# Kinetic Studies of the Branched Chain Amino Acid Preferring Peptidase Activity of the 20S Proteasome: Development of a Continuous Assay and Inhibition by Tripeptide Aldehydes and *clasto*-Lactacystin $\beta$-Lactone

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ABSTRACT: We have developed an assay to continuously monitor the branched amino acid preferring peptidase (BrAAP) activity of the proteasome. This assay is based on the hydrolysis of the flourogenic peptide, Abz-Gly-Pro-Ala-Leu-Ala-Nba (Abz is 2-aminobenzoyl and Nba is 4-nitrobenzylamide) which is cleaved exclusively at the Leu-Ala bond by the 20S proteasome with a $k_c/K_m$ value of 13 000 $M^{-1}$ $s^{-1}$. Hydrolysis of this peptide is accompanied by an increase in fluorescence intensity ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 415 nm) due to release of the internally quenched 2-aminobenzoyl fluorescence that accompanies diffusion apart of the hydrolysis products, Abz-Gly-Pro-Ala-Leu and Ala-Nba. Using this assay, we examined inhibition of the BrAAP activity of the proteasome by a series of tripeptide aldehydes, Z-Leu-Leu-Xaa-H. When Xaa = Phe, (*p*-Cl)Phe, and Trp we observe biphasic or partial inhibition of the BrAAP activity. In contrast, when Xaa = Nva and Leu, simple inhibition kinetics are observed and allow us to calculate $K_i$ values of 120 nM and 12 nM, respectively. The inhibitors that exhibit simple inhibition kinetics for BrAAP activity are also approximately equipotent for inhibition of the chymotrypsin-like (ChT-L) and peptidyl−glutamyl peptide hydrolyzing (PGPH) activities, dissociation constants varying by less than 25-fold, whereas the inhibitors that exhibit biphasic inhibition kinetics for BrAAP activity are >300-fold more potent for inhibiting ChT-L activity than for PGPH activity. Inactivation of the BrAAP activity of the proteasome by *clasto*-lactacystin $\beta$-lactone is also biphasic. $\beta$-Lactone inactivates approximately 60% of the BrAAP activity rapidly, with kinetics indistinguishable from its inactivation of the chymotrypsin-like activity. The remaining 40% of the BrAAP activity is inactivated by $\beta$-lactone at a 50-fold slower rate, with kinetics indistinguishable from its inactivation of the PGPH activity. These results suggest a mechanism in which hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba (i.e., BrAAP activity) occurs at two different active sites in the 20S proteasome, and that these two active sites are the same ones that catalyze the previously described ChT-L and PGPH activities.

The 20S proteasome is a large, multimeric proteolytic enzyme (MW = 700 kDa) found in high concentration in all mammalian cells (*1*−*5*). This enzyme is the catalytic core of the larger 26S proteasome (MW = 2000 kDa) that degrades ubiquitinated proteins to peptides and ubiquitin chains (*1*, *2*, *5*). The 26S proteasome catalyzes the final step of the ubiqutin−proteasome pathway of protein degradation which has been shown to be the principal pathway for intracellular protein turnover (*1*, *3*) and for the processing of a number of important cellular proteins (*3*); such as, cyclins (*6*), tumor suppressor protein p53 (*7*−*9*), and latent nuclear factor $\kappa$B (*10*, *11*).

A unique feature of the 20S proteasome is its broad $P_1$ specificity[1] (*2*). This enzyme has been shown to efficiently hydrolyze peptide substrates such as Suc-Leu-Leu-Val-Tyr-AMC,[2] Z-Leu-Leu-Arg-AMC, and Z-Leu-Leu-Glu-2NA and hydrolysis of these substrates has been the basis for the definition of chymotryptic, tryptic and peptidylglutamyl peptide hydrolyzing activities of the proteasome, respectively (*2*, *13*). In addition, a number of other hydrolytic activities of the proteasome have also been described, such as the branched chain amino acid preferring activity or BrAAP (*14*) and the small neutral amino acid preferring activity or SNAAP (*14*).

As part of a program to discover inhibitors of enzymes of the ubiquitin−proteasome pathway, we specifically targeted the chymotryptic activity of the 20S proteasome for mechanism-based inhibitor design and are developing several classes of inhibitors including peptide aldehydes. It was clearly of some interest to us to determine if these compounds

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[1] The nomenclature for the amino acid residues of the substrate ($P_n$, ..., $P_3$, $P_2$, $P_1$) and corresponding protease active subsites to which they bind ($S_n$, ..., $S_3$, $S_2$, $S_1$) is that of Schecter and Berger (*12*).

[2] Abbreviations: Suc, *N*-succinyl; AMC, 7-amino-4-methylcoumarinamide; 2NA, 2-naphthylamide; Z, benzyloxycarbonyl; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; pAB, *p*-aminobenzoate; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; Nva, norvaline; BrAAP, branched chain amino acid preferring; ChT-L, chymotrypsin-like; SNAAP, small neutral amino acid preferring; PGPH, peptidyl−glutamyl peptide hydrolyzing; FI, fluorescence intensity.

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inhibit other proteasome activities, such as the BrAAP activity. However, we recognized that if we wanted to accurately study the inhibition of this activity we needed to first develop a method to continuously monitor hydrolysis of a BrAAP substrate. In this paper, we report the first continuous assay for the BrAAP activity of the 20S proteasome. This assay is based on the hydrolysis of the fluorogenic substrate Abz-Gly-Pro-Ala-Leu-Ala-Nba. We used this assay to study inhibition of this proteasome activity by several peptide aldehydes and found that peptide aldehydes with large $P_1$ residues (e.g., Phe or Trp) are partial inhibitors of the BrAAP activity. We also studied the interaction of the 20S proteasome with the natural product, irreversible proteasome inhibitor *clasto*-lactacystin $\beta$-lactone (*15*), and found that it also behaves as a partial inhibitor of BrAAP activity. Combined, these data support a mechanism for partial inhibition involving hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba at two distinct active sites in the 20S proteasome.

## EXPERIMENTAL SECTION

*General.* Buffer salts were purchased from Sigma Chemical Co (St. Louis, MO). Suc-Leu-Leu-Val-Tyr-AMC was purchased from Bachem (Philadelphia, PA). Abz-Gly-Pro-Ala-Leu-Ala-Nba was a custom synthesis product from Enzyme Systems Products (Dublin, CA) and was purified to >98% by HPLC before use. 20S proteasome was purified from rabbit skeletal muscle as previously described (*16*) and stored at −80 °C as aliquots of a 1 mg/mL stock solution in a pH 7.6 buffer of 25 mM HEPES and 1 mM DTT.

*Synthesis of Peptide Aldehydes.* Z-blocked dipeptides (Bachem) were coupled to the appropriate L-amino acid-derived alcohol (Bachem) with TBTU (MeCN, 0−25 °C for 60 min), and the resultant tripeptide alcohol was oxidized to their corresponding aldehydes with Dess−Martin periodiane (*17*). Chemical purity was judged to be greater than 98% by proton NMR and HPLC analysis.

*Synthesis of clasto-Lactacystin β-Lactone. clasto*-Lactacystin $\beta$-lactone was prepared using the method of Uno (*18*). Structure was confirmed by proton NMR and fast atom bombardment mass spectrometry. The purity of the material was assessed at >95% by HPLC.

*Reaction Product Analysis.* Abz-Gly-Pro-Ala-Leu-Ala-Nba (95 $\mu$M) was reacted with 37 nM purified rabbit psoas 20S proteasome at 37 °C in a pH 8 buffer containing 20 mM HEPES, 0.5 mM EDTA, and 0.035% w/v SDS. After 4 h the reaction was stopped by addition of one volume of 2 M guanidine HCl. The guanidine and dodecyl sulfate form an insoluble salt that was removed by centrifugal filtration using an Ultrafree-MC (Millipore) 0.22 $\mu$M filter unit. A 50 $\mu$L aliquot of the filtrate was subjected to reverse-phase HPLC on a 3.9 × 150 mm Delta Pak C18 column (Millipore). The column was equilibrated at 1 mL/min, 40 °C, with water containing 0.06% v/v TFA. The peptides were eluted with a linear gradient of acetonitrile (0.05% TFA) increasing at 1%/min from 2 to 44 min after injection. The column eluent was monitored for absorbance at 214 nm and fluorescence of the Abz group ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 415 nm) with in-line detectors. Peptides eluting from the column were collected manually and analyzed by mass spectrometry as described below.

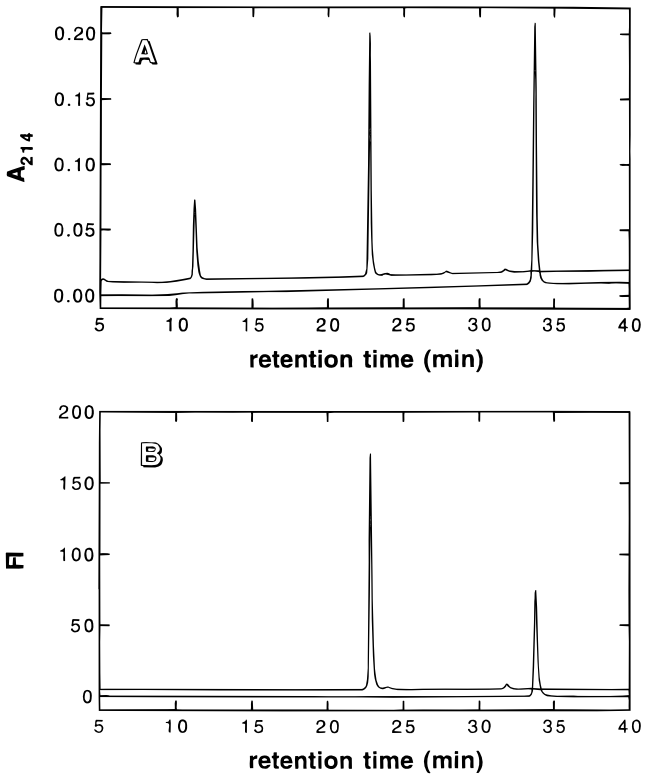


FIGURE 1: High-pressure liquid chromatographic analysis of reaction products of the 20S proteasome catalyzed hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba. Abz-Gly-Pro-Ala-Leu-Ala-Nba (95 $\mu$M) was reacted with 37 nM purified rabbit psoas 20S proteasome for 4 h at 37 °C in a pH 8 buffer containing 20 mM HEPES, 0.5 mM EDTA, and 0.035% w/v SDS. An aliquot corresponding to 2.4 nmols of substrate was subjected to HPLC as described in Experimental Procedures. The control was substrate incubated as above in the absence of enzyme. Panel A: UV chromatograms of digested and control samples. Panel B: Fluorescence chromatograms of digested and control samples ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 415 nm). The peaks at retention times 11.5, 23 and 34 min were identified as $H_2$N-Ala-Nba, Abz-Gly-Pro-Ala-Leu-OH, and Abz-Gly-Pro-Ala-Leu-Ala-Nba, respectively.

*Mass Spectral Analysis of Reaction Products.* Thioglycerol (3 $\mu$L) was added to the peptide solutions, and the solvent was evaporated in a vacuum centrifuge. The resulting peptide solutions in thioglycerol were applied to a probe, ionized by fast atom bombardment, and analyzed in a VG 30-250 quadrupole mass spectrometer. Alternatively, the peptide solutions were ionized with an electrospray source configured to the same instrument. Both techniques yielded equivalent results. Molecular ions with masses corresponding to the parent peptide, Abz-Gly-Pro-Ala-Leu-Ala-Nba (M + H = 682) and the N-terminal digestion product, Abz-Gly-Pro-Ala-Leu-OH (M + H = 477) were observed in the spectra of the appropriate HPLC fractions (see Figure 1, and Results). The molecular ion of the C-terminal digestion product, $H_2$N-Ala-Nba (M + H = 224) was not detected with either ionization mode.

*Assay of 20S Proteasome Activities and Inhibition by β-Lactone or Peptide Aldehydes.* In a typical kinetic run for measurement of the BrAAP activity of the 20S proteasome, 2.00 mL of assay buffer (20 mM HEPES, 0.5 mM EDTA, 0.035% SDS, pH 7.8) and Abz-Gly-Pro-Ala-Leu-Ala-Nba in DMSO were added to a 3 mL fluorescence cuvette and the cuvette, was placed in the jacketed cell holder of a Hitachi 2000 fluorescence spectrophotometer. Reaction

temperature was maintained at 37.0 ± 0.02 °C by a circulating water bath. After the reaction solution had reached thermal equilibrium (~5 min), 2−10 $\mu$L of the stock enzyme solution was added to the cuvette. Reaction progress was monitored by the increase in fluorescence emission at 415 nm ($\lambda_{ex}$ = 340 nm) that accompanies cleavage of the peptide and release of internal quenching. For each kinetic run, 200−1000 data points, corresponding to {time, FI} pairs, were collected by a microcomputer interfaced to the fluorescence spectrophotometer.

Measurement of the ChT-L, PGPH, and trypsin-like activities of the proteasome were performed in a manner similar to measurement of the BrAAP activity. The substrates for these activities are Suc-Leu-Leu-Val-Tyr-AMC, Bz-Val-Gly-Arg-AMC, and Z-Leu-Leu-Glu-AMC, respectively, and the release of the cleavage product, AMC, was monitored at 440 nm ($\lambda_{ex}$ = 380 nm). Bz-Val-Gly-Arg-AMC is not compatible with SDS, therefore measurement of the trypsin-like activity was performed using the 20S proteasome activator, PA28, instead of SDS to activate the proteasome.

When the hydrolysis of substrate reached steady state, inhibitor ($\beta$-lactone, peptide aldehydes) was added at an appropriate concentration and the reaction progress curve monitored. The rate constant for inactivation, $k_{obs}$, by $\beta$-lactone was determined by a nonlinear least-squares fit of the data to the equation for time dependent or slow binding inhibition (*19*):

$$\{\text{fluorescence}\} = v_i t\left(\frac{v_c - v_i}{k_{obs}}\right)(1 - \exp(-k_{obs}t)) \quad (1)$$

where $v_c$ is the initial velocity of the enzyme which slowly decays to $v_i$, the final steady-state velocity with a first-order rate constant, $k_{obs}$.

The dissociation constant, $K_i$, for reversible inhibitors was determined by nonlinear least-fit of the data to the expression

$$\frac{v_i}{v_c} = \frac{1}{1 + \frac{[I]}{K_i}} \quad (2)$$

where [I] is the inhibitor concentration. In all cases, the concentration of substrate [S] ≪ $K_m$, so that $K_{i,app} \approx K_i$.

## RESULTS

*Development of a Continuous Assay for the BrAAP Activity of the 20S Proteasome.* The BrAAP activity of the 20S proteasome is typically measured in a coupled, stopped-time assay in which rate-limiting concentrations of the proteasome cleave Z-Gly-Pro-Ala-Leu-Ala-pAB to produce Z-Gly-Pro-Ala-Leu and Ala-pAB (*14*). Excess aminopeptidase N hydrolyzes Ala-pAB to Ala and pAB which is quantified by diazotization. While suitable for many studies, this assay does not offer the precision or accuracy that is required for detailed kinetic and mechanistic studies. We felt it was critical to develop a means to continuously monitor hydrolysis of a BrAAP substrate.

To this end, we employed the general strategy of Nishino and Powers (*20*) for designing internally quenched protease substrates and prepared Abz-Gly-Pro-Ala-Leu-Ala-Nba. This peptide has the amino acid sequence of the common BrAAP substrate, Z-Gly-Pro-Ala-Leu-Ala-pAB (*14*), but has the fluorescent N-terminal blocking group, 2-aminobenzoate, that is internally quenched by the C-terminal blocking group, 4-nitrobenzylamide. Complete hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba results in a 3.7-fold increase in fluorescence intensity at 415 nm ($\lambda_{ex}$ = 340 nm). This was determined in an experiment in which 20 $\mu$M Abz-Gly-Pro-Ala-Leu-Ala-Nba was reacted with 10 $\mu$M porcine pancreatic elastase to rapidly effect total hydrolysis of the substrate at the Ala-Leu bond.

When reacted with the 20S proteasome we found that Abz-Gly-Pro-Ala-Leu-Ala-Nba was indeed found to be a substrate for this enzyme and, as predicted, is hydrolyzed exclusively at the Leu-Ala bond. The chromatograms of Figure 1 demonstrate that 20S proteasome-catalyzed hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba results in the production of two distinct peptide products even after prolonged incubation with high concentrations of proteasome, demonstrating there is a single cleavage site in this substrate. The substrate and the two products were collected from chromatographic experiments and subjected to mass spectral analysis. Molecular ions with masses corresponding to the parent peptide (M + H = 682) and the N-terminal digestion product, Abz-Gly-Pro-Ala-Leu-OH (M + H = 477) were observed in the spectra of the HPLC fractions which eluted at 34 and 23 min, respectively (Figure 1). The C-terminal digestion product, $H_2$N-Ala-Nba, was not detected by MS; however the product which has a retention time of 11.5 min did not have fluorescent properties and we conclude that this product is $H_2$N-Ala-Nba.

Kinetic analysis of 20S proteasome-catalyzed hydrolysis of Abz-Gly-Pro-Ala-Leu-Ala-Nba demonstrated that initial, steady-state velocities are linear with enzyme concentrations ranging from 0.5 to 20 nM ([S] = 4 $\mu$M). Also, steady-state velocities linearly correlate with substrate concentration from 2 to 20 $\mu$M ($r^2 > 0.99$). Concentrations of Abz-Gly-Pro-Ala-Leu-Ala-Nba greater than 20 $\mu$M are insoluble in our assay buffer. The slope of the linear dependence of $v_s$ on [S] allows us to calculate a $k_c/K_m$ value of 13 000 $M^{-1}$ $s^{-1}$ and to provide the following limits: $K_m \geq 100$ $\mu$M and $k_c \geq 1$ $s^{-1}$.

*Inhibition of the BrAAP Activity of the 20S Proteasome by Tripeptide Aldehydes.* Compounds of general structure Z-Leu-Leu-Xaa-H are known inhibitors of the proteasome (*21*, *22*) and have been characterized most often for their ability to inhibit the ChT-L activity of this enzyme. $K_i$ values for the inhibition of the 20S proteasome-catalyzed hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC for five tripeptide aldehydes, where Xaa = Nva, Leu, Phe, (*p*-Cl)Phe, and Trp, are summarized in Table 1. These $K_i$ values were determined at concentrations of Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC that are at least 5- to 10-fold lower than the substrate's $K_m$, so they reflect true dissociation constants for the complex of inhibitor and enzyme. These $K_i$ values were determined by nonlinear least-squares fit of the data to eq 2 (see Experimental Section).

Table 1: Inhibition of Peptidase Activities of the 20S Proteasome by Z-Leu-Leu-Xaa-H[a]

| | ChT-L[b] | PGPH[c] | BrAAP[d] | | | |
|---|---|---|---|---|---|---|
| Xaa | $K_i$ (nM) | | $f_1$ | $f_2$ | $K_1$ (nM) | $K_2$ (nM) |
| Nva | 25 | 580 | | | 120 | |
| Leu | 4.0 | 50 | | | 12 | |
| Phe | 5.0 | 5000 | 0.76 | 0.24 | 2.2 | 330 |
| (p-Cl)Phe | 5.6 | 12000 | 0.68 | 0.32 | 17 | 4200 |
| Trp | 7.7 | 2600 | 0.79 | 0.21 | 7.5 | >10000 |

[a] All reactions were conducted at 37 °C in a pH 7.8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.035% SDS, and <1% DMSO. [b] $K_i$ values for inhibition of the ChT-L activity of the 20S proteasome were measured with 2−4 $\mu$M Suc-Leu-Leu-Val-Tyr-AMC and 0.5−2 nM 20S proteasome and calculated by nonlinear least-squares fit to eq 2. [c] $K_i$ values for inhibition of the PGPH activity of the 20S proteasome were measured with 2−4 $\mu$M Z-Leu-Leu-Glu-AMC and 0.5−2 nM 20S proteasome and calculated by nonlinear least-squares fit to eq 2. [d] $K_i$ values for inhibition of the BrAAP activity of the 20S proteasome were measured with 2−10 $\mu$M Abz-Gly-Pro-Ala-Leu-Ala-Nba and 1−10 nM 20S proteasome. For Xaa = Nva and Leu, $K_i$ values were calculated by nonlinear least-squares fit to eq 2, while for Xaa = Phe, (pCl)Phe, and Trp, $K_i$ values were calculated by nonlinear least-squares fit to the mechanism-independent expression of eq 3.

When studied as inhibitors of the BrAAP activity of the 20S proteasome, Z-Leu-Leu-Nva-H and Z-Leu-Leu-Leu-H were found to have $K_i$ values of 120 ± 11 nM and 12 ± 1 nM, respectively (see Figure 2A). In contrast to this simple behavior, Z-Leu-Leu-Phe-H, Z-Leu-Leu-(*p*-Cl)Phe-H, and Z-Leu-Leu-Trp-H are partial inhibitors of the proteasome (Figure 2B−D). The inhibition data for these compounds require the expression of eq 3:

$$\frac{v_i}{v_c} = \frac{f_1}{1+\frac{[I]}{K_1}} + \frac{f_2}{1+\frac{[I]}{K_2}} \qquad (3)$$

This is a mechanism-independent expression for partial inhibition where $f_1$ and $f_2$ are fractional contributions from two inhibitor-sensitive catalytic components. When combined with inhibitor, these two components form complexes that have dissociation constants of $K_1$ and $K_{2,app}$, respectively. The inhibition data of Figure 2B-D can be fit to the expression of eq 3 to provide the values that are summarized in Table 1. The values for $K_{2,app}$ are uncertain since the highest inhibitor concentration tested was lower than the estimated values of $K_{2,app}$.

*BrAAP-Selective Tripeptide Aldehyde Inhibitors.* It was of some interest to determine if a tripeptide based on the $P_3$-$P_1$ sequence of the substrate Abz-Gly-Pro-Ala-Leu-Ala-Nba would be a potent and BrAAP-selective inhibitor of the proteasome. To this end, we prepared Z-Pro-Ala-Leu-H and determined $K_i$ values for the chymotryptic and BrAAP activities of the 20S proteasome (Figure 3). To explore the effects of systematic transformation of Z-Leu-Leu-Leu-H into Z-Pro-Ala-Leu-H, we also prepared and tested Z-Leu-Ala-Leu-H. $K_i$ values are all summarized in Table 2.

We see that while Z-Pro-Ala-Leu-H is more selective[3] for the BrAAP activity than is either Z-Leu-Leu-Leu-H or Z-Leu-Ala-Leu-H, it is several orders of magnitude less potent than either of these compounds. We see that Z-Pro-Ala-Leu-H inhibits the BrAAP activity 150-times less potently than Z-Leu-Leu-Leu-H, even though Z-Pro-Ala-Leu-H contains structural features that define this acitivity. Specifically, Z-Pro-Ala-Leu-H contains a Pro at $P_3$ and a small, aliphatic residue at $P_2$ which have been argued to direct peptides to the proteasome's BrAAP site (*24*).

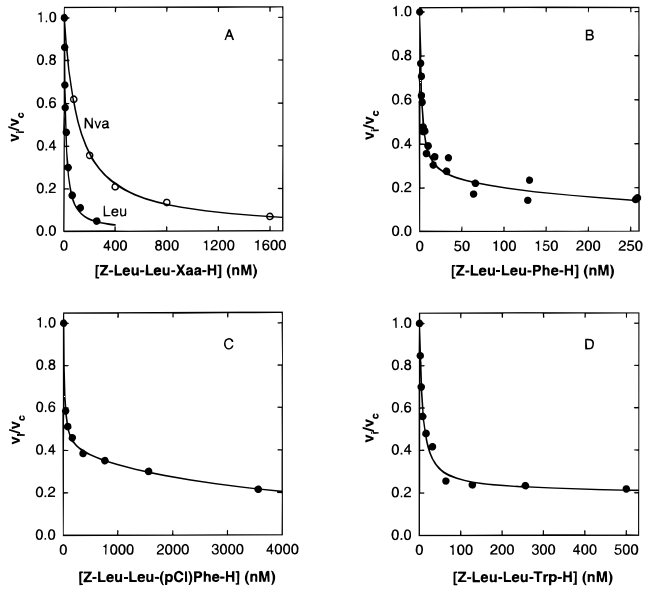


FIGURE 2: Inhibition of the BrAAP Activity of 20S proteasome by tripeptide aldehydes. Kinetic experiments were conducted at 37 °C in a pH 8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.05% SDS, and <1% DMSO. 2 $\mu$M ≤ [Abz-Gly-Pro-Ala-Leu-Ala-Nba] ≤ 10 $\mu$M; 1 nM ≤ [20S proteasome] ≤ 10 nM. In all cases, $v_i/v_c$ is the parameter plotted on the *y*-axis. Panel A: The lines drawn through the data points for inhibition by Z-Leu-Leu-Nva-H and Z-Leu-Leu-Leu-H were drawn using eq 2 and the parameters of Table 1. Panel B: The data shown are from two independent experiments. Panels B, C, and D: The lines drawn through the data points were drawn according to eq 3 and the best fit parameters of Table 1.

*Inhibition of the BrAAP Activity of the 20S Proteasome by clasto-Lactacystin β-Lactone.* The natural product lactacystin has been shown to be an inhibitor of the 20S proteasome (*15*). Studies from Fenteany and co-workers (*15*) demonstrated time-dependent inactivation of the ChT-L, trypsin-like, and PGPH activities of the proteasome. We have previously shown that the active form of lactacystin is *clasto*-lactacystin $\beta$-lactone (*25*), and now report that this molecule can also inactivate the BrAAP activity of the 20S proteasome. Figure 4 shows the residual activities of the 20S proteasome after incubation with various amounts of $\beta$-lactone. In this experiment 20S proteasome in complex with saturating concentrations of the PA28 activator was aliquoted. Each aliquot was treated with different amounts of $\beta$-lactone for 30 min at room temperature. The residual activity of each aliquot was measured against four peptide substrates. In agreement with the initial findings (*15*), the ChT-L activity was more rapidly inactivated by the $\beta$-lactone than PGPH or trypsin-like activities. Here the result is manifest by the observation that a stoichiometry of ap-

[3] Even greater BrAAP-selectivity was observed for the tetrapeptide Z-Gly-Pro-Ala-Leu-H which inhibits the chymotryptic and BrAAP activities of the proteasome with $K_i$ values of 470 $\mu$M and 14 $\mu$M, respectively (*23*). However, comparison of these results with ours is difficult since Vinitsky et al. (*23*) used unactivated proteasome in their study. Although we know that potency of the inhibitors would increase with SDS-activated proteaseome, we are not sure if the selectivity would change.

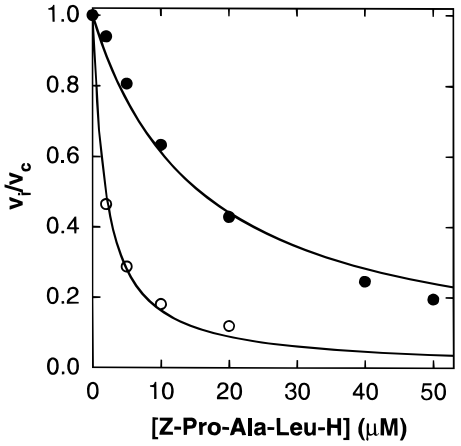


FIGURE 3: Inhibition of the chymotryptic and BrAAP activities of the 20S proteasome by Z-Pro-Ala-Leu-H. Kinetic experiments were conducted at 37 °C in a pH 8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.035% SDS, and <1% DMSO. Filled circles: [Suc-Leu-Leu-Val-Tyr-AMC] = 10 $\mu$M, [20S proteasome] = 0.5 nM. The line through the points was drawn using eq 2 and $K_{i,app}$ = 15.8 ± 1.2 $\mu$M. Open circles: [Abz-Gly-Pro-Ala-Leu-Ala-Nba] = 10 $\mu$M, [20S proteasome] = 8 nM. The line through the points was drawn using eq 2 and $K_i$ = 1.94 ± 0.13 $\mu$M.

Table 2: Inhibition of Peptidase Activities of the 20S Proteasome by Tripeptide Aldehydes[a]

| inhibitor | $K_i$ (nM) chymotrypsin[b] | $K_i$ (nM) BrAAP[c] | BrAAP selectivity ratio[d] |
|---|---|---|---|
| Z-Leu-Leu-Leu-H | 4 | 12 | 0.3 |
| Z-Leu-Ala-Leu-H | 7 | 64 | 0.1 |
| Z-Pro-Ala-Leu-H | 11000 | 1900 | 5.8 |
| Z-Leu-Leu-Glu-H | 27000 | 5200 | 5.2 |

[a] All reactions were conducted at 37 °C in a pH 7.8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.05% SDS, and <1% DMSO. [b] $K_{i,app}$ values for inhibition of the chymotryptic activity of the 20S proteasome were measured with 10 $\mu$M Suc-Leu-Leu-Val-Tyr-AMC and 0.5−2 nM 20S proteasome and calculated by nonlinear least-squares fit to eq 2. $K_i$ was calculated from the experimental observed $K_{i,app}$ value and the expression, $K_i = K_{i,app}/(1 + [S]/K_{m,app})$ where $K_{m,app}$ = 20 $\mu$M. [c] $K_i$ values for inhibition of the BrAAP activity of the 20S proteasome were measured with 2−10 $\mu$M Abz-Gly-Pro-Ala-Leu-Ala-Nba and 1−10 nM 20S proteasome. $K_i$ values were calculated by nonlinear least-squares fit to eq 2. [d] Selectivity ratio = $K_{i,ChT}/K_{i,BrAAP}$.

proximately 2.5 mol of lactone per mole of 20S particle inactivated >90% of the ChT-L activity but less than 15% of either PGPH or trypsin-like activities (Figure 4). Interestingly, a fraction of the BrAAP activity (~60%) could be inactivated with low amounts of $\beta$-lactone in parallel with ChT-L activity. One model to account for this result would involve a single site of lactone modification causing inactivation of ChT-L activity *and* inactivation of ~60% of total BrAAP activity. The remainder of the BrAAP activity (~40%) could be inhibited by prolonged incubation times at much higher $\beta$-lactone concentrations similar to the PGPH activity (data not shown).

The $\beta$-lactone reacts with the eukaryotic proteasome by acylating the side chain hydroxyl group of Thr[1] of subunit X (*15*, *26*). By analogy to the structure and mechanism of the *Thermoplasma* proteasome (*27*, *28*) and the *Rhodococcus* proteasome (*29*, *30*), this residue (i.e., Thr[1] of subunit X) functions as the catalytic nucleophile for the chymotryptic activity of eukaryotic proteasomes and forms a hemiacetal with the aldehyde function of peptide aldehyde inhibitors of

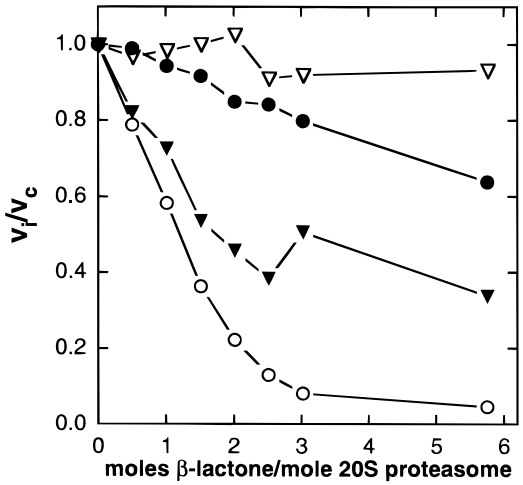


FIGURE 4: Residual activity of the chymotrypsin-like (open circles), BrAAP (filled triangles), PGPH (filled circles), and trypsin-like (open triangles) activities of the 20S proteasome treated with varying amounts of $\beta$-lactone. 0.35 $\mu$M 20S proteasome in the presence of a 4-fold excess of PA28 was incubated with different concentrations of $\beta$-lactone (0−2 $\mu$M) for 30 min at room temperature. Residual ChT-L, PGPH, and BrAAP activities were measured using the relevant substrates in buffer at 37 °C containing 20 mM HEPES, pH 7.8, 0.5 mM EDTA, and 0.035% SDS. The trypsin-like activity was measured using PA28 for activation.[6]

this activity. Although the mechanism by which lactacystin inactivates other peptidase activities of eukaryotic proteasomes has not been established, it is clear from the kinetics that the mechanism involves additional sites of modification (*15*). As a result of this difference it is possible to create conditions for proteasome modification with $\beta$-lactone that effect a nearly complete inhibition of the ChT-L activity while retaining a substantial portion (~40%) of the total BrAAP activity (Figure 4). Also, since the modification with $\beta$-lactone masks the functional group that would form a hemiacetal with an aldehyde inhibitor, this modification should greatly diminish the affinity of the tripeptide aldehyde inhibitors for binding in the active site that corresponds to the ChT-L activity (see Table 1) (*30*). Figure 5 shows the effect that $\beta$-lactone mediated inactivation of the chymotryptic activity has on the ability of Z-Leu-Leu-(pCl)Phe-H to inhibit the BrAAP activity. The bi-phasic nature of the titration curve observed with unmodified enzyme (Figure 5, closed circles; see also Figure 2C and Table 1) was almost completely abolished when the ChT-L activity was first inactivated by $\beta$-lactone modification (Figure 5, open circles). These data suggest that the component of the BrAAP activity that is most sensitive to Z-Leu-Leu-(pCl)Phe-H (Table 1) may correspond to the active site that catalyzes the ChT-L activity.

Approximately, 40% of the BrAAP activity is independent of the active site that catalyses the ChT-L activity. The data in Figure 6 suggest that this portion of the BrAAP activity corresponds to the active site that catalyses the PGPH activity. Z-Leu-Leu-Glu-H and Z-Pro-Ala-Leu-H were synthesized as inhibitors of the PGPH and BrAAP activities, respectively, based on the sequence of their peptide substrates. With unmodified enzyme both compounds are more potent inhibitors of the PGPH activity, even though Z-Pro-Ala-Leu-H was synthesized based on the substrate of the BrAAP activity. When the ChT-L activity was first inactivated with $\beta$-lactone, the titration curves for the PGPH and

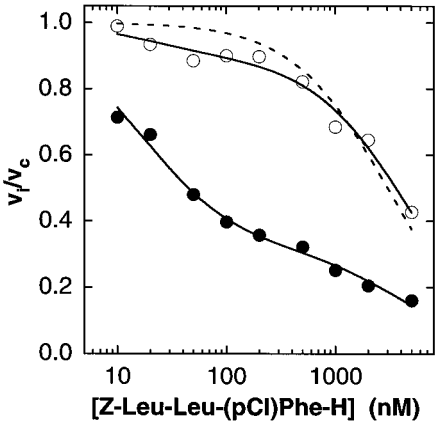


FIGURE 5: Inhibition of the BrAAP activity of $\beta$-lactone modified 20S proteasome by Z-Leu-Leu-(pCl)Phe-H. Kinetic experiments were conducted at 37 °C in pH 8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.035% SDS, and 4.8 $\mu$M Abz-Gly-Pro-Ala-Leu-Ala-Nba. Closed circles: Control [20S proteasome] = 2.3 nM. The solid line represents the best fit of the data points to eq 3, and the derived parameter estimates were $f_1 = 0.68$, $K_1 = 17$ nM, $f_2 = 0.32$, $K_2 = 4200$ nM. Open circles: 20S proteasome in storage buffer (50 mM HEPES, pH 7.8, 1mM DTT) was incubated for 20 min at room temperature with 16 $\mu$M *clasto*-lactacystin $\beta$-lactone, then diluted into assay buffer at a final concentration of 2.3 nM. The chymotryptic activity of the enzyme preparation was <2% of control, and the residual BrAAP activity was 42% of control. The solid line represents the best fit of the data points to eq 3, and the derived parameter estimate were $f_1 = 0.11$, $K_1 = 18$ nM, $f_2 = 0.89$, $K_2 = 4500$ nM. The dotted line represents a fit of the data to a simple binding isotherm with $K_{i,app} = 3000$ nM.

remaining BrAAP activity were similar with the peptide aldehyde inhibitors, Z-Leu-Leu-Glu-H and Z-Pro-Ala-Leu-H. The dissociation constants for these inhibitors post $\beta$-lactone modification are shown in Table 3. Since Z-Leu-Leu-Glu-H and Z-Pro-Ala-Leu-H are poor inhibitors of the chymotrypsin-like activity (Table 2), in the absence of $\beta$-lactone modification poor inhibition of the total BrAAP activity is observed. The rate of inactivation of the PGPH and remaining BrAAP activity by $\beta$-lactone after inactivation of the ChT-L activity by $\beta$-lactone are identical (Table 3), suggesting that these activities are catalyzed by the same active site.

## DISCUSSION

*A Continuous Assay for the BrAAP Activity of the 20S Proteasome.* In this study, we used the general strategy of Nishino and Powers (*20*) for the design of fluorogenic endopeptidase substrates to develop a new substrate and assay for the BrAAP activity of the 20S proteasome. Abz-Gly-Pro-Ala-Leu-Ala-Nba was prepared, and we found that it is cleaved exclusively at the Leu-Ala bond with a $k_c/K_m$ value of 13 000 $M^{-1}$ $s^{-1}$. This value compares favorably with the $k_c/K_m$ value of 11 000 $M^{-1}$ $s^{-1}$ for hydrolysis of Z-Gly-Pro-Ala-Leu-Ala-pAB by SDS-activated 20S proteasome isolated from cow pituitaries (*24*). Together, these data suggest that the activity of the 20S proteasome that we investigate with Abz-Gly-Pro-Ala-Leu-Ala-Nba is the same activity that Orlowski and others have described using Z-Gly-Pro-Ala-Leu-Ala-pAB. Thus, any mechanistic conclusions that we draw using this new continuous assay do indeed describe the classic BrAAP activity.

*Partial Inhibition of the BrAAP Activity of the Proteasome by Peptide Aldehydes.* In the studies reported herein, we find that while Z-Leu-Leu-Nva-H and Z-Leu-Leu-Leu-H are simple inhibitors of the BrAAP activity of the 20S proteasome, peptide aldehydes with large $P_1$ residues, Phe, (*p*-Cl)-Phe, and Trp, are partial inhibitors of this activity. In this section, we discuss several possible explanations for these results; some of these relate to the degree of purity and homogeneity of our enzyme preparation, while others relate to the proteasome's complex kinetic mechanism.

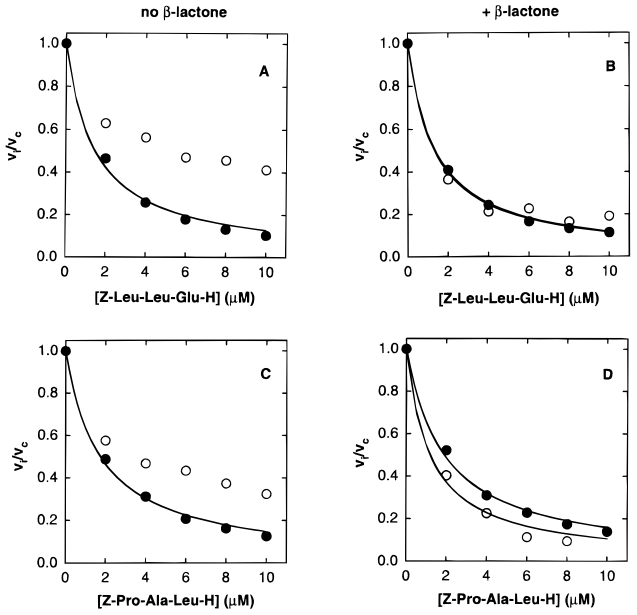


FIGURE 6: Inhibition of the BrAAP (open circles) and PGPH (filled circles) activities of the 20S proteasome by Z-Leu-Leu-Glu-H and Z-Pro-Ala-Leu-H prior to (panels A and C) and post (panels B and D) inactivation of the chymotrypsin-like activity with $\beta$-lactone. 0.5 $\mu$M 20S proteasome was incubated at room temperature for 15 min with 1.1 $\mu$M $\beta$-lactone to selectively inactivate the chymotrypsin-like activity. Kinetic experiments were performed at 37 °C in pH 7.8 buffer containing 20 mM HEPES, 0.5 mM EDTA, and 0.035% SDS. The line through the points was drawn using eq 2. $K_{i,app} = 1.3$ $\mu$M for inhibition of both activities by Z-Leu-Leu-Glu-H post-modification of the chymotrypsin-like activity with $\beta$-lactone. $K_{i,app} = 1.2$ $\mu$M and 1.4 $\mu$M for inhibition of the BrAAP and the PGPH activities, respectively, by Z-Pro-Ala-Leu-H post-modification with $\beta$-lactone.

Table 3: Kinetics of PGPH and BrAAP Inactivation after Inhibition of the Chymotrypsin-like Activity with $\beta$-Lactone[a,b]

| inhibitor | $K_i$ (nM) PGPH[c] | $K_i$ (nM) BrAAP[d] |
|---|---|---|
| Z-Leu-Leu-Leu-H | 14 | 11 |
| Z-Leu-Leu-Glu-H | 1300 | 1300 |
| Z-Pro-Ala-Leu-H | 1200 | 1400 |
| $\beta$-lactone[e] | 400 $M^{-1}$ $s^{-1}$ [f] | 400 $M^{-1}$ $s^{-1}$ |

[a] All reactions were conducted at 37 °C in a pH 7.8 buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.05% SDS, and <1% DMSO. [b] 0.5 $\mu$M 20S proteasome was incubated with 1.1 $\mu$M $\beta$-lactone at room temperature for 15 min to selectively inactivate the chymotrypsin-like activity. [c] $K_i$ values for inhibition of the PGPH activity of the 20S proteasome were measured with 10 $\mu$M Z-Leu-Leu-Glu-AMC and 0.5−2 nM $\beta$-lactone-modified 20S proteasome and calculated by nonlinear least-squares fit to eq 2. [d] $K_i$ values for inhibition of the BrAAP activity of the 20S proteasome were measured with 10 $\mu$M Abz-Gly-Pro-Ala-Leu-Ala-Nba and 1−10 nM $\beta$-lactone-modified 20S proteasome and calculated as before. [e] Measurement of the inactivation rates of the PGPH and BrAAP activities with $\beta$-lactone was performed after blocking of the chymotrypsin-like activity with $\beta$-lactone. Measurement of the second-order inactivation constants are described in Experimental Section eq 1. [f] $k_{obs}/[I]$.

The possibility of impure or heterogeneous enzyme preparations must be considered whenever anomalous enzyme kinetic behavior is being explained. This is especially true for the proteasome for which skeptical investigators have at one time or another attributed all of its multiple activities to either contaminating proteases or heterogeneous subunit populations (*5*, *30*). If, for example, our enzyme preparation is impure and Abz-Gly-Pro-Ala-Leu-Ala-Nba is hydrolyzed by the 20S proteasome and a contaminating protease, partial inhibition will be observed if these two enzymes bind inhibitors with different affinities. We must also consider the possibility that while our proteasome preparations are pure, they are composed of heterogeneous proteasome particles. In this case, partial inhibition will arise if the BrAAP activities of the different particles have different sensitivities to the aldehyde inhibitors. However, we believe that neither of these mechanisms accounts for observations of multiple activities and partial inhibition. The proteasome literature provides ample evidence documenting that these activities all exist in proteasome preparations from many species and tissues (*5*, *13*). Furthermore, the presence of the various activities is independent of the state of purity of the proteasome (*13*). Thus, it is highly unlikely that multiple activities and partial inhibition of the proteasome arise from impure proteasome preparations. Equally uncompelling are arguments of proteasome subunit heterogeneity since the proteasome literature strongly supports homogeneous particles (*5*, *13*).

Possibly the strongest evidence supporting homogeneous proteasome particles comes from two recent reports: molecular genetic experiments in yeast (*31*) and crystallization and X-ray diffraction studies of the 20S proteasome from bovine liver (*32*). In the former paper, Chen and Hochstrasser (*31*) demonstrate that mutant particles of yeast 20S proteasome contain the same 14 different subunits as the wild-type enzyme. This indicates that these proteasome particles comprise a uniform population of heterooligomeric complexes rather than a mixture of particles of variable subunit composition. And in the latter paper, Morimoto et al. (*32*) report the crystallization of bovine liver 20S proteasome. While the ability to crystallize strongly suggests that 20S proteasome particles exist as a single population, the authors go on to report that the enzyme does indeed have a uniform and highly ordered structure.

If we now assume that the 20S proteasome used in this study is pure and homogeneous in its subunit composition, we must advance other explanations for partial inhibition. As we discuss in detail below, these mechanisms incorporate the documented kinetic complexity of the proteasome that arise from this enzyme's ability to bind substrates and inhibitors at multiple sites.

Recently the crystal structure of the 20S proteasome from yeast at 2.4 Å resolution was solved by Groll and co-workers (*33*). Only three of the seven different $\beta$-subunit types carried an active site N-terminal threonine residue, $\beta$1/Pre3, $\beta$2/Pup1, and $\beta$5/Pre2, and these subunits alone were found to bind the tripeptide aldehyde, calpain inhibitor I (Acetyl-Leu-Leu-norLeu-H). Co-crystallization with lactacystin identified that the $\beta$5-subunit carried the chymotrypsin-like activity. More recently, Arendt and Hochstrasser (*34*), through mutational analysis of the N-terminal threonine on $\beta$2/Pup1 and $\beta$1/Pre3 to alanine, identified $\beta$2/Pup1 as the source of trypsin-like activity and $\beta$1/Pre3 as the PGPH activity containing subunit. Although BrAAP activity was not assayed for, it is clear from the crystal structure that only three $\beta$-type subunits carry active site nucleophiles, suggesting that the BrAAP activity must be contained on one or a combination of $\beta$1, $\beta$2, and $\beta$5.

One mechanism to explain the partial inhibiton of the BrAAP activity by certain tripeptide aldehydes states that the proteasome possesses two independent active sites and that both of these sites can hydrolyze Abz-Gly-Pro-Ala-Leu-Ala-Nba and bind peptide aldehydes. Partial inhibition arises for such a mechanism if an inhibitor is bound at the two sites with different affinities. We observe that Abz-Gly-Pro-Ala-Leu-Ala-Nba is hydrolyzed exclusively at the Leu-Ala bond. Interestingly, Z-Leu-Leu-Leu-H (MG132), having Leu in the $P_1$ position, is a potent inhibitor of the ChT-L, PGPH, and BrAAP activities. Clearly, the $\beta$-subunits responsible for these activities can all bind Leu in the $S_1$ cleft. Arendt and Hochstrasser (*34*) mutated the N-terminal threonine in the yeast proteasome $\beta$2/Pup1 and $\beta$1/Pre3 $\beta$-subunits eliminating cleavage after basic and acidic residues, respectively, in small peptide substrates; however, neither mutation had significant effects on ubiquitin-dependent proteolysis in vivo and had little effect on cell growth and viability. This suggests that the active site responsible for the ChT-L activity can compensate for overall proteolysis when the other activities are inhibited. No direct correlation has ever been made between proteasome active sites operationally defined by small peptide substrates and cleavage sites in polyubiquitinated proteins.

Inhibition of the BrAAP activity by Z-Leu-Leu-Nva-H and Z-Leu-Leu-Leu-H yielded simple titration curves for inhibition which may argue against a two active site mechanism unless both active sites possess similar binding affinities for these inhibitors. With other aldehyde inhibitors Z-Leu-Leu-Phe-H, Z-Leu-Leu-(pCl)Phe-H, and Z-Leu-Leu-Trp-H, partial inhibition is observed. The data from the titration curves could be fit to a mechanism-independent expression for partial inhibition which yielded dissociation constants for two inhibitor sensitive catalytic components of the proteasome, one component being at least 2 orders of magnitude more sensitive to the inhibitor than the other. It was observed that both Z-Leu-Leu-Nva-H and Z-Leu-Leu-Leu-H, which showed simple relatively potent inhibition of the BrAAP activity, also inhibited the ChT-L and PGPH activities with dissociation constants which varied by less than 25-fold. The inhibitors which gave biphasic inhibition of the BrAAP activity, however, were significantly more potent against the ChT-L activity than the PGPH activity, the difference in the dissociation constants being greater than 300-fold. Therefore, one possible explanation for the biphasic inhibition of the proteasome's ability to cleave the BrAAP substrate is that the active sites responsible for the chymotrypsin-like and PGPH activities ($\beta$5 and $\beta$1) can also cleave after the leucyl residue in the BrAAP substrate and that the subunit carrying the ChT-L activity contributes to 60−80% of the BrAAP activity and the subunit carrying the PGPH activity contributes to 20−40% of the BrAAP activity. AEBSF which irreversibly inhibits the trypsin-like activity ($k_{obs}$/[I] = 30 $M^{-1}$ $s^{-1}$) does not inhibit the chymotrypsin-like PGPH or BrAAP activities ($k_{obs}$/[I] < 0.1 $M^{-1}$ $s^{-1}$), suggesting that the $\beta$-subunit responsible for the trypsin-like activity ($\beta$2) is

distinct from the BrAAP activity containing subunit (unpublished data). Orlowski et al. (*14*), also showed that leupeptin, a potent and selective inhibitor of the trypsin-like activity, had no effect on the cleavage of four BrAAP substrates.

Further evidence that the proteasome subunit responsible for the chymotrypsin-like activity is partially responsible for the BrAAP activity is illustrated by the selectivity data in Table 2. Z-Pro-Ala-Leu-H was synthesized as a potential inhibitor of the BrAAP activity based on the $P_3-P_1$ sequence of its substrate. As expected, Z-Pro-Ala-Leu-H is a more selective inhibitor of the BrAAP activity than the ChT-L activity; however, it is much less potent against the BrAAP activity than Z-Leu-Leu-Leu-H and Z-Leu-Ala-Leu-H, two inhibitors with less similarity to the BrAAP substrate sequence. The two latter aldehydes are also much more potent inhibitors of the ChT-L activity than Z-Pro-Ala-Leu-H. Thus, the explanation we favor is that compounds potent against the ChT-L activity also show potent inhibition of the BrAAP activity because the active site responsible for ChT-L activity (i.e., $\beta$5) is also responsible for 60−80% of BrAAP activity.

*Partial Inhibition of the BrAAP Activity of the Proteasome by clasto-Lactacystin β-Lactone.* *clasto*-Lactacystin $\beta$-lactone inactivates multiple eukaryotic proteasome peptidase activities, ChT-L, trypsin-like, PGPH, and BrAPP, but with different rates of inactivation. It is most potent against the proteasome's chymotrypsin-like activity having a second-order rate constant for inactivation of $\sim$15000 $M^{-1}$ $s^{-1}$ for the SDS-activated 20S proteasome employed in this study[4] (data not shown). Proteasome modified with increasing amounts of $\beta$-lactone was assayed for the residual activity against the four substrates which operationally define the four activities. From extrapolation, it was estimated that 2.5 mol of $\beta$-lactone are required to inhibited the ChT-L activity (Figure 4). The yeast crystal structure (*33*) revealed that only two $\beta$-subunits (one per ring) are capable of binding $\beta$-lactone. The fact that $\beta$-lactone hydrolyzes in alkaline aqueous solution[5] suggests that this value of 2.5 mol is an overestimation and the actual binding stoichiometry is closer to 2 mol of $\beta$-lactone per mol of 20S particle. The BrAAP activity followed similar stoichiometry of binding with $\beta$-lactone as the ChT-L activity; however, its inhibition was incomplete in that when 95% of the ChT-L activity was inactivated, only 60% of the BrAAP activity was inactivated. The PGPH and trypsin-like activities remain essentially uninhibited. The fact that the inactivation of the BrAAP activity parallels the inactivation of the ChT-L activity but is partial suggests that only a fraction ($\sim$60%) of the total BrAAP activity is carried on the same site as the ChT-L activity.

Modification of the proteasome with sufficient $\beta$-lactone to completely inactivate the ChT-L activity while not affecting the trypsin-like or PGPH activities allows us to study the inhibition kinetics of the fraction of the BrAAP activity not carried on the $\beta$5 subunit. Z-Leu-Leu-(pCl)-Phe-H shows biphasic inhibition of the unmodified proteasome's BrAAP activity, 68% of the total activity being inhibited with a $K_i$ of 17 nM and the remaining BrAAP activity having a $K_i$ of 4.2 $\mu$M (Figure 5, and Table 1). This biphasic nature disappears after prior modification of the $\beta$5 subunit with $\beta$-lactone (Figure 6). Approximately 40% of the original BrAAP activity remains and the titration curve with Z-Leu-Leu-(pCl)Phe-H could be fit to a simple binding isotherm ($K_i = 4.5$ $\mu$M), suggesting that a single active site is responsible for this fraction of the BrAAP activity. The $K_i$ of 12 $\mu$M for the PGPH activity with Z-Leu-Leu-(pCl)-Phe-H is of similar magnitude to that of this BrAAP activity, suggesting the same active site.

Upon modification of the 20S proteasome with sufficient $\beta$-lactone to block the ChT-L activity, the inhibition titration curves for the PGPH and BrAAP activities by Z-Leu-Leu-Glu-H and Z-Pro-Ala-Leu-H can be superimposed on one another and the measured dissociation constants for each of the activities are similar (Figure 6 and Table 3). The inactivation rates of both activities with $\beta$-lactone post-inhibition of the ChT-L activity with $\beta$-lactone (i.e., modification of the $\beta$5 subunit) are also identical. Both the PGPH and the BrAAP activities, which have different inhibition profiles when the proteasome is unmodified, follow similar kinetics when the $\beta$5 subunit is blocked suggesting that the same active site is involved in cleaving the substrates which operationally define both activities. In other words, both the $\beta$5 and the $\beta$1 subunits of the 20S proteasome are capable of cleaving after the leucine residue in the BrAAP substrate.

The subunit or subunits of the proteasome responsible for the BrAAP and SnAAP activities has been the center of some debate. The solution of the yeast proteasome structure and the identification of only 3 $\beta$-subunits per ring carrying an N-terminal threonine and the fact that only these three subunits had peptide aldehyde inhibitor bound suggested that the substrate operationally defining the BrAAP and SNAAP activities are in fact hydrolyzed by one or more of these three subunits. We have shown through inhibitor studies that the BrAAP substrate is cleaved by the active sites responsible for cleaving the ChT-L and PGPH substrates. Thus, an explanation of BrAAP activity at the molecular level need not involve additional active sites or additional catalytic mechanisms. Rather, this explanation sits well within the context of proteasome enzymology (*35*), genetics (*34*), and structural biology (*33*) that have been elucidated over the last 20 years.

[4] Previously, we reported a value of 25 000 $M^{-1}$ $s^{-1}$ for inactivation of the ChT-L activity by $\beta$-lactone. These experiments utilized rabbit reticulocyte 20S activated by the protein activator PA28 instead of detergent. In all other respects the assay conditions are identical. Thus, the difference in $\beta$-lactone potency most likely reflects differences between detergent activated 20S and the 20S−PA28 complex.

[5] *clasto*-Lactacystin $\beta$-lactone hydrolyses to its dihydroxy acid in aqueous solution at pH 8.0, 37 °C, with a first-order rate constant of 0.0009 $s^{-1}$ (*25*).

[6] Measurement of the trypsin-like activity was done with PA28 for activation instead of SDS. It is of course impossible to measure the trypsin-like activity of SDS-activated proteasome since the detergent SDS precipitates small Arg-containing peptides such as those typically used in measuring this activity.

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