirmed by a different approach in which the wild type and two mutant ClpP1P2 complexes were isolated by native gel electrophoresis, and the peptidase

ClpP1P2 Cleavage Specificity and Boronate Inhibitors

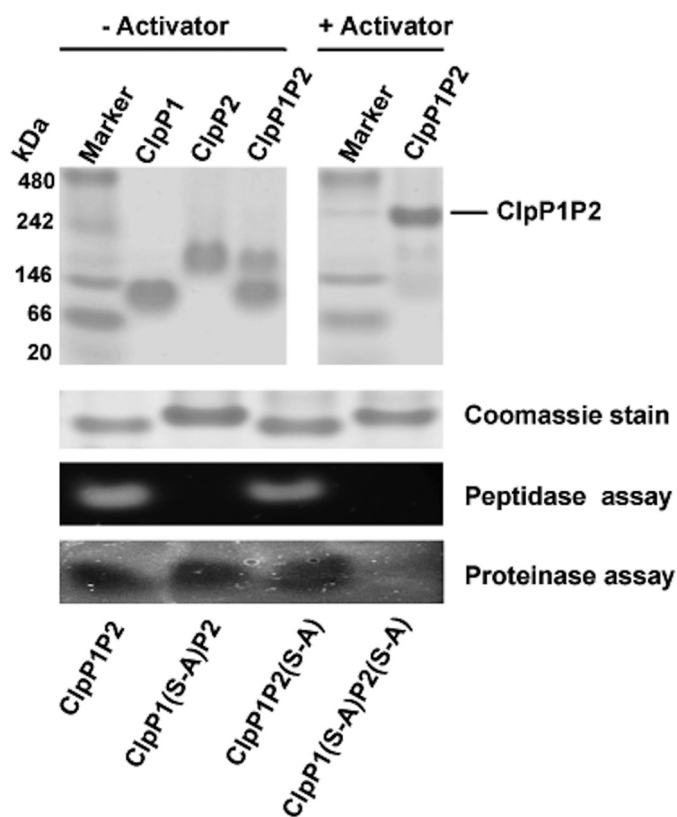


FIGURE 3. Isolation of WT or mutant forms of ClpP1P2 complexes in native gel and determining their enzymatic activity. ClpP1 and ClpP2 (3 μg of each) were mixed together in buffer A to form WT ClpP1P2 or mutant ClpP1P2(S-A) or ClpP1(S-A)P2 complexes and isolated using the native PAGE in the presence or absence of the peptide activator Z-LL. The peptidase (with Ac-PKM-amc) and proteinase (with GFPssrA) activities of the enzyme were then determined directly in the gel as described under "Experimental Procedures."

activity against Ac-Pro-Lys-Met-amc and proteinase activity against GFPssrA in the presence of ClpX were determined directly in the gel (Fig. 3). As expected, the peptidase activity was detected only in the complexes containing active ClpP1, whereas the proteinase activity was detected in complexes containing either active ClpP1 or ClpP2.

It is noteworthy that both ATPases also accelerated tripeptide cleavage by ClpP1P2 and thus must be influencing ClpP1P2 structure/conformation and not just protein unfolding and translocation. ClpC1 increased the degradation of Ac-Pro-Lys-Met-amc about 1.5-fold and ClpX by 3.5-fold. With the longer peptide substrate, Mca-Gly-His-Gln-Gln-Tyr-Lys-Met-Lys-Dpa(2,4-dinitrophenol)-amide (where Mca is (7-methoxycoumarin-4yl)acetyl and Dpa is diaminopropionic acid), ClpC1 caused an approximately 2-fold increase, and ClpX caused an 8-fold increase in degradation rate. This activation was observed when ClpP1P2(S-A) was used in place of ClpP1P2 but not for ClpP1(S-A)P2. As was found for protein breakdown (1), the activation of tripeptide degradation by the ATPases was observed only in the presence of the activator peptides. Thus, although the ClpP2 active site cannot cleave tripeptide substrates but can digest proteins for reasons that are not clear, the association with the ATPase is, by itself, not sufficient to activate the ClpP2 active site against peptides. Presumably, the

presence of the protein substrate somehow exposes activity of ClpP2.

Synthesis of Tripeptide Boronate Inhibitors of ClpP1P2—Our major goal in defining the specificity of ClpP1P2 was to lay the basis for the designing of new inhibitors that may selectively kill Mtb cells. Of the many peptide derivatives that inhibit serine proteases, a potent class is peptides with a boronic-based electrophile in their C termini (26–28). These highly specific transition state inhibitors can bind to the active sites of both serine and threonine proteases. For example, the peptide boronate proteasome inhibitor, bortezomib (Velcade), is now used as the preferred treatment for multiple myeloma (27). Using our best substrates identified in Table 1, we synthesized a number of corresponding boronate derivatives. Some of these peptide boronates (e.g. Ac-Pro-Lys-boroMet (Table 3, #1), Ac-His-Lys-boroMet (Table 3, #2), and Ac-Ala-Lys-boroMet (Table 3, #3)) inhibited the peptidase activity of ClpP1P2 with a K_i of $<1 \mu\text{M}$ (Table 3). To test how the nature of the N-terminal blocking group might affect inhibitor potency, we synthesized several tripeptide boronates with an N-terminal picolinoyl group in place of the acetyl one. The inhibitory activity of picolinoyl-Ala-Lys-boroMet (Table 3, #6) and picolinoyl-Pro-Lys-boroMet (Table 3, #7) had similar or somewhat greater activities to those of the corresponding acetyl compounds. Interestingly, picolinoyl-Trp-Lys-boroMet (Table 3, #5) was a very potent inhibitor ($K_i = 0.18 \mu\text{M}$). Because the dipeptide substrates Ac-Lys-Met-amc and Ac-Trp-Met-amc are rapidly cleaved by ClpP1P2 (Table 2), we also synthesized dipeptide boronate Lys-boroMet with several types of N-terminal blocking groups. As shown in Table 3, several dipeptide boronates were the most effective inhibitors of ClpP1P2 activity, especially *N*-(2-(3,5 difluorophenyl)acetyl)-Lys-boroMet (Table 3, #11), $K_i = 65 \text{ nM}$.

Although peptide boronic acid inhibitor may often dissociate extremely slowly from their target enzymes (e.g. bortezomib from proteasomes), inhibition is nevertheless usually fully reversible. Thus, when ClpP1P2 was incubated with the inhibitors for 2 h at room temperature, enzyme activity could be restored to $>90\%$ of the original activity by either centrifugation using filters with 10,000 M_r cut off or simply by dilution.

Effects of Peptide Boronate Inhibitors on ClpP1 and ClpP2 Activity—We then tested how the peptide boronate inhibitors affect peptide and protein degradation by ClpP1P2 complexes and by each of its two types of active sites. In hydrolyzing the peptide substrates, ClpP1P2(S-A) was even more sensitive to the peptide boronate inhibitor than WT ClpP1P2 (Fig. 7A). This result is in agreement with our earlier finding (Ref. 1, Fig. 6A) that ClpP1 active sites catalyzed most of hydrolysis of the tripeptide substrates. ClpP1(S-A)P2, whose peptidase activity was only 1% that of WT ClpP1P2 (Fig. 6A), was much less sensitive to boronate inhibitors. Thus its IC_{50} against Ac-PKM-amc was >10 times higher than that of the WT enzyme (Fig. 7A). Together, these findings indicate that the peptide boronates inhibit preferentially ClpP1 and apparently have much lower affinity to ClpP2 for reasons that are not clear.

Because both ClpP1 and ClpP2 active sites contribute to protein degradation (Fig. 6B) and the ClpP2 active site has low affinity for the tripeptide substrates (Fig. 6A), we initially anticipated that the peptide boronate inhibitors would have little

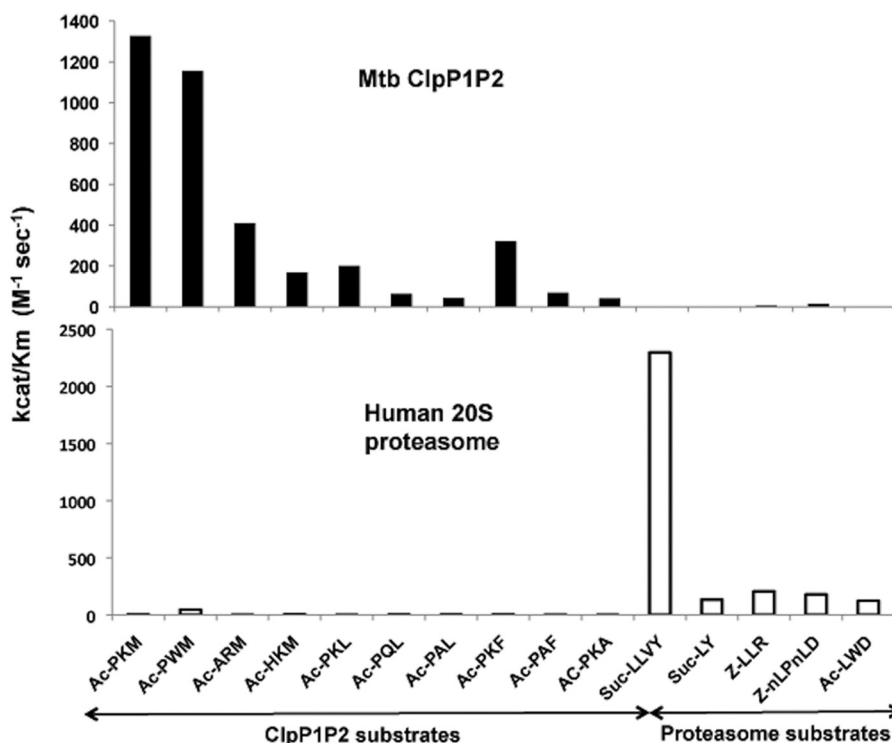


FIGURE 4. **Cleavage preferences of Mtb ClpP1P2 are different from those of human proteasomes.** Specificity constants k_{cat}/K_m were determined using 70 nM of ClpP1P2 or 8.5 nM concentrations of human proteasomes at peptide-amc substrate concentration of 10 μ M.

effect on protein degradation. Surprisingly, FITC-casein degradation by ClpC1P1P2 and GFP_{ssrA} degradation by ClpXP1P2 were both inhibited by peptide boronates (Fig. 7, A and B), although these agents showed 10 times lower potency against proteins than against Ac-PKM-amc, presumably reflecting the lower ability to inhibit ClpP2. Accordingly, protein degradation by the complex with only ClpP1 active was inhibited most strongly (Fig. 7A). With only ClpP2 active, the enzyme was inhibited by the boronates ~2 times worse than the WT ClpP1P2, further indicating that ClpP2 is also important in protein degradation by the complex (Fig. 7A). These observations predict that some peptide boronates, if taken up by Mtb, should block ClpP1P2-dependent protein degradation and be toxic to these cells.

Peptide Boronates Are Toxic to Mtb but Not to Other Bacteria or Mammalian Cells—To test the effects of these peptide boronates on the growth of Mtb cells, we used the resazurin cytotoxicity assay. Many of these potent inhibitors of ClpP1P2 were very effective against Mtb. We did not see a direct correlation between the potency of the inhibitors *in vitro* (K_i value) and cytotoxicity. For instance, the boronate derivative of the best substrate, Ac-Pro-Lys-boroMet (Table 3, #1; $K_i = 0.46 \mu$ M), had an $MIC_{50} = 12 \mu$ M, whereas a relatively less potent inhibitor, Ac-Ala-Lys-boroMet (Table 3, #3; $K_i = 0.8 \mu$ M), showed higher cytotoxicity with $MIC_{50} = 3 \mu$ M (Table 3). Also, the very potent inhibitor, picolinoyl-Trp-Lys-boroMet (Table 3, #5; $K_i = 0.18 \mu$ M) had an $MIC_{50} = 3 \mu$ M against Mtb (Table 3). This lack of a direct correlation is not surprising as cytotoxicity must depend also on drug uptake and metabolic stability in bacteria and on the inhibitor's capacity to block the degradation of certain cell proteins (not peptide substrates) and thus on the degree of inhibition of ClpP1P2, which these peptidase assays do not assess.

As noted above, some dipeptide boronates are also very potent inhibitors of ClpP1P2. Interestingly, the dipeptide boronate, picolinoyl-Lys-boroMet (Table 3, #8), was as effective as its tripeptide derivative against ClpP1P2 peptidase activity ($K_i = 0.34 \mu$ M) but appeared somewhat less toxic to cells ($MIC_{50} = 6 \mu$ M) (Table 3). The dipeptide (Table 3, #9) with *N*-(3-phenyl)propanoyl instead of piconoyl was more potent with $K_i = 0.23 \mu$ M and $MIC_{50} = 3 \mu$ M. Thus, dipeptide derivatives can also be used as anti-Mtb agents. Several other types of N-terminal blocking groups were also tested that appeared to increase inhibitory potency of the dipeptide Lys-boroMet but reduced its toxicity against Mtb. In the case of *N*-(phenylmethanesulfonyl)-Lys-boroMet (Table 3, #14), both inhibitory efficiency and toxicity dramatically decreased. Thus, the blocking group is an important determinant of cytotoxicity, presumably by affecting inhibitor uptake or metabolism.

All these boronate inhibitors tested in the viability assay were only toxic to Mtb. At the same time, even at 200 μ M concentration, they did not affect the growth of *E. coli* or *S. aureus*, whose ClpP are not essential for viability (Table 3). It is noteworthy that only some of these boronates (e.g. Ala(1-naphthyl)-Lys-boro-Leu) that were toxic to Mtb had an effect on growth of *M. smegmatis* (probably due to the differences in permeability between these two related species).

We also tested whether some of these potent boronate inhibitors affected mammalian cells. When myeloma cells (MM1.S) were grown overnight in the presence of 10 μ M *N*-(picolinoyl)-Ala-Lys-boroMet, *N*-(picolinoyl)-Pro-Lys-boroMet, and Ala(1-naphthyl)-Lys-boroLeu, no inhibition of growth was observed, whereas *N*-(picolinoyl)-Trp-Lys-boroMet caused only slight inhibition (25%). By contrast, the proteasome inhibitor bortezomib in parallel experiments caused 50% inhibition of growth

ClpP1P2 Cleavage Specificity and Boronate Inhibitors

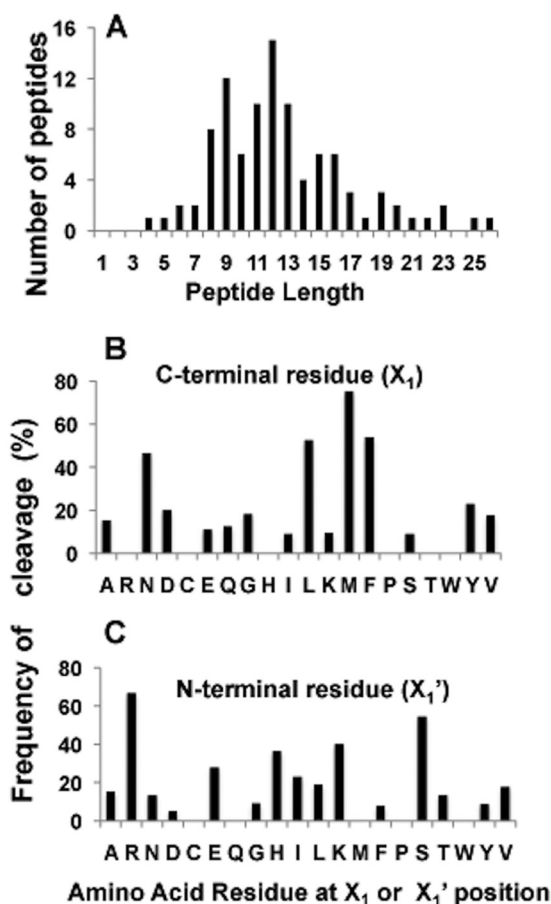


FIGURE 5. Size distribution of peptide products and cleavage preferences for ClpP1P2. A, size distribution of individual peptides generated after cleavage of GFP_{ssrA}. Protein substrate was digested by ClpP1P2 in buffer A containing 1 μ M GFP_{ssrA}, 75–100 nM ClpP1P2, 300–400 nM ClpX, and 2 mM Mg-ATP. The produced peptides were separated by centrifugal filter (10 kDa) and analyzed using Liquid LS-MS/MS. B and C, cleavage preferences for X_1 and X_1' positions during GFP_{ssrA} degradation. The number of cleavages after and before a given amino acid is presented as a percent of the total number of that amino acid in the protein sequence. Peptides were generated and analyzed as in A.

at 5 nM. The weak effect of peptide boronate inhibitors on mammalian cells could be in part explained by the relatively lower potency of these inhibitors against human mitochondrial ClpP (Table 3).

Peptide Boronates Inhibit ClpP1P2 Function in Vivo—To confirm that these peptide boronate inhibitors actually can enter in mycobacteria and inhibit ClpP1P2, we tested whether these inhibitors could slow the degradation of GFP-ssrA by ClpP1P2 in Mtb. A strain constitutively expressing GFP-ssrA was incubated with *N*-(3-phenyl)propanoyl-Lys-boroMet, a potent ClpP1P2 inhibitor with 3 \times MIC (Table 3, #9). The accumulation of the substrate could be observed much earlier (within 10 h) than when the toxic effects of inhibitor become evident (5 days). The fluorescence of GFP was followed from 3 to 42 h during logarithmic growth at 37 $^{\circ}$ C and normalized to cell number. Although hardly any GFP fluorescence was detected in the absence of the inhibitors (the same baseline was observed for the strain without GFP_{ssrA}), GFP fluorescence markedly increased after 10 h of incubation with *N*-(3-phenyl)propanoyl-Lys-boroMet (Table 3, #9) and stayed at the elevated

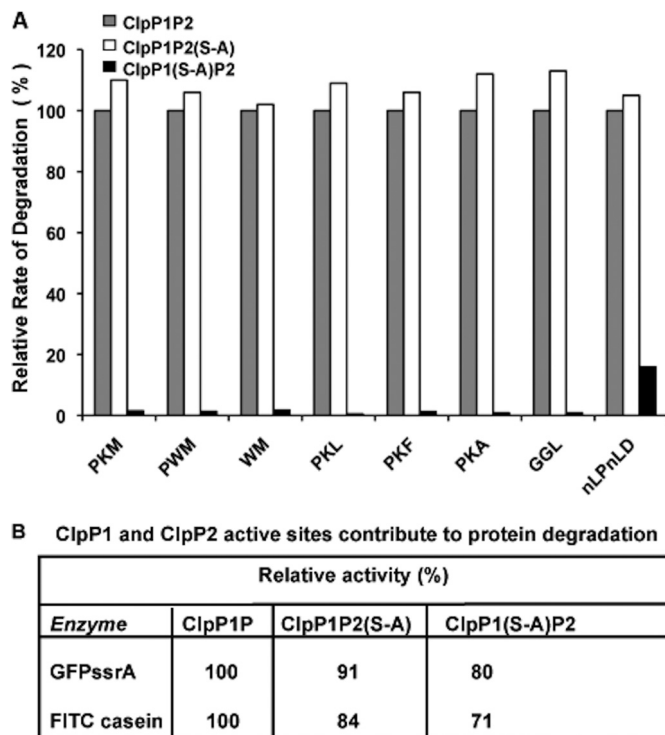


FIGURE 6. ClpP1 accounts for nearly all of the peptidase activity, although both ClpP1 and ClpP2 subunits contribute to protein breakdown. A, degradation of peptide-amc substrates by WT ClpP1P2 and mutant ClpP1P2(S-A) and ClpP1(S-A)P2 mutant complexes. To determine the contribution of each type of active site in peptide degradation, the peptidase activity of ClpP1P2 complexes was measured against the best peptide substrates as in Fig. 1A. The activity of the WT ClpP1P2 against each substrate was taken as 100%. B, degradation of protein substrates by WT ClpP1P2, ClpP1P2(S-A), and ClpP1(S-A)P2 mutant complexes. To determine the ClpP1 and ClpP2 contributions to protein degradation, the cleavage of fluorescent protein substrates, GFP_{ssrA} with ClpX present and FITC-casein with ClpC1 present, were measured, as described under “Experimental Procedures.” The rates of degradation by the WT ClpP1P2 are taken as 100%.

levels for at least 40 h (Fig. 8, A and B). These data indicate that the ClpXP1P2 is indeed a target of this inhibitor *in vivo*.

Similar experiments were also carried out with non-pathogenic fast-growing-related species *M. smegmatis* to determine the concentration dependence of the inhibition. A strain constitutively expressing GFP-ssrA (2) was incubated with different concentrations of either Ala(1-naphtyl)-Lys-boroLeu (Table 3, #4), which is the most potent agent against *M. smegmatis* (with an MIC₅₀ = 1.5 μ M), and an MIC₅₀ against Mtb of 25 μ M or *N*-(2-(3,5-difluorophenyl)acetyl)-Trp-boroMet (Table 3, #12), which had an MIC₅₀ = 12 μ M against both Mtb and *M. smegmatis*. After 6 h in the presence of both inhibitors at concentrations above the MIC₅₀, the fluorescence of GFP markedly increased (Fig. 8C). These findings indicate that in both species of mycobacteria ClpP1P2 represents a target for boronate inhibitors.

DISCUSSION

Distinct Specificity of Peptide Bond Hydrolysis by ClpP1P2—Although the ClpP family of proteases has been known for almost 30 years and its biological roles, ATP dependence, and molecular architecture extensively studied, its cleavage site specificity had not been investigated previously. To develop

TABLE 3

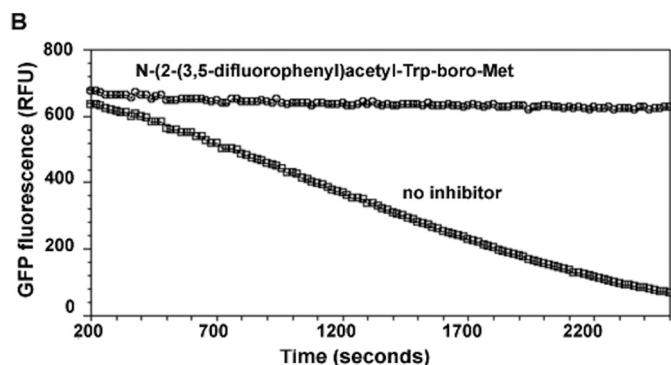
Selected potent boronates inhibit ClpP1P2 stronger than human mitochondrial ClpP and prevent the growth of Mtb

Inhibitor	K_i		MIC ₅₀ ^a
	Human	Mtb	
		μM	μM
#1, Ac-Pro-Lys-boroMet	2.4	0.46	12
#2, Ac-His-Lys-boroMet	6.8	0.78	6
#3, Ac-Ala-Lys-boroMet	2.1	0.80	3
#4, Ala(1-naphtyl)-Lys-boroLeu	1.46	3.2	12
#5, N-(Picolinoyl)-Trp-Lys-boroMet	0.96	0.18	3
#6, N-(Picolinoyl)-Ala-Lys-boroMet	1.7	0.24	3
#7, N-(Picolinoyl)-Pro-Lys-boroMet	4.2	0.82	24
#8, N-(Picolinoyl)-Lys-boroMet	5.8	0.34	6
#9, N-(3-Phenyl)propanoyl-Lys-boroMet	0.9	0.23	3
#10, N-(Benzyl)-Lys-boroMet	2.4	0.18	6
#11, N-(2-(3,5-Difluorophenyl)acetyl)-Lys-boroMet	0.75	0.065	6
#12, N-(2-(3,5-Difluorophenyl)acetyl)-Trp-boroMet	0.34	0.29	12
#13, N-(1H-benzo(b)thiophene-7-carbonyl)-Lys-boroMet	0.4	0.12	12
#14, N-(Phenylmetanesulfonyl)-Lys-boroMet	11.6	13	200

^a MIC of all listed inhibitors against *E. coli* and *S. aureus* were >200 μM .

A Peptide and protein degradation by ClpP1P2, ClpP1P2(S-A) and ClpP1(S-A)P2 are affected differently by boronate inhibitor

Enzyme	IC ₅₀ (μM)		
	ClpP1P2	ClpP1P2(S-A)	ClpP1(S-A)P2
Substrate			
Ac-PKM-amc	0.14	0.09	1.4
GFP-ssrA	5.8	1.8	12.4
FITC-casein	3.2	1.6	8.7

FIGURE 7. A, peptide boronates inhibit the ClpP1 activity more strongly than the ClpP2 activity. Effects of peptide boronate inhibitor (N-(1H-benzo(b)thiophene-7-carbonyl)-Lys-boro-Met) on degradation of peptide (Ac-Pro-Lys-Met-amc) and protein (GFPssrA and FITC-casein) substrates by WT ClpP1P2, ClpP1P2(S-A), and ClpP1(S-A)P2 complexes are presented as IC₅₀ values. Enzymatic activity of ClpP1P2 complexes was measured as described under "Experimental Procedures." B, substrate-based boronate derivatives inhibit protein degradation by ClpP1P2. N-(2-(3,5-difluorophenyl)acetyl)-Trp-boroMet (20 μM) prevents the degradation of GFPssrA by Mtb ClpP1P2 (the degradation assay was carried out as in Fig. 5A). RFU, relative fluorescence units.

specific inhibitors of ClpP1P2 that may serve as lead compounds in drug development, we first defined the protease's sequence preference using a tripeptide diversity library. ClpP1P2 strongly preferred Met over other hydrophobic amino acids (Leu > Phe > Ala) in the X₁ position (Fig. 1). This preference for cleavages after Met was also observed in an analysis of the peptides generated during protein degradation by the ClpXP1P2 complex (Fig. 5). Interestingly, during maturation of ClpP from *E. coli* (29) and Mtb (1), their N-terminal sequence are autolytically cleaved after Met to produce the mature pro-

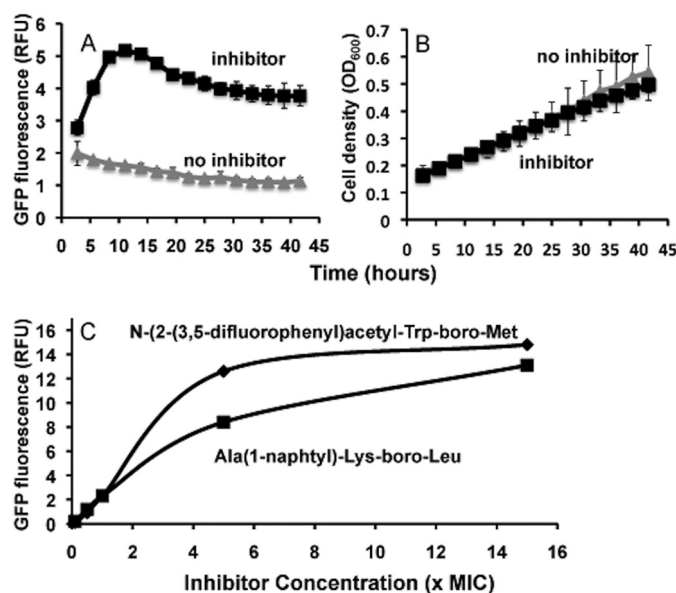


FIGURE 8. ClpP1P2 is the target for boronate inhibitors in mycobacteria. A, GFPssrA substrate accumulates in Mtb in the presence of ClpP1P2 inhibitor. H37Rv strain expressing PMV261::GFP-ssrA was incubated with and without 10 μM concentrations of inhibitor (N-(3-phenyl)propanoyl)-Lys-boroMet. Fluorescence measurements were normalized by cell density. Fluorescence measurements were taken every 2.75 h for 42 h. Error bars represent 95% confidence intervals of triplicate experiment. B, growth curves for Mtb in the presence and absence of the inhibitor. C, ClpP1P2 is the target for boronate inhibitors in *M. smegmatis*. *M. smegmatis* constitutively expressing GFP-ssrA was grown to stationary phase then diluted to an A₆₀₀ = 0.003 and dispensed into a sterile flat-bottom 96-well plate. Peptide boronates (Ala(1-naphtyl)-Lys-boroLeu (Table 3, #4) or N-(2-(3,5-difluorophenyl)acetyl)-Trp-boroMet (Table 3, #12) at different concentrations were added to wells. The fluorescence of GFP was measured after 6 h at 37 °C and normalized to cell density. RFU, relative fluorescence units.

teins. This specificity appears quite unusual; in fact, we know of no endoprotease with a similar preference for cleavages after Met.

The substrates for Mtb ClpP1P2 identified during the screening of a tripeptide library have greatly improved the assay of this family of enzymes. For example, Ac-Pro-Lys-Met-amc and Ac-Pro-Trp-Met-amc have specificity constant k_{cat}/K_m values 1000 times higher than those of Suc-Leu-Tyr-amc (the most widely used substrate for 25 years) and >40 times higher than those of the Z-Gly-Leu-Leu-amc, which we used initially to characterize Mtb ClpP1P2 (1). Most of these new ClpP1P2

ClpP1P2 Cleavage Specificity and Boronate Inhibitors

substrates are also very rapidly hydrolyzed by ClpPs from *E. coli*, *B. subtilis*, and *S. aureus* (Table 2). Although the substrate library screened most possible tripeptides of the form $X_1-X_2-X_3$ -amc, cleavage specificity of some serine proteases is determined in part by residues both preceding and following the scissile bond (30) (e.g. the hepatitis C virus NS3/4 protease). Interestingly, the protein GFP was most frequently cleaved before a positively charged residue or a Ser (Fig. 5C). Thus, the nature of the X' position may influence cleavage rates.

The two Clp ATPases, ClpX and ClpC, clearly promote degradation of different types of cell proteins. In the presence of ClpX, ClpP1P2, like ClpPs from other species, degraded GFPssrA but not GFP lacking the ssrA tag. Although the ClpC1P1P2 complex does not support ATP-dependent degradation of GFPssrA, under similar conditions it did catalyze degradation of casein, which was not a substrate for ClpXP1P2. The proteolytic sites of most if not all ATP-dependent proteases like ClpPs or proteasomes are compartmentalized within a distinct proteolytic chamber. Therefore, protein substrates transported into the chamber cannot exit readily and are hydrolyzed in a processive manner to produce a spectrum of peptide products of different lengths (1, 31, 32). The peptides released during GFPssrA degradation by ClpXP1P2 varied in length from 4 to 26 residues (Fig. 5A), with most ranging between 8 and 16. Thus the mean size of the products is significantly larger than that generated by the archaeal or mammalian proteasome, where most peptide products are <8 residues long (33). Because product size seems to depend on a kinetic competition between further cleavages and diffusion of peptides out of the proteolytic chamber, the larger size of products probably reflects the easier diffusion of larger peptides out of the 2-ring ClpP than of the 4-ring proteasomes. Interestingly, homologous homooligomeric ClpPs exist in either an extended state with open active sites and closed equatorial pores or in a compressed state with closed active sites and open pores for product release (34).

Roles of ClpP1 and P2 in Proteolysis—Although the presence of both ClpP1 and ClpP2 is essential for enzymatic activity, their active sites contribute to the degradation process in very different manners. Although the ClpP1P2(S-A) complex was as active as the wild type enzyme against tripeptide substrates, ClpP1(S-A)P2 showed only 0.2–1% of its activity (Fig. 6A). Thus, ClpP2 lacks activity against these types of tripeptides, although Z-nLPnLD-amc was hydrolyzed by ClpP1(S-A)P2 slightly faster than other substrates. Interestingly, in another bacterium containing ClpP1 and ClpP2 subunits, *Listeria monocytogenes*, the relative roles of ClpP1 and ClpP2 were quite different, with ClpP2 complexes proteolytically active by themselves, whereas ClpP1 subunits were only activated in the mixed ClpP1P2 complex (35). Despite their very different activities in tripeptide degradation, surprisingly, we found that both ClpP1 and ClpP2 subunits made major contributions to protein degradation. Thus, ClpP2 clearly plays a catalytic and not just a structural role, although its active sites for some reason appear latent except when Clp ATPases and a protein substrate were present. Perhaps the low peptidase activity of ClpP2 is associated with the requirement for additional contacts with the substrate on both ends of the scissile bond. Alternatively, the

ClpC1 or ClpX ATPases and/or the protein substrate may induce the proteolytically active conformation in ClpP2. In human mitochondrial ClpP, the association with ClpX alters the conformation of ClpP to promote the formation of the active tetradecamer (5), and substrate binding is known to alter the conformations of the Clp ATPases (23). In Mtb, unlike most bacteria, ClpX and ClpC1 are both essential for viability and infectivity, which makes them attractive targets as we noted previously (36). In fact, two novel natural product antibiotics have been identified recently that act on ClpC1 and cause selective killing of mycobacteria (19, 37). For example, the cyclic peptide lassomycin uncouples ATPase activity from ATP-dependent proteolysis by ClpC1P1P2 (19).

While we were preparing this manuscript for publication, Schmitz and Sauer (38) also reported studies of the degradation of GFPssrA by Mtb ClpXClpP1P2 complex. They confirmed our findings (1) that the active form of the protease is the mixed ClpP1P2 complex and also observed that inactivation of ClpP1 or ClpP2 still allows rapid GFPssrA breakdown. However, they reported that GFPssrA could also be degraded slowly by ClpC1P1P2, perhaps because they used 10-fold more protease in their degradation assay than were used here. Another clear inconsistency is that those authors observed peptide degradation only in the presence of both the ATPase and the activator (which they designated by the pharmacological or physiological term, “agonist”), whereas in our studies peptides were efficiently degraded in the presence of the dipeptide activator alone.

Peptide Boronate Inhibitors and Therapeutic Potential—This characterization of cleavage specificity of Mtb ClpP1P2 indicated a novel active site preference and strongly suggested that synthesis of substrate-based inhibitors would be a valuable approach for development of selective anti-Mtb agents. We have focused on peptide boronates because many potent and highly specific peptide boronic acid inhibitors have been synthesized for serine and threonine proteases (26). For example, bortezomib is a potent, highly selective inhibitor of human proteasomes that is now the preferred treatment worldwide for multiple myeloma, and two other boro-peptides are presently in clinical trials. Peptide boronates were shown to inhibit the Mtb proteasome and Lon from *Salmonella enterica* and mitochondria (28, 39). Unlike ClpP or the proteasome, Lon is inhibited by these agents and other covalent inhibitors only in the ATP-bound state, which is necessary for its active site formation.

Although many peptide boronates have been synthesized previously, these agents with a methionine in the X_1 position are a novel type of protease inhibitors and appear relatively selective for ClpP family members (40). The ability of these substrate-based inhibitors to block protein hydrolysis as well as to stop Mtb growth strongly suggests that cleavage specificity of ClpP1P2 is an accurate reflection of enzyme function *in vivo*. However, a clear limitation of this approach was apparently the surprising lack of activity of ClpP2 on the tripeptide substrates, and because our tripeptide boronates were synthesized based upon substrate preference, it is not surprising that they are less potent in blocking ClpP2-catalyzed protein breakdown than peptide hydrolysis. Presumably, if these peptide boronates were

more potent against ClpP2, they would be even more effective against protein substrates and in killing Mtb.

Our most potent substrate-based boronate derivatives and their analogs inhibited the peptidase activity of ClpP1P2 with K_i of $<1 \mu\text{M}$, with some as low as 65 nM. Most importantly, they blocked Mtb growth with MIC_{50} value from 3 to 12 μM (Table 3), apparently through their abilities to inhibit ClpP1P2. Accordingly, these inhibitors did not affect the growth of bacteria where ClpP is not essential, *E. coli* and *S. aureus*. Moreover, in Mtb and *M. smegmatis*, these agents blocked ClpP1P2 activity as they caused GFP_{ssrA} to accumulate (Fig. 8) in a similar way as was observed upon ClpP1P2 depletion genetically (2). Not surprisingly, the efficacy of these peptide boronates in preventing mycobacterial growth correlated only roughly with their potency against peptide substrates, presumably because cytotoxicity requires blocking the degradation of endogenous proteins, whose accumulation is toxic.

Although we utilized here a blocked tripeptide-amc library, the dipeptide derivatives (Ac-Trp-Met-amc and Ac-Lys-Met-amc) were also very good substrates for ClpP1P2. Consequently several blocked dipeptide boronates were synthesized. They were found to be as effective as their tripeptide analogs against ClpP1P2 and showed similar or slightly lower toxicity against Mtb (Table 3). The N-terminal blocking group also had significant effects on inhibitor efficacy. Curiously, use of a picolinoyl group in place of the standard acetyl group resulted in a higher affinity for ClpP1P2 in the case of Ala-Lys-boro-Met with similar cytotoxicity, but this substitution reduced both inhibitory activity and cytotoxicity in the case of Pro-Lys-boro-Met for reasons that are unclear. Many further modifications are clearly possible to further enhance potency *in vitro* and efficacy *in vivo*, which presumably is limited by rates of drug uptake and metabolism as well as their limited ability to inhibit the ClpP2 and ClpP1 active sites.

Unlike other eubacteria, Mtb and other actinomycetes in addition to ClpP1P2 express a 20 S proteasome (11) that is much simpler in organization than proteasomes of eukaryotes and has quite different cleavage preferences (41–43). Unlike ClpP1P2, the Mtb proteasome is not essential but appears to be important for survival in the macrophage (24) and protection against free radicals (44). Peptide boronates that inhibit with high affinity ClpP1P2 and proteasomes could, therefore, be even more potent anti-Mtb agents, although this possibility does not seem feasible, as our data show that these two enzymes have quite different cleavage preferences (Fig. 4). A successful agent against Mtb would have to have little or no effect on important mammalian enzymes. It is noteworthy that the cleavage preferences of ClpP1P2 are quite different from those of the human proteasome, the main protein degradation system in human cytosol. The best ClpP1P2 substrates (Ac-Pro-Lys-Met-amc and Ac-Pro-Trp-Met-amc) are quite poor proteasomal substrates, and the best proteasomal substrates are cleaved very slowly by ClpP1P2 (Fig. 4). Therefore, these inhibitors are unlikely to affect protein degradation in human cells. Indeed, peptide boronates did not affect the growth of mammalian cells tested here.

These peptide boronates are also potent inhibitors of other ClpP family members, and inhibitors of *S. aureus* ClpP are

potentially of therapeutic interest. Even though ClpP is not essential in *S. aureus*, Lewis and co-workers recently showed that ClpP-deficient *S. aureus* were more sensitive to standard antibiotics and less able to enter the persister state than wild type strains (45). Because *S. aureus* infections are common in hospitals and difficult to treat with conventional antibiotics, inhibitors of ClpP may prove valuable in fighting *S. aureus* infections. These findings on enzyme specificity provide a rational basis for synthesis of more potent substrate-based inhibitors of ClpP1P2 from Mtb and perhaps other bacteria. Clearly, the success achieved thus far in generating anti-Mtb agents validates this target choice, biochemical rationale, and synthetic strategy for development of new types of agents cytotoxic for Mtb.

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