We have studied the mechanism of inhibition of the recombinant Rhodococcus proteasome by four different chemical classes of active site-directed small molecule inhibitors. Clasto-lactacystin β-lactone is a time-dependent inhibitor of the Rhodococcus proteasome's ability to hydrolyze Suc-Leu-Leu-Val-Tyr-AMC, a substrate for this proteasome's single type of active site, and proceeds with a $k_{\text{inact}}/[I]$ of 1.700 s$^{-1}$. Using peptide mapping of tryptic digest, LC/MS, and amino acid sequence analysis, we have established that the Oγ of the hydroxyl group on the N-terminal threonine of the β-subunit is the sole modification made by the β-lactone.

Active site titrations of the Rhodococcus proteasome with reversible peptide aldehydes show the expected stoichiometry of one inhibitor molecule per β-subunit. Prior modification with β-lactone completely abrogates the binding of peptidyl boronic acid inhibitors, suggesting that these inhibitors also inactivate the enzyme by reacting with the Oγ moiety on Thr1. High performance liquid chromatography analysis of peptidyl vinyl sulfone-modified intact Rhodococcus proteasome β-subunit and its tryptic peptides suggests that the peptidyl vinyl sulfone modifies a residue in the N-terminal 20 amino acids. This modification is also blocked by prior treatment with β-lactone.

The proteasome is a multi-proteinase complex which occurs in all eukaryotic cells. In 1989, the proteasome was also discovered to exist in prokaryotes, namely in the archaeabacterium, Thermoplasma acidophilum (1). The quaternary structure of the proteasome is conserved between both eukaryotes and prokaryotes, having a barrel-shaped structure composed of four 7-subunit members stacked on top of one another. However, the complexity of the subunit composition within this structure is different between eukaryotic proteasomes and their prokaryotic counterparts. The archaeabacterial proteasome has a relatively simple structure, consisting of only two types of subunits, α and β. The α-subunits form the outer rings of the cylindrical stack, whereas the β-subunits form the two inner rings (2). All enzymatic activity has been shown to be associated with the β-subunits, the α-subunits having regulatory and targeting functions.

Eukaryotic proteasomes, on the other hand, are much more complex. They consist of 14 different, although related types of subunits, which can be divided into seven α types and seven β types (3). Eukaryotic proteasomes have three well characterized peptidase activities, namely, “chymotrypsin-like” (P1 residue is a Leu or Tyr in substrate), “trypsin-like” (Arg in P1 position), and “peptidylglutamyl-peptide hydrolyzing” (Glu in P1 position) (4). Other activities such as “branch-chain amino acid preferring” and “small neutral amino acid preferring” activities have also been identified (5). Inhibitor studies provide convincing evidence that some of these activities are catalyzed at different active sites but the exact correspondence of peptidase activities to active sites in eukaryotic proteasomes is unknown. Thermoplasma proteasomes which contain only one type of active site exhibit a much more limited ability to hydrolyze tri- and tetra-peptides (1). Nevertheless, similar to eukaryotic proteasomes, the archaeabacterial proteasome exhibits indiscriminate cleavage preference when presented with unfolded polypeptide substrates (6). Seemüller and co-workers (7) have identified the N-terminal threonine residue of the β-subunit of the T. acidophilum proteasome as the catalytic nucleophile, using site-directed mutagenesis techniques. The crystal structure of this enzyme in complex with a tripeptide aldehyde inhibitor lends support to this. The aldehyde function of the inhibitor was situated at Thr1 of the β-subunit (8).

Recently, the first characterization of a eubacterial proteasome was undertaken by Tamura and co-workers (9). The proteasome from the nocardioform actinomycete Rhodococcus erythropolis strain N186/21 was found to have two types of α (α1, 31 kDa and α2, 29 kDa) and two types of β-subunits (β1, 25 kDa and β2, 24 kDa), arranged in the same quaternary structure as proteasomes from eukaryotic sources. Similar to the Thermoplasma proteasome, the peptidase activity of the Rhodococcus proteasome is limited to tri- and tetrapeptides which are substrates for chymotrypsin-like activity.

Proteasomes isolated from Rhodococcus are composed of these four subunit types, according to a α2β1β2α2 stoichiometry (10). It is unclear whether the subunits take random positions in their respective α or β rings or whether the subunits segregate into four homoligomeric rings. Nevertheless, coexpression of the genes for one of the α-type and one of the β-type subunits in Escherichia coli leads to the production of enzymatically functional proteasomes. Here we have employed these recombinant proteasomes in kinetics and biophysical studies to discern the mechanisms of four different chemical classes of inhibitors. These inhibitors include peptidyl aldehydes, peptidyl boronic acids, peptidyl vinyl sulfones, and the natural product, clasto-lactacystin β-lactone. The data suggest that a common mechanistic element for all of the inhibitors is their reaction with the proposed catalytic nucleophile, Oγ of Thr1 on the β-subunit.
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**EXPERIMENTAL PROCEDURES**

**Synthesis of Clasto-lactacystin β-Lactone—Clasto-lactacystin β-lactone** was prepared by the method of Uno et al. (11). The structure of the synthesized material was confirmed by proton magnetic resonance spectroscopy and fast atom bombardment-mass spectrometry. The purity of the material was assessed >95% by HPLC.

**Synthesis of Dansyl-Phe-Leu-B(OH)₂ and Morph-Nal-Leu-Leu-B(OH)₂—Rhodococcus** was prepared by coupling (1R)-S-pineniold 3-ammonium trifluoroacetate 3-methylbutan-1-borionate (12) to N-boc-β-(1-naphthyl)-l-alanine using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl ammonium tetrafluoroborate (TBTO) in the presence of disopropylethylamine. Removal of the t-butocy carbonyl protecting group with HCl/LiCl, coupling of the primary amine with 4-morpholinecarbonyl chloride, and final removal of the pinanediol protecting group using 2-methylpropanehydroborate afforded MNBL. N-Dansyl-phenylalanine-1-leucine boronic acid (DFBL) was performed in a similar manner by coupling (1R)-(S)-pinanediol 3-ammonium trifluoroacetate 3-methylbutan-1-borionate to dansyl-phenylalanine using TBTO in the presence of disopropylethylamine in anhydrous N,N-dimethylformamide followed by the deprotection of pinanediol using 2-methylpropanehydroborate. The tripeptide aldehyde, Z-Leu-Leu-OH followed by reduction with lithium aluminum hydride.

**Synthesis of Z-Leu-Leu-Phe-H—The tripeptide aldehyde, Z-Leu-Leu-Phe-H** was prepared by conventional methods, coupling the Weinreb amide of L-phenylalanine to the commercially available protected dipeptide Z-Leu-Leu-OH followed by reduction with lithium aluminum hydride.

**Synthesis of Z-Leu-Leu-(CH)₂SO₂R—Condensation of the anion of diethyl methylsulfonylmethylphosphatate generated with sodium hydride in anhydrous tetrahydrofuran and N-benzoyloxycarbonyl-l-leucine-l-leucine-1-leucinal yielded the trans-a,β-ethylenic methylsulfone of N-(benzoyloxycarbonyl)-l-leucine-l-leucine-l-leucinal (Z-Leu-Leu-(CH)₂SO₂R).**

**Assay of Rhodococcus Proteasome 20 S Activity and Inhibition by 1-β-Lactone and Pyroglutamyl Boronic Acid—1 μg of Rhodococcus proteasome was incubated with 10 μM Suc-Leu-Leu-Val-Tyr-AMC in 2 mL of 20 mM HEPES, 0.5 mM EDTA, pH 8.0, at 37 °C, in a 3-mL fluorescence cuvette, and the cuvette placed in the jacketed cell holder of a Hitachi F-2000 Fluorescence Spectrophotometer, fitted with a magnetic stirring device to allow continuous stirring. The progress curves for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC were measured by monitoring the production of AMC (λex = 440 nm, λem = 530 nm), and the enzyme activity was determined by a linear fit of the data, fluorescence (F) versus time (t). When the hydrolysis of substrate reached steady state, inhibitor was added at an appropriate concentration, and the fluorescence progress curve measured. The fluorescence versus time data were collected and processed using LAB CALC (Galactic) software. AMC values were determined by a non-linear least-squares fit of the data to the equation for time-dependent or slow binding inhibition (13).

**Fluorescence = \[v_0 + [(v_m - v_0)k_{inhib}] [1 - \exp(-k_{inhib}t)] \] (Eq. 1)**

where \(v_0\) is the initial velocity which slowly decays to the final steady state velocity, \(v_m\), with a first-order rate constant, \(k_{inhib}\).

**K_v** values for reversible inhibitors were calculated using the expression for competitive inhibition,

**K_app = [I]/(v_0/v_0 - 1) \] (Eq. 2)**

\(K_v\) is the dissociation constant for the inhibitor with the enzyme, \(v_0\) is the inhibitor concentration, \(v_m\) is the rate of substrate hydrolysis by the enzyme, \(v_{inhib}\) is the steady-state inhibited hydrolysis rate. In all cases, [S] ≪ \(K_v\) so that \(K_{app} = K_v\).

**Active Site Titrations of Rhodococcus Proteasome by Activity Measurements**—1.5 μg of Rhodococcus proteasome (β₁β₄₁₄) in 150 μL of 20 mM HEPES, 0.5 mM EDTA, pH 8.0, was incubated for 1 h at 37 °C, with a range of concentrations of the inhibitor, Z-Leu-Leu-Phe-H. Suc-Leu-Leu-Val-Tyr-AMC at 10 μM was added (in 3 μL of dimethyl sulfoxide), and the rate of substrate hydrolysis measured, as outlined previously. Microfluorocuvettes were used to cater for the small volumes used in this assay. A plot of \(v/v_0\) versus [I] was fit to the equation for tight binding enzyme inhibition (14),

**v/v₀ = (1/2[E]₀)[(1) - [K_v] + ([I] + [K_v] - [E]₀)² + 4K_v[E]₀²] \] (1/2) \] (Eq. 3)**

where \(v_v\) is substrate hydrolysis rate by enzyme, \(v_m\) is substrate hydrolysis rate in the presence of inhibitor, [I] = inhibitor concentration, \([E]₀\) = active site concentration, \(K_v\) = dissociation constant of the enzyme-inhibitor complex.

**Active Site Titrations of Rhodococcus Proteasome by Direct Binding Measurements—Rhodococcus (β₁β₄₁₄) proteasome was concentrated using Centricon filters (M_cutoff = 10,000 Da) to a final protein concentration of 6.37 mg/mL (as determined by the Bio-Rad protein assay). The stock enzyme was diluted 1:100 in 400 μL of 20 mM HEPES, 0.5 mM EDTA, pH 7.6 (25 μg per proteasome particle). Dansyl-Phe-Leu-B(OH)₂ was titrated into the enzyme solution (0.5 μL aliquots of 100 or 400 μM solutions) and the fluorescence was monitored on a Hitachi model F4500 at the peak fluorescence for the bound probe at 536 nm (λ_exc = 292 nm) after each addition. The fluorescence at 536 nm was plotted against [DFBL]. The active site titrations with MNBL were performed in a similar manner, except that the fluorescence was monitored at 357 nm (λ_exc = 295 nm).

**Labeling of the Proteasome with Clasto-lactacystin β-Lactone—Approximately 375 μg/mL (β₁β₄₁₄) and (β₂β₄₁₄) in 20 mM HEPES, 0.5 mM EDTA, pH 8.0, were incubated with 40 μM Clasto-lactacystin β-lactone for 30 min at 37 °C. Control enzyme samples were incubated with an equivalent volume of acetone. Residual peptidase activity was determined to ensure complete modification of the proteasome active sites.**

**Separation of the Proteasome Subunits**—The active β₁β₄₁₄ and β₂β₄₁₄ proteasomes were separated by reverse-phase HPLC using a 6 × 150-mm Shodex Shpak D-013 column run on a Waters (Milipore Inc., Millford, MA) HPLC system. The column was equilibrated at a flow rate of 1.5 mL/min with 80% 0.06% (v/v) trifluoroacetic acid in water, 20% 0.05% trifluoroacetic acid in water, 20% 0.05% trifluoroacetic acid in acetone at 40 °C. Following injection of 50 μg of total protein in a 100-μL volume, elution of the subunits was achieved, using a 1% min linear gradient (20–50%) of acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The elution of the subunits was monitored with an in-line UV absorbance detector at 214 nm, and the individual subunits collected manually in final volumes of approximately 1.5 μL.

**Tryptic Digestion and Peptide Mapping of Rhodococcus Proteasome Subunits**—HPLC fractions collected as described (typically 20–25 μg of protein in 1.5 mL of acetonitrile/water) were evaporated to dryness in a vacuum centrifuge. 2 μL of 10% (w/v) Lubrol PX detergent (Calbiochem) was added prior to the evaporation to prevent adsorption of the polypeptide to the walls of the vessel. The dried samples were reconstituted with 100 μL of 1% (w/v) ammonium bicarbonate. Tryptsin (0.2 μg) was added to the polypeptide solution and digestion was allowed to proceed overnight at 37 °C. 10 μL of 1% trifluoroacetic acid was added to stop the reaction following this 15–20-h incubation. Reverse-phase HPLC analysis of the resulting peptide digests was performed using a 2 × 150-mm Delta-Pak C-18 300 Å, 5 μm column, at 40 °C in 0.05% trifluoroacetic acid in water at a flow rate of 1.0 mL/min. Elution was achieved with a 1% min linear gradient of acetone (0–60%) containing 0.05% (v/v) trifluoroacetic acid.

**N-Terminal Amino Acid Sequencing**—Peptides were subjected to automated Edman degradation on an Applied BioSystems Inc. Model 470A sequencer with a Model 120A analyzer, using standard manufacturer's programming and chemicals.

**Liquid Chromatography-Mass Spectrometry (LC/MS)**—The molecular weights of the individual Rhodococcus subunits were determined by using a combination of liquid chromatography and electrospray ionization mass spectrometry. The subunits were separated by HPLC and then introduced by loop injection into a VG 30–250 quadrupole mass spectrometer (Micromass Inc., Altrincham, United Kingdom) and ionized by electrospray. The mass analyzer was scanned over the m/z range of 600–1,600 at a rate of 10 scans, and the data transformed automatically using the manufacturer's standard LAB-BASE™ protocol. The molecular masses of the subunits were determined from the mass spectra.
RESULTS AND DISCUSSION

Inactivation of Recombinant Rhodococcus Proteasome with Clasto-lactacystin β-Lactone—Incubation of the recombinant Rhodococcus proteasomes (β₃α₁)₁₄ and (β₃a₂)₁₄, with clasto-lactacystin β-lactone, resulted in a time dependent loss in the enzyme’s ability to hydrolyze Suc-Leu-Leu-Val-Tyr-AMC. As is readily apparent from the progress curve in Fig. 1 panel A, the inactivation of Rhodococcus protease by β-lactone shows complex kinetics. The inactivation is partial and at longer times the progress curve bends upward indicating reactivation of inactive enzyme. As shown previously, the β-lactone hydrolyzes to the inactive dihydroxy acid with a t₁/₂ of approximately 13 min under the assay conditions (15). This would explain the partial nature of the inhibition in this experiment and in fact complete inhibition can be obtained with higher lactone concentrations (data not shown). The second phenomenon, reactivation of enzyme, is presumably due to hydrolysis of the covalent acyl-enzyme formed between the lactone and the active site nucleophile (see below). This reactivation phenomenon is also observed with the lactone modified rabbit skeletal muscle proteasome and it is a relatively slow process with a t₁/₂ of approximately 20 h at pH 8 and 37 °C.

Fig. 1, panel B, shows the β-lactone concentration dependence of the apparent first-order rate constant for inactivation. Up to a β-lactone concentration of 10 μM, the rate of inactivation increased in proportion to lactone concentration giving no indication of saturable binding. kₜₐₙ₉[II] was estimated to be 1.700 M⁻¹ s⁻¹ for both types of recombinant proteasomes. This rate of inactivation is approximately 10 times slower than that obtained for the chymotrypsin-like activity of rabbit psoas muscle 20 S proteasome-PA28 complex, under similar assay conditions.

To probe the mechanism of inhibition by β-lactone, modified and unmodified Rhodococcus proteasome were subjected to LC/MS. Table I shows the masses of the individual subunits, α₁, β₁, α₉, and β₈, as determined using LC/MS and compares them with calculated values. The measured masses are identical to the calculated values within experimental error. Table I also shows the mass of control and β-lactone modified α₁ and β-subunits from both (β₃α₁)₁₄ and (β₃a₂)₁₄. The difference in the masses of the modified and unmodified β-subunits were 211 ± 7 atomic mass units for β₁ and 215 ± 8 atomic mass units for the β₂-subunit. These values are identical, within experimental error, to the mass increment of 213 that would result from a single site modification with β-lactone, i.e. one molecule of β-lactone reacts per β-subunit. No change in the mass of either α-subunit was observed, suggesting no modification of the α-subunits occurs.

Rhodococcus (β₃α₁)₁₄ proteasome was reacted with varying amounts of β-lactone and the residual peptidase activity was determined. Aliquots of the same samples were subjected to LC/MS to estimate the percentage modification of the β-subunits. Fig. 2 shows the percentage residual activity and the percentage of active sites modified as functions of the [β-lactone]. Residual activity and fractional modification mirror one another, suggesting a simple correlation between the number of β-subunits modified and loss of activity.

Identification of the β-Lactone-modified Residue on the β-Subunit of the Recombinant Rhodococcus Proteasome—Both β-lactone-modified and unmodified (β₃α₁)₁₄ proteasome were subjected to reverse phase HPLC to separate the subunits and these individual subunits were subjected to digestion with trypsin. The resulting digestion products were separated by reverse-phase HPLC on a C-18 column (Fig. 3). The peptide

| TABLE I | Mass spectral analysis of recombinant Rhodococcus proteasomes before and after modification with clasto-lactacystin β-lactone |
|---------|----------------------------------------------------------------------------------------------------------------------|
| Subunit | Calculated (atomic mass units) | Measured (atomic mass units) | Difference (Mod - con) |
| α₁      | 28,181 ± 5 | 28,179 ± 5 | 2 ± 6 |
| β₁      | 24,952 ± 5 | 24,951 ± 5 | 211 ± 7 |
| α₉      | 27,728 ± 3 | 27,727 ± 3 | 0 ± 4 |
| β₂      | 24,940 ± 6 | 24,941 ± 6 | 215 ± 8 |

*The calculation was based on the amino acid sequences from Tamura et al. (9) and the values for isotope abundance-weighted average masses of amino acid residues from Feng et al. (16).
Inhibition of Rhodococcus Proteasome

The α and β subunits of control and β-lactone modified recombinant Rhodococcus proteasomes were separated by reverse-phase HPLC and individually subjected to four cycles of automated Edman degradation. Quantitation of the PTH-derivatives appearing at each cycle was based on external standards (see "Experimental Procedures").

Maps of the α-subunits from the modified and unmodified samples were identical (data not shown). In the tryptic peptide map, the fraction of modified β-subunits were determined for each sample as described under "Experimental Procedures." A single difference between the peptide maps was observed. The peak at 34.5 min shifts to a longer retention time of 37.3 min post-treatment with β-lactone. These results suggested that the β-lactone modification of the β-subunit occurred in the N-terminal region of the polypeptide. Modified and unmodified Rhodococcus proteasomes (β2α1)14 and (β2α2)14 were then separated into their individual subunits by reverse-phase HPLC. The individual subunits were collected and subjected to N-terminal sequencing. The results of four cycles of automated Edman degradation are shown in Table II. Both α-subunits, modified and unmodified, yielded similar quantities of the phenylthiohydantoin (PTH)-derivative of Thr,3 after the first cycle of Edman degradation, followed by the PTH-derivatives of Met, Pro, and Tyr. In the case of the β-subunits, however, the first cycle of Edman degradation of the modified β-subunit did not produce an identifiable PTH-derivative, whereas the unmodified β-subunit yielded the expected derivative of Thr. Further cycles yielded similar quantities of Thr (2nd cycle), Ile (3rd cycle), and Val (4th cycle) for both the modified and unmodified β-subunits. These results suggested that Thr1 had been modified by clasto-lactacystin β-lactone, thereby preventing it from forming an identifiable derivative. That the recoveries of the PTH-derivative in subsequent cycles were similar to controls further suggests that the Oy residue of Thr2 was the site of modification. This is because the free α-amino group is required for progression of the Edman degradation.

The N-terminal threonine of the β-subunit has also been identified as the catalytic nucleophile in the Thermoplasma proteasome and in the β-subunit, Subunit X, of eukaryotic proteasomes which are treated with β-lactone (17). Seemüller and co-workers (7) used site-directed mutagenesis techniques to show that deletion or mutation of the N-terminal threonine of the β-subunit resulted in proteasomes that folded correctly but were completely inactive. One exception was the mutation of Thr1 → Ser, suggesting that the Thr3 functions in a manner analogous to active site serines in serine proteases (18).

Lactacystin and the related β-lactone inactivate multiple eukaryotic proteasome hydrolyzing activities, chymotrypsin-like, trypsin-like, and pepidylglutamyl-peptide hydrolyzing, but with different rates of inactivation. This observation supports the theory that the three activities arise from different active sites. Fenteany and co-workers (17) employed [3H]lactacystin to modify eukaryotic proteasomes and isolate the modified subunits by reverse-phase HPLC. One of the β-subunits was rapidly modified, and upon digestion with trypsin, two peptides within this subunit were found to be labeled with radioactivity. One of these peptides was identified as the N-terminal tryptic peptide, the Thr3 being the modified residue. It

The sequence of the Rhodococcus α-subunit published in Tamura et al. (9) starts with Met which is removed during expression in E. coli. Our sequence and LC/MS results are consistent with this.

### Table II

| Subunit | 1st cycle | 2nd cycle | 3rd cycle | 4th cycle |
|---------|-----------|-----------|-----------|-----------|
| β1 (control) | 15 | 11 | 16 | 12 |
| β1 (modified) | 2 | 12 | 14 | 14 |
| α2 (control) | 13 | 15 | 8 | — |
| α2 (modified) | 10 | 9 | 8 | 11 |
| β2 (control) | 9 | 8 | 8 | 7 |
| β2 (modified) | 2 | 8 | 10 | 9 |

3 The sequence of the Rhodococcus α-subunit published in Tamura et al. (9) starts with Met which is removed during expression in E. coli. Our sequence and LC/MS results are consistent with this.
is assumed that this modification is the one that inactivates the enzyme, by analogy to Thermoplasma studies which suggest that this is the catalytic nucleophile (7). Additionally, the structures of thermoplasma and yeast proteasome have been solved with bound β-lactone (19, 20). The structures show close proximity of the β-lactone to the N-terminal threonine of all the T. acidophilium β-subunits and to the two copies of the β5 subunits of the yeast enzyme. It was proposed that the carbonyl C4 of β-lactone and the Oγ of Thr1 form a covalent ester linkage. Our results support these conclusions since modification of this residue alone in Rhodococcus proteasome is necessary and sufficient to completely inactivate the enzyme.

Active Site Titrations of Recombinant Rhodococcus Proteasomes—An active site titration of the Rhodococcus recombinant proteasome was performed using the reversible peptide aldehyde inhibitor, Z-Leu-Leu-Phe-H. Fig. 4 shows the fractional residual activity of the proteasome after incubation with varying amounts of Z-Leu-Leu-Phe-H. The data were fit to the equation for tight binding enzyme inhibition (see “Experimental Procedures,” Equation 3). The dissociation constant $K_i$ for the inhibitor was determined independently as 4 nM. A best fit of the data yielded 26.7 pmol of active sites/1.5 μg of proteasome particle for ($\beta_1\alpha_1$)$_{14}$, and 26.1 pmol of active sites per 1.5 μg of proteasome particle for ($\beta_2\alpha_2$)$_{14}$.

was proposed that a reversible covalent hemiacetal forms between the Oγ of Thr1 and the aldehyde function.

The quantum yield of the dansyl fluorophore is sensitive to its environment. In aqueous solution, its fluorescence intensity is strongly quenched, due to interactions with the environment causing decay of the excited state by non-radiative processes. However, in a non-polar or rigid environment, the fluorescence intensity of these molecules is enhanced (21). We employed this property of the dansyl moiety in the proteasome inhibitor, dansyl-Phe-Leu-B(OH)$_2$ (Fig. 5) to perform a direct titration of the active sites in the recombinant Rhodococcus proteasome ($\beta_2\alpha_2$)$_{14}$. DFLB is a potent time-dependent inhibitor of the Rhodococcus proteasome ($K_{i,\text{app}}$) [I] $= 56,000$ nM$^{-1} s^{-1}$) having a $K_i$ of <1 nM. Free in aqueous solution, the fluorescence of the inhibitor’s dansyl moiety is highly quenched. However, upon binding to the active site of the enzyme, the fluorescence of the inhibitor is enhanced approximately 15-fold. Fig. 5 shows the fluorescence intensity measured at 536 nm (ex$_s$ = 329 nm) versus the amount of DFLB added to 25.5 μg of Rhodococcus proteasome particles. A breakpoint is observed after the addition of 0.47 nmol of DFLB, so that addition of more DFLB results in a shallower slope for the increase in fluorescence. The fluorescence intensity up to the addition of 0.47 nmol of DFLB is due to inhibitor binding to the active sites of the proteasome. Beyond this point, the fluorescence increased in direct proportion to the free inhibitor. The intersection of the two slopes occurs at a DFLB concentration of 0.45 nmol. The data therefore suggest that there are 0.45 nmol of active sites per 25.5 μg of proteasome particle or 0.94 active sites per β-subunit. Within the limits of experimental error, the estimation of 0.94 active sites is close to the expected value of one active site per β-subunit. The experiment was repeated using thyroglobulin, a protein of similar molecular mass as the proteasome, but nonspecific for the inhibitor. No inhibitor binding was observed and the fluorescence intensity was proportional to the fluorescence of the free inhibitor. When the Rhodococcus proteasome was modified with β-lactone to an extent to inhibit all of the active sites, and then titrated with DFLB, no inhibitor

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**FIG. 4.** Active site titration of Rhodococcus proteasome with Z-Leu-Leu-Phe-H. 1.5 μg of Rhodococcus proteasome was incubated with varying amounts of Z-Leu-Leu-Phe-H. The plot of $v/v_0$ versus moles of inhibitor for both recombinant proteasomes was fit to the equation for tight binding enzyme inhibition (see “Experimental Procedures,” Equation 3). The dissociation constant $K_i$ for the inhibitor was determined independently as 4 nM. A best fit of the data yielded 26.7 pmol of active sites/1.5 μg of proteasome particle for ($\beta_1\alpha_1$)$_{14}$, and 26.1 pmol of active sites per 1.5 μg of proteasome particle for ($\beta_2\alpha_2$)$_{14}$.

**FIG. 5.** Active site titration of Rhodococcus proteasome with DFLB. Titration of increasing amounts of DFLB into 25.5 μg of unmodified and β-lactone-modified Rhodococcus proteasome was performed as described under “Experimental Procedures.” Fluorescence of the dansyl moiety of the inhibitor, DFLB, was monitored at 536 nm ($\lambda_{\text{ex}}$ = 329 nm). After the addition of 0.45 nmol of DFLB to 25.5 μg of proteasome, the fluorescence of the dansyl was no longer enhanced. An active site concentration of 0.45 nmol/per 25.5 μg of proteasome particles was estimated. When the proteasome had been previously modified with β-lactone or when a nonspecific protein thyroglobulin was substituted for the proteasome no enhanced fluorescence was observed.
binding was observed.

In contrast to DFLB, the naphthyl fluorescence of another peptidyl boronic acid inhibitor of the proteasome, MNLB (Fig. 6, panel A), is completely quenched upon binding to a Rhodococcus active site. The reason for this quenching is unclear. Presumably, the naphthyl moiety in the MNLB interacts with some “quencher” in the Rhodococcus proteasome. There are no tryptophan residues present so the quencher is likely to be tyrosine or some ionic species (21). The rate of association of MNLB with the Rhodococcus proteasome was determined to be 12,800 $M^{-1} s^{-1}$, measured directly from a fit of this data to the integrated rate expression for a second-order reaction (22).

The fluorescence of the naphthyl moiety was no longer integrated rate expression for a second-order reaction (22) (Fig. 6, panel A). Complete inhibition of the proteasome with $\beta$-lactone completely abrogates binding of boronic acid-derived inhibitors, suggesting that both inhibitors bind to the same site. Boronic acid-derived inhibitors have been shown to covalently react with the $O\gamma$ residue of the active site serine of thrombin to yield a tetrahedral boron adduct mimicking the tetrahedral intermediate in substrate hydrolysis (18). By analogy we propose that boronic acid-based inhibitors interact in a similar manner with the O$\gamma$ on the N-terminal Thr of the $\beta$-subunit of the proteasome.

Abrogation of peptidyl inhibitor binding to the proteasomal active sites is therefore more likely to occur close to the active site rather than by disrupting interactions of the peptide portion of the inhibitor at the S1, S2, and S3 specificity pockets. Peptidyl vinyl sulfones based on substrate selectivity have been synthesized by Palmer and co-workers and are potent inhibitors of disease-associated cysteine proteases such as cathepsins B, L, and S (23). This class of inhibitors have also been shown to be irreversible, active site-directed inhibitors of the proteasome (24, 25). We found that the peptidyl vinyl sulfone, Z-Leu-Leu-Leu-(CH)$_2$SO$_2$Me inhibits the Rhodococcus proteasome ($k_{mub}/K_i$ of 150 $M^{-1} s^{-1}$).

We reacted unmodified and $\beta$-lactone modified Rhodococcus proteasome with Z-Leu-Leu-Leu-(CH)$_2$SO$_2$Me and separated

FIG. 6. Active site titration of Rhodococcus proteasome with MNLB. Panel A, titration of increasing amounts of MNLB into 21.3 $\mu$g of unmodified and $\beta$-lactone modified Rhodococcus proteasome particles was performed as described under “Experimental Procedures.” Fluorescence at 337 nm ($\lambda_{em} = 293$ nm) was recorded after equilibrium had been reached. The fluorescence of the naphthyl moiety was no longer quenched beyond the addition of 0.4 nmol of inhibitor, yielding an active site concentration of 0.4 nmol/21.3 $\mu$g of proteasome particle. No quenching was observed when the active sites had been previously modified with an excess of $\beta$-lactone. Panel B, progress curve for the association of MNLB with Rhodococcus proteasome. The rate of association was determined to be 12,800 $M^{-1} s^{-1}$, measured directly from a fit of this data to the integrated rate expression for a second-order reaction (22).

estimated to be $5.5 \times 10^{-5} M^{-1} s^{-1}$ ($t_{1/2} = 3.5$ h). The dissociation constant calculated using these parameters is 4.3 nm, and agrees with the $K_i$ of 5.8 nm estimated from an enzyme inhibition assay (data not shown).

Since there are no tryptophan residues in the Rhodococcus proteasome, observation of the naphthyl fluorescence was readily achieved ($\lambda_{ex} = 293$ nm; $\lambda_{em} = 337$ nm). Incubation of 21.3 $\mu$g of Rhodococcus proteasome particles with increasing amounts of MNLB resulted in quenching of the naphthyl fluorescence until all the active sites were filled. Further additions of MNLB resulted in a fluorescence increase in proportion to the unbound inhibitor. The “breakpoint” was observed at 0.4 nmol of MNLB which is equivalent to 1.0 active sites per $\beta$-subunit. Again, when the proteasome’s active sites were blocked with $\beta$-lactone, no quenching of the MNLB was observed upon titration with the inhibitor (Fig. 6, panel A).

Peptidyl vinyl sulfones based on substrate selectivity have been synthesized by Palmer and co-workers and are potent inhibitors of disease-associated cysteine proteases such as cathepsins B, L, and S (23). This class of inhibitors have also been shown to be irreversible, active site-directed inhibitors of the proteasome (24, 25). We found that the peptidyl vinyl sulfone, Z-Leu-Leu-Leu-(CH)$_2$SO$_2$Me inhibits the Rhodococcus proteasome ($k_{mub}/K_i$ of 150 $M^{-1} s^{-1}$).

We reacted unmodified and $\beta$-lactone modified Rhodococcus proteasome with Z-Leu-Leu-Leu-(CH)$_2$SO$_2$Me and separated

FIG. 7. Modification of the Rhodococcus proteasome with $\beta$-lactone blocks the active site directed binding of vinyl sulfone inhibitors. Modification of the Rhodococcus proteasome with vinyl sulfone inhibitors results in a shifted retention time for the $\beta$-subunit upon separation from the $\alpha$-subunit by reverse-phase HPLC. With prior modification with $\beta$-lactone the shift in the retention time of the $\beta$-subunit was prevented. Modification with $\beta$-lactone alone causes no such shift.
the α- and β-subunits by HPLC, as outlined under “Experimental Procedures” (Fig. 7). The β-subunit of peptidyl vinyl sulfone-modified enzyme has a longer retention time, suggesting the inhibitor covalently reacts with this subunit. Prior modification of the proteasome with β-lactone prevented the retention time shift indicating that β-lactone blocks reaction of the vinyl sulfone moiety of the inhibitor. No shift in the retention time of the α-subunits was observed, suggesting that it is not modified by the inhibitor. From peptide mapping of the tryptic digest of the vinyl sulfone-modified β-subunits, we found that the N-terminal tryptic peptide of the β-subunit was the modified peptide (data not shown). These two observations additionally suggest that the peptidyl vinyl sulfone may inhibit the proteasome by modification of the Oγ of Thr1.

Scheme 1 summarizes our working hypothesis for the mechanisms of the inhibitors studied. Clasto-lactacystin β-lactone, peptidyl mimetic aldehydes, boronic acids, and vinyl sulfones may all involve a common element for inhibition of the proteasome, that is reacting with the catalytic nucleophile, the Oγ on Thr1.

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Scheme 1. Proposed mechanism of proteasome inhibition by different classes of inhibitors.