Although the structure of the 20 S proteasome from Thermoplasma acidophilum has been elucidated, its enzymatic properties have not been explored in depth. Thermoplasma proteasomes, which contain one type of active site, exhibit not only "chymotrypsin-like" activity (as reported), but also some "post-glutamyl!" and "trypsin-like" activities. Like eukaryotic proteasomes, its activity can be stimulated by SDS, Mg\(^{2+}\), and also guanidine HCl, but not urea. The enzyme was strongly inhibited by novel peptide aldehydes with hydrophobic P4 residues, and was rapidly inactivated by 3,4-dichloroisocoumarin (DCI). DCI modified the N-terminal threonine of the catalytic \(\beta\)-subunit, the presumed active site nucleophile.

To define how proteins are degraded, casein was derivatized with fluorescein isothiocyanate to facilitate detection of released products by the proteasome. Many fluorescent peptides were generated, but the relative amounts of different peptides were independent of the duration of the reaction. The rate of disappearance of protein substrates paralleled the rate of appearance of fluorescent peptides. Upon activation by SDS, guanidine, heat (55 °C), or partial inhibition with DCI, proteasomes still functioned processively, but generated a different pattern of peptides under each condition. Thus, processivity is an inherent feature of the 20 S proteasome, not requiring all active sites or ATP hydrolysis.

The 20 S proteasome is a 700-kDa barrel-shaped proteolytic complex (1–4), which in eukaryotic cells plays an essential role in the ATP-ubiquitin-dependent pathway for protein breakdown (1, 5–11). The archaeal Thermoplasma acidophilum contains a homologous 20 S protease, which is also composed of four superimposed rings and has similar subunit composition and quaternary structure (1, 12–14). Extensive studies by Baumeister and colleagues (14–16) established that both the archaeal and eukaryotic particles are composed of two types of subunits, \(\alpha\) and \(\beta\), seven of which are present in each ring (i.e., \(\alpha_2\beta_2\beta_2\alpha_2\)). While the eukaryotic proteasome contains seven distinct \(\alpha\)- and \(\beta\)-subunits (1), the archaeal particle contains only one type each of \(\alpha\)- and \(\beta\)-subunits.

The proteasome's catalytic mechanism has long been unclear, because its subunit sequences are not related to those of known proteases and its sensitivity to inhibitors differs from those of other proteases (14, 17, 18). Certain of its properties (e.g., sensitivity to DCI\(^1\)) resemble those of serine proteases, but it is resistant to agents that covalently modify such enzymes (e.g., peptide chloromethylketones). The recent x-ray diffraction analysis by Lowe et al. (19) indicated that the central chamber of the Thermoplasma proteasome contains 14 active sites, which were identified as the sites of binding of the inhibitor, Ac-LLeLnL-al. The aldehyde group of this inhibitor was associated with the N-terminal threonine residues on each \(\beta\)-subunit (19). It was, therefore, proposed that the hydroxyl group of this threonine functions as the reactive nucleophile catalyzing peptide bond cleavage. Site-directed mutagenesis (20) showed that replacement of this threonine by an alanine prevented activity, but its replacement by a serine residue still allowed activity. Strong support for this model came from the finding that lactacystin, an antibiotic from streptomycyes, inhibits the mammalian proteasome by covalently modifying the terminal threonine residues (21, 22).

Despite this detailed knowledge about the structure of the Thermoplasma proteasome, its enzymatic properties have not been extensively studied. The cloning of both Thermoplasma subunit genes and their expression as active particles in Escherichia coli greatly facilitate such studies (23). Unlike eukaryotic proteasomes, which exhibit several different peptidase activities against model peptides (24–27), the archaeal proteasome has been reported to cleave model substrates only after large hydrophobic residues. However, when archaean proteasomes were incubated with polypeptides for extended periods, they cleaved virtually all types of peptide bonds (28). The archaean proteasome has also been claimed to differ from eukaryotic particles in not being activated by low concentrations of SDS (14).

To learn more about how the archaenal proteasome digests proteins, we undertook a systematic reexamination of its specificity, using a variety of fluorogenic peptide substrates, and explored whether inhibitors or activators of the mammalian proteasome also influence its activity. The present findings on the archaenal particle differ in many respects from those in the literature (14, 17). Prior studies showed that the archaenal proteasome degrades polypeptides to peptides ranging in size between 4 and 14 amino acids (28). However, it was not established whether these small peptides were initial products or were generated by multiple rounds of substrate digestion. We demonstrate here that Thermoplasma proteasome, unlike traditional proteases, cleaves proteins in a highly processive manner, i.e. it degrades the protein to oligopeptides before attacking another protein molecule.

\(^1\)The abbreviations used are: DCI, 3,4-dichloroisocoumarin; Ac, acetyl; Amc, 7-amino-4-methylcoumarin; Boc, tert-butyloxycarbonyl; CM, carboxymethyl; FITC, fluorescein isothiocyanate; Me, methylated; nL, norleucine; R-R'-R''-al, peptide aldehydes (where \(R\) indicates amino acid residues); Sue, succinyl; Z, benzoyloxycarbonyl; HPLC, high performance liquid chromatography; IGF, insulin-like growth factor; PAGE, polyacrylamide gel electrophoresis; Bis-Tris, bis/2-hydroxyethylaminotris(hydroxymethyl)methane.
MATERIALS AND METHODS

Peptide substrates were purchased from Bachem (Switzerland) and Ni²⁺-NTA-agarose from QIAGEN (Dusseldorf, Germany). Recombinant human IGF-1 was a kind gift from Dr. W. Prouty (Lilly). Other chemicals were purchased from Sigma and were of the highest purity available.

Purification of Recombinant Proteasomes—The archaeal proteasome was purified from *E. coli* BL21(DE3) strain, transformed with a high copy plasmid PRSET5a containing the genes for the wild-type *T. acidophilum*, α-subunit and β-subunit with a His₆ tag at its C terminus. A 2-liter culture was grown to mid-logarithmic phase, and the pellet resuspended in three volumes of 50 mM Tris-HCl (pH 7.5). After lysis with a French press, the extract was centrifuged at 100,000 × *g* for 1 h, and the supernatant was applied to a 2.5-mL Ni²⁺-agarose column in 50 mM Tris-HCl buffer (pH 7.5). The column was washed with 10 volumes of this buffer, and then with the same buffer containing 50 mM imidazole. The enzyme was eluted with 2.5 mL of 250 mM imidazole. Protein concentrations were measured by the method of Bradford (29).

Peptidase Activity—Except where noted, hydrolysis of the various fluorogenic peptide substrates was determined by continuously monitoring the fluorescence of the formed 7-amido-4-methylcoumarin (excitation wavelength, 370 nm; emission, 480 nm). The assays were performed in 50 mM Tris-HCl buffer (pH 7.5) in a 0.75-ml final reaction volume. Peptide stocks were prepared in 100% Me₂SO, whose final concentration in the assay never exceeded 4%, which did not affect proteasome activity. The rates of substrate hydrolysis were determined during the linear portions of the curves (between 2 and 5 min).

To determine *K*ₘ for peptide aldehydes, we used the “progress curves” method, in which the inhibitor is added to the reaction at steady state (30). *K*ₘ(app) was calculated from *V/V*ₐₕ = 1 + [I]/*K*ₘ(app), where *V*ₐₕ is the rate of substrate hydrolysis in the absence and *V* is the presence of an inhibitor. The true equilibrium constant was calculated from *K*ₘ = *K*ₘ(app) /[I]ᵢₐₕ, *K* for each compound was measured with 3–6 different inhibitor concentrations in multiple experiments.

Preparation of Protein Substrates—To facilitate analysis of the peptides generated by proteolysis, 25 mg of β-casein in 2.5 mL of 0.2 M sodium borate buffer (pH 9.8) was mixed with 5 mg of FITC in 0.25 mL of ethanol. After 1 h, the modified protein was separated from the free FITC on a Sephadex G-25 column. Analysis of the absorbance spectra indicated that 5–6 FITC residues were bound to each casein molecule. The FITC-casein was lyophilized and stored at −20 °C, and before each use was repurified using the Sephadex G-25 column.

Bovine α-lactalbumin and recombinant human IGF-1 were reduced and carboxymethylated to insure denaturation and prevent sulfhydryl bond formation. The lyophilized proteins were dissolved in a degassed solution containing 40 mM dithiothreitol and 8 mM urea in 0.17 M sodium phosphate buffer (pH 7.4). The concentration of protein was adjusted to give a 5–6 mM concentration of disulfide bonds. After 30 min at room temperature, the mixture was chilled on ice, and a 2 M solution of sodium iodoacetate was added to give a final concentration of 120 mM. The reaction was performed for 1 h at 0 °C in the dark, followed by immediate removal of the urea and excess reagents with a Sephadex G-25 column (PD-10, Pharmacia Biotech Inc.). The modification was monitored by reverse-phase HPLC and by estimation of free thiol groups at stationary phase, the cells were collected by centrifugation, and the pellet resuspended in three volumes of 50 mM Tris-HCl (pH 7.5). After lysis with a French press, the extract was centrifuged at 100,000 × *g* for 1 h, and the supernatant was applied to a 2.5-mL Ni²⁺-agarose column in 50 mM Tris-HCl buffer (pH 7.5). The column was washed with 10 volumes of this buffer, and then with the same buffer containing 50 mM imidazole. The enzyme was eluted with 2.5 mL of 250 mM imidazole. Protein concentrations were measured by the method of Bradford (29).

Enzyme Purification—The Thermoplasma proteasome was purified to homogeneity by affinity chromatography on Ni²⁺-agarose from *E. coli* carrying a high copy plasmid that contains the mature wild-type α-gene and β-gene with a His₆ tag on the C terminus of its β-subunit (23). From a 2-liter culture grown on Luria Broth at 30 °C, 13 mg of the enzyme were obtained with an 80% yield of total enzyme activity, as assayed with Suc-LLVY-Amc. SDS-PAGE revealed the presence of two very closely migrating bands of approximately 25 kDa. Microsequencing showed that the first 10 N-terminal amino acids of these polypeptides matched perfectly the reported N termini of α- and β-subunits of the enzyme (15), and thus initiating methionines were quantitatively removed. On native PAGE, a single protein band was evident, which showed peptidase activity against Suc-LLVY-Amc in a gel-overlay assay (data not shown).

Activators of Peptide Hydrolysis—The Thermoplasma proteasome was purified from *E. coli* carrying a high copy plasmid that contains the mature wild-type α-gene and β-gene with a His₆ tag on the C terminus of its β-subunit (23). From a 2-liter culture grown on Luria Broth at 30 °C, 13 mg of the enzyme were obtained with an 80% yield of total enzyme activity, as assayed with Suc-LLVY-Amc. SDS-PAGE revealed the presence of two very closely migrating bands of approximately 25 kDa. Microsequencing showed that the first 10 N-terminal amino acids of these polypeptides matched perfectly the reported N termini of α- and β-subunits of the enzyme (15), and thus initiating methionines were quantitatively removed. On native PAGE, a single protein band was evident, which showed peptidase activity against Suc-LLVY-Amc in a gel-overlay assay (data not shown).

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particles behave in a similar fashion as eukaryotic proteasomes. After activation with 0.02% SDS for 10 min, a 1000-fold dilution into buffer lacking SDS immediately reduced activity by 50%, but it still exceeded that in untreated preparations. Like SDS, the chaotrope, guanidine HCl, could activate the enzyme up to 3-fold at 22 °C. Concentrations of guanidine as low as 25 mM enhanced activity, which reached a sharp maximum at 0.5 M and fell at higher concentrations. Surprisingly, the proteasome was still fully active in the presence of 3 M guanidine. By contrast, urea, for reasons that are unclear, was unable to activate the enzyme and inhibited peptidase activity above 1 M (Fig. 2b).

It appears likely that high temperatures, SDS, guanidine, and Mg2+ all stimulate peptidase activity by causing similar conformational changes in the proteasome, since no further activation was observed when these conditions were combined (e.g., SDS and high temperatures). In fact, when these stimulatory factors were combined, peptidase activity often was less than with either treatment alone. For example, although 0.02% SDS normally stimulates activity 2-fold at 22 °C, it inactivated proteasomes that had been previously activated by incubation with Mg2+ (50 mM). Additionally, following activation by incubation at 55 °C, concentrations of Mg2+, which stimulate peptidase activity (50 mM) at 22 °C, inhibited the enzyme by 30%.

**Enzyme Inhibitors**—Because the proteasome functions by a novel mechanism involving an active site threonine, we investigated the effects of several standard and novel inhibitors of the Thermoplasma proteasome. The inhibitors of metalloproteases, EDTA (5 mM) and o-phenanthroline (5 mM); of serine proteases, 1,1- tosylamido-2-phenylethyl chloromethyl ketone (1 mM) and phenylmethanesulfonyl fluoride (10 mM); and of cysteine proteases, p-hydroxy-mercuri phenylsulfonic acid (1 mM) and E-64 (50 mM); as well as the peptide aldehydes, chymostatin (50 μM), antipain (50 μM), and leupeptin (50 μM), did not reduce peptide cleavage significantly. Additionally, no activation was seen with dithiothreitol (1 mM). As reported previously (17), the enzyme was sensitive to the peptide aldehyde, Ac-LLnL-al (calpain inhibitor 1), which binds to its β-subunits at the presumed peptidase site (19). Recently, we synthesized several very potent, new peptide aldehyde inhibitors of the eukaryotic proteasome. Among them, N-benzophenone derivatives of LLnL-al or LLF-al were also potent inhibitors of the Thermoplasma proteasome (Table I). N-benzophenone-LLnL-al at 50 μM also inhibited degradation of the protein substrate, FITC-casein, by 90%. These findings resemble our observations with mammalian proteasomes, where the presence of the large hydrophobic N-benzophenone residue in the P4 position also dramatically enhanced inhibitor efficiency.

Peptide chloromethylketones irreversibly inhibit serine and cysteine proteases by covalently modifying functional groups in the active site. Although the related peptide chloromethylketones, N-benzophenone-e-biotinyl-KLLF-CH2Cl and N-benzophenone-e-biotinyl-KAAF-CH2Cl, at 50 μM reduce the peptidase activity by 70–90%, 1000-fold dilution of the enzyme into buffer fully restored the original activity. Thus, the inhibition by these chloromethylketone inhibitors of proteasomes is reversible and cannot involve alkylation of the active site (19).

DCI is an irreversible inhibitor of serine proteases, which reacts covalently with the hydroxyl group of the active site serine (33). DCI also inhibits proteasomes from mammals and archaeabacteria (17, 18) and appears to be a mechanism-based inhibitor, since Mg2+, which stimulates peptidase activity of the Thermoplasma proteasome (Fig. 1) increased similarly its susceptibility to DCI (Fig. 3). Recent studies have indicated that the hydroxyl group on the N-terminal threonine residue of the β-subunits functions as the active nucleophile in peptide bond cleavage (19, 20). To test whether this residue also reacts with DCI, the β-subunit was isolated by SDS-PAGE, and the N termini of the DCI-treated and control enzymes were sequenced by Edman degradation. In the untreated proteasome, the N-terminal sequence began with three threonine residues (Table II). By contrast, over 80% of the DCI-treated enzyme lacked the N-terminal threonine, apparently due to a chemical modification, which did allow Edman degradation of subsequent residues. Surprisingly, DCI also modified 15–20% of the second Thr. Since all subsequent residues were identical, DCI must have altered the threonine’s hydroxyl group but left the amino group intact, allowing complete Edman degradation to proceed. In addition, after treatment with DCI, SDS-PAGE revealed two high molecular weight minor bands, which seem to have resulted from cross-linking of subunits. DCI thus appears to modify active site threonine residues in a similar fashion, as lactacystin or clasto-lactacystin β-lactone (21, 22) alter the eukaryotic proteasomes β-subunits. However, when the archaeal proteasomes were incubated with either lactacystin (50 μM) or its more potent derivative, β-lactone (10 μM), at concentrations that markedly inhibit rabbit (22) or yeast (34) 20 S and 26 S particles, very little or no inhibition was observed.

**Cleavage of Different Peptide Substrates**—Proteasomes from eukaryotic organisms possess at least three distinct activities, which preferentially cleave different peptide substrates at the carboxyl side of large hydrophobic, acidic, or basic amino acids (3, 24–27). By contrast, the archaeal proteasome, which contains only one type of β-subunit, has been reported to exhibit only “chymotrypsin-like” activity against model peptides (14, 17), even though in prolonged incubations, it can cleave a large variety of peptide bond in a polypeptide substrate (28). Therefore, we have reexamined the capacity of the Thermoplasma proteasome to hydrolyze different fluorogenic peptides that are widely used to characterize the mammalian particle. In accord with prior observations, the proteasome showed appreciable “chymotrypsin-like” activity, as measured with Suc-LLVY-Amc...
tested a number of other fluorogenic peptides as substrates. The proteasome preferentially, but actually has broader specificity than reported previously. To confirm this conclusion, we added some "trypsin-like" activity was observed with Boc-LRR-Amc, which was cleaved at about half the rate of the acidic substrate. In addition, separation of the a- and b-subunits by SDS-polyacrylamide gel electrophoresis, the proteins were transferred to an Immobilon-P membrane (Millipore) and microsequenced.

**Table II**

Table of amino acids in the N termini of b-subunit after treatment of proteasome with DCI

| Position | Residue | Yield Before treatment | Yield After treatment |
|----------|---------|-----------------------|-----------------------|
| 1        | Thr     | 98                    | 18a                   |
| 2        | Thr     | 103                   | 81a                   |
| 3        | Thr     | 105                   | 98                    |
| 4        | Val     | 104                   | 102                   |
| 5        | Gly     | 95                    | 97                    |
| 6        | Ile     | 98                    | 107                   |
| 7        | Thr     | 103                   | 105                   |
| 8        | Leu     | 99                    | 97                    |
| 9        | Lys     | 102                   | 96                    |

a Different from the sequence in untreated proteasomes, which agreed exactly with published sequence.

(Fig. 4a), and at 55 °C, exhibited a $K_m = 39 \mu M$ and a $V_{max} = 28$ nmol/mg/min. Surprisingly, it also showed significant "post-glutamyl hydrolase" activities, as assayed with Z-LLE-Amc (Fig. 4b). At 100 μM, this substrate was cleaved at approximately 7–8% of the rate of the chymotryptic substrate. In addition, some "trypsin-like" activity was observed with Boc-LRR-Amc, which was cleaved at about half the rate of the acidic substrate (Fig. 4b). Thus, its active site cuts after large hydrophobic groups preferentially, but actually has broader specificity than reported previously. To confirm this conclusion, we tested a number of other fluorogenic peptides as substrates (100 μM). Appreciable activity was found with other hydrophobic peptides that are also cleaved by the "chymotryptic site" on eukaryotic particles (Table III). However, shorter peptides with a tyrosine residue in the P1 position (Suc-LY-Amc) were not degraded. Other peptides with an acidic P1 residue were also cleaved but much less rapidly than the hydrophobic peptides. Hydrolysis of other substrates with a basic P1 position was even slower. Clearly the rate of cleavage is determined not just by the P1 residue, but is also influenced by preceding residues, as was also suggested by findings with inhibitors (Table I).

**Processive Degradation of FITC-Casein**—Two methods were developed to test whether the proteasome degrades polypeptides by a processive mechanism, i.e. retains cleaved protein substrates and makes multiple cleavages before product release. To study the mode of digestion of protein substrates, a fluorescent derivative of casein was prepared, which contained 5–6 FITC residues/casein molecule. After incubation of proteasomes with this substrate, the fluorescent peptides formed were analyzed by reverse-phase HPLC. A large number of products was obtained, indicating that the enzyme cleaved the protein substrate at multiple sites (Fig. 5). These reactions were linear for at least 6 h when FITC-casein present was added in large molar excess, to ensure that the fraction of substrate consumed never exceeded 50%, as estimated by the disappearance of the substrate peak (which had a retention time of 25 min, and is not evident on the chromatograms shown). As the peptide products accumulated with longer incubation periods, the absolute levels of the different peaks increased in parallel, and the ratio of their areas did not change with time (Fig. 5). Thus, while consuming casein molecules, the proteasome generates the same characteristic pattern of products, and does not release partially digested polypeptides that are attacked at later times. In other words, the proteasome degrades casein in a processive fashion. In contrast, when similar experiments were carried out with the conventional endopeptidases, chymotrypsin or trypsin, the pattern of peptides generated varied markedly with time (Fig. 6). Thus, these enzymes initially produced fragments of FITC-casein, which were degraded further at later times, as expected for non-processive proteinases that detach from the polypeptide substrates after each cleavage.

It is also noteworthy that the spectra of peptides generated by the proteasome did differ when the particle was incubated under different conditions that enhanced peptidase activity. For example, incubation at 22 °C with SDS or guanidine HCl and incubation at 55 °C not only enhanced activity (Fig. 2), but also led to a distinct pattern of peptide products (Fig. 5). Although the precise pattern of peptides formed differed somewhat under each condition, the proteasome functioned in a processive manner in each case. Interestingly, when FITC-casein degradation was allowed to proceed overnight, so that the protein
substrates were completely consumed, the pattern of peptide products differed from those found during incubations of 15–120 min. Thus, when no protein substrate is available, the proteasome can digest further some of the peptides released earlier.

Processive Degradation of Various Proteins—Additional evidence that the proteasome functions in a highly processive manner was obtained by comparing in a single experiment the rates of disappearance of the protein substrate and the appearance of the reaction products. The amount of FITC-casein degraded was determined from the area under its peak on the HPLC, and fluorescamine was used to measure the generation of new amino groups formed as a result of cleavage of peptide bonds in the substrate. If the proteasome had initially released polypeptide intermediates that were degraded further in subsequent proteolytic rounds, then cleavage of these intermediates would have generated new amino groups without further consumption of FITC-casein. However, the ratio of new peptides generated (i.e. new amino groups) to the substrate molecule consumed did not increase during the reaction period (Fig. 7A). Thus, as peptides accumulated, they were not hydrolyzed further during incubations of up to 6 h.

To determine if these results were a specific consequence of our use of FITC-casein as the substrate, similar experiments were performed with casein reductively methylated (Me-casein) to increase the sensitivity of the fluorescamine assay. The disappearance of these Me-casein molecules coincided exactly with the appearance of new peptide products (i.e. fluorescamine-reactive residues) (Fig. 7B). These findings together with those in Fig. 5 demonstrate that the proteasome degrades different forms of casein in a highly processive fashion. To establish whether processivity is a general feature of proteasome action, additional experiments were performed with other denatured proteins as substrates, lactalbumin and insulin-like growth factor, after reduction and carboxymethylation to prevent disulfide bond formation. With these polypeptides, the rates of substrate consumption and new peptide generation were also proportional to each other at all times studied (Fig. 7, C and D). Moreover, with the proteasome, but not chymotrypsin (data not shown), the amount of peptide products was 8–25-fold higher than amount of substrate consumed (Fig. 7, A–D), indicating that multiple cuts were made in each protein molecule. Thus, the processivity is an inherent feature of the proteasome and independent of the polypeptide degraded.

To test if all of the proteasome’s active sites are essential for this processive behavior, the enzyme was incubated with the reversible inhibitor, N-benzophenone-LLnL-al, or the irreversible inhibitor, DCI. When the proteasome was competitively inhibited by N-benzophenone-LLnL-al by 50%, it continued to function in a processive manner, and the observed peptide pattern was similar to that of the untreated enzyme. Only the intensities of the peaks obtained were reduced by this agent, indicating a slower rate of protein digestion (data not shown). However, when the particle was treated with DCI (such that it lost 50% of its activity against Suc-LLVY-Amc), the pattern of peptides generated was different from that generated with the untreated enzyme. Interestingly, the DCI-treated enzyme showed a marked decrease in the amount of products that eluted in the first part of the HPLC gradient, while showing an increase in the generation of peaks that eluted later. Despite these changes in peptides generated, the new pattern was independent of the length of incubation. Thus, the DCI-treated enzyme still degraded casein in a processive fashion.
with DCI, the teolytic activity enhanced reaction with DCI, and after reaction hydroxyl group on the N-terminal threonine of the nucleophilic attack on the peptide bond is initiated by the inner chamber (19). Recent studies have suggested that the active site, which are symmetrically distributed within the unineukaryotic particle, it contains only one type of studies of the mechanism of the 20 S proteasome; for example, were 6 M and 20 M with FITC-casein (A); 22 M and 45 M with Me-casein (B); 96 M and 60 M with Me-CM-lactalbumin (C); 390 M and 80 M with Me-CM-IGF-1 (D). The substrate concentrations exceeded by at least 3-fold the estimated $S_{5,0}$ values. Newly formed amino groups were measured with fluorescamine, and the amount of substrate cleaved was calculated from the peak area on HPLC. All reactions were in 50 mM Bis-Tris propane-HCl (pH 7.5).

**DISCUSSION**

The Thermoplasma proteasome offers many advantages for studies of the mechanism of the 20 S proteasome; for example, unlike the eukaryotic particle, it contains only one type of active site, which are symmetrically distributed within the inner chamber (19). Recent studies have suggested that the nucleophilic attack on the peptide bond is initiated by the hydroxyl group on the N-terminal threonine of the $\beta$-subunit (19, 20). Further support for this mechanism comes from our finding (Table II) that DCI, which is known to react with active sites of serine proteases (33), also modifies this N-terminal threonine residue. Interestingly, mutagenesis of this threonine to a serine allows activity and enhances the sensitivity of the proteasome to DCI. Most likely, the threonine hydroxyl reacts with the heterocyclic DCI ring, which functions as a peptide analog. Accordingly, $\text{Mg}^{2+}$ concentrations that stimulated proteolytic activity enhanced reaction with DCI, and after reaction with DCI, the $\beta$-subunit was still sensitive to Edman degradation; therefore, the amino group of the threonine was not modified. Curiously, the N-terminal $\beta$-subunit of Thermoplasma (as well as eukaryotic proteasomes) contain 2 or 3 adjacent threonine residues, and DCI also modified to a limited extent the second threonine. Possibly, some DCI may be transferred from the first to the second threonine by a transesterification. Alternatively, the second threonine may also have some nucleophilic activity. Curiously, the archaeal particle was not inhibited by lactacystin or the corresponding $\beta$-lactone, which inactivates eukaryotic proteasomes by modifying this terminal threonine. Presumably, the substrate binding site of the archaeal proteasome differs from that in the eukaryotic proteasome, and these inhibitors do not react with the different mammalian $\beta$-subunits at very different rates (21).

Like serine and sulfhydryl proteases, the proteasome is sensitive to certain peptide aldehydes, such as Ac-LiLAl, which was shown to bind to the active site threonine, apparently forming a hemiacetal transition state (19). We have synthesized several much more potent peptide aldehydes inhibitors of both the archaeal (Table I) and mammalian particles. These inhibitors contain large hydrophobic residues at the P4 position. Presumably, the increasing hydrophobicity in this position allows the inhibitor to bind more effectively to the hydrophobic cleft in the active site (19). It is also noteworthy that peptide chloromethylketones, which covalently modify active sites of serine or sulfhydryl proteases, inhibit the proteasome only irreversibly. This difference is probably because the proteasome’s active site lacks a modifiable histidine or sulfhydryl residue (19).

In contrast to prior reports (17), we found that the Thermoplasma particle is relatively inactive when isolated but can be activated by ionic detergents (e.g., SDS), as has been found with eukaryotic proteasomes. The prior failure of investigators (17) to find the activation by SDS may be due to their addition of SDS to proteasome isolated from cultures grown at 70 °C, when they are already activated. The chaotropic agent, guanidine HCl, and $\text{Mg}^{2+}$ also enhance peptidase activity, and this stimulation by $\text{Mg}^{2+}$ was seen at concentrations found in cells (1–5 mM) and was completely reversed upon removal of $\text{Mg}^{2+}$. Since the peptide concentrations used were high (100 M), this activation must reflect an increase in catalytic activity ($V_{\text{max}}$), probably due to an altered conformation of the particle. Presumably, these different treatments enhance peptide hydrolysis by a similar mechanism, since their effects were not additive.

**Processivity and Sites of Cleavage**—We have demonstrated, using two very different approaches, that the 20 S proteasome, unlike traditional proteolytic enzymes, degrades proteins in a highly processive manner. This particle digests proteins to

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**FIG. 7.** The kinetics of degradation of different proteins by proteasomes at 55 °C. Dark squares, substrate degraded; open squares, peptide products. The concentrations of substrates and enzyme were 6 M and 20 M with FITC-casein (A); 22 M and 45 M with Me-casein (B); 96 M and 60 M with Me-CM-lactalbumin (C); 390 M and 80 M with Me-CM-IGF-1 (D). The substrate concentrations exceeded by at least 3-fold the estimated $S_{5,0}$ values. Newly formed amino groups were measured with fluorescamine, and the amount of substrate cleaved was calculated from the peak area on HPLC. All reactions were in 50 mM Bis-Tris propane-HCl (pH 7.5).

**FIG. 8.** Pattern of fluorescent peptides generated from FITC-casein (10 M) at 55 °C by control proteasomes (0.2 M (a) and by proteasomes (0.2 M) partially inactivated with 1 mM DCI (b). After modification, the excess inhibitor was removed by Centricon ultrafiltration. The modified enzyme had 50% of the control activity against Suc-LLVY-Amc.

$^2$ T. N. Akopian, B. Gilbert, R. Rando, and A. L. Goldberg, manuscript in preparation.
small peptides before attacking another protein molecule. All experiments were carried out with a large substrate excess, and degradation occurred at linear rates (as measured by either loss of substrate or appearance of products). Under these conditions, the proteins were digested preferentially over peptides produced by the particle, and the generation of peptide products occurred with identical kinetics to the disappearance of the four protein substrates. In very long incubations (>20 h), when the protein substrate was depleted, these peptide products could be digested further (data not shown). However, in vivo, peptides released by proteasomes never accumulate, because of rapid hydrolysis to amino acids by cytosolic peptidases.

The absence of polypeptide intermediates in proteolysis suggests that the proteasome cleaves the protein substrate at multiple sites like a bread-cutter without the release of partially degraded intermediates, although such intermediates may be generated and channelled to other active sites within the proteasome without release, as suggested by Dick et al. (35) from studies of insulin B-chain degradation. Certain structural features of the proteasome may contribute to this processivity. For example, to reach the proteolytic sites, substrates must traverse the very narrow opening in the α-ring, the outer chambers, and the narrow opening to the inner chamber, all of which may also prevent polypeptide release during the course of proteolysis. These findings have been obtained with denatured, relatively small proteins (8, 14, or 24 kDa), and it remains possible that with larger proteins with greater tertiary structure, polypeptide fragments may be released, as occurs during the processing of NF-κB p105 (36) and perhaps β-galactosidase by the 26 S proteasome (1).

The factors that determine the sites of cleavage of a protein in such a mechanism are complex. One determinant is the specificity of the active sites, which clearly prefer large hydrophobic residues in the P1 position. Although the archaeal proteasome was reported to be exclusively “chymotrypsin-like” (14, 17), we also found significant activity against some peptides with acidic or basic residues in the P1 position, which were cleaved at 5–10% of the rate of the most rapidly digested substrate (Suc-LLVY-Arg). Our results thus are consistent with the ability of these particles to cleave a wide variety of peptide bonds in polypeptides (28). Moreover, the residues distant from the P1 position seem to influence cleavage rates. Our finding that hydrophobic residues in the P4 position greatly enhance binding of peptide aldehyde inhibitors strongly suggests that a hydrophobic residue in this position stimulates substrate binding to the catalytic site. 2

Another important determinant of cleavage sites must be the distance between the active sites within the central chamber. Of particular interest was the finding that DCI-treated proteasomes, which contain statistically half the number of active sites, generated a smaller variety of peptide products from FITC-casein than the normal particles. Thus, the number of active sites seems to influence the pattern of peptides generated. Presumably, the products of the DCI-treated particles are on the average larger than normal, because the average distances between active sites are greater. Analysis of the sizes of these various peptides should critically test this hypothesis. By contrast, the competitive inhibitor, N-Benzohexenone-LLeu-al, which should not decrease the number of active sites, but reduced their efficiency, did not alter the products generated. Another determinant of peptide size and number should be the transition time of substrates within the central proteolytic chamber. Accordingly, conditions that enhance peptidase activity (e.g., SDS, guanidine HCl, 55 °C) all altered the pattern of peptide produced. Incidentally, many studies with mammalian proteasomes have utilized SDS-activated proteasomes, on the assumption that they function in an identical manner to native particles. This assumption is clearly not valid for the archaeal proteasome.

Using similar approaches, we have found that the 20 S and 26 S mammalian proteasomes also function in a processive fashion. 3 There has been appreciable interest in the nature of the peptides released by mammalian proteasomes, because of their role in the generation of antigenic peptides presented on major histocompatibility complex class I molecules, and because γ-interferon alters its subunit composition and peptidase activities (1, 37, 38). In degrading a protein processively, the proteasome releases a characteristic set of peptides in stoichiometric amounts. Consequently, any peptide epitope appropriate for antigen presentation will be generated at a rate proportional to the rate of breakdown of that protein, as seems to occur in vivo (39). The generation of antigenic peptides would be a much less reliable process if they were produced by a non-processive mechanism involving multiple cleavages by conventional proteases.

It appears likely that processivity is a characteristic feature of intracellular proteolysis that has important consequences. For example, complete degradation of proteins can explain why in vivo, despite the continuous turnover of cell proteins, partially digested proteins do not accumulate in cells. Although not a property of any known extracellular proteins, processive behavior has previously been documented for two ATP-dependent proteases, La (Lon) and ClpAP (Ti) (40–42), both of which function in intracellular proteolysis in E. coli. With protease La or ClpAP, processivity is linked to ATP hydrolysis. However, processivity also is an inherent feature of the 20 S proteasome, which does not require ATP hydrolysis or additional proteins.

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REFERENCES
1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
2. Wilk, S. (ed) (1994) Enzyme and Protein, Vol. 47, pp. 187–369, S. Karger, Basel, Switzerland
3. Orlowski, M. (1990) Biochemistry 29, 10989–10997
4. Rivett, A. J., Savory, P., and Djaballah, H. (1994) Methods Enzymol. 244, 331–350
5. Rubin, D., and Finley, D. (1995) Curr. Biol. 5, 854–858
6. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 6, 761–807
7. Hochstrasser, M. (1995) Curr. Opin. Cell Biol. 7, 215–223
8. Rechsteiner, M. (1991) Cell 66, 615–618
9. Peters, J.-M. (1994) Trends Biochem. Sci. 19, 377–382
10. Goldberg, A. L. (1995) Science 268, 522–523
11. Ciechanover, A. (1994) Cell 79, 13–21
12. Kleinschmidt, J., Hugle, B., Grund, C., and Franke, W. (1983) Eur. J. Cell Biol. 32, 143–156
13. Baumeister, W., Dahlmann, B., Hegerl, R., Kopp, F., Kuehn, L., and Pfeifer, G. (1988) FEBS Lett. 241, 239–245
14. Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., and Baumeister, W. (1989) FEBS Lett. 251, 125–131
15. Zweck, P., Gruzi, A., Puhler, G., Dahlmann, B., Lottspeich, F., and Baumeister, W. (1992) Biochemistry 31, 964–972
16. Lupas, A., Kuster, A., and Baumeister, W. (1993) Proc. Natl. Acad. Sci. 90, 7273–7276
17. Dahlmann, B., Kuehn, L., Gruzi, A., Zweck, P., and Baumeister, W. (1992) Eur. J. Biochem. 208, 789–797
18. Orlowski, M., Cardozo, C., and Michaud, C. (1993) Biochemistry 32, 1563–1572
19. Lowe, J., Stock, D., Jap, B., Zweck, P., Baumeister, W., and Huber, R. (1995) Science 265, 533–539
20. Seemuller, E., Lupas, A., Stock, D., Lowe, J., Huber, R., and Baumeister, W. (1995) Science 268, 579–582
21. Feng, W., Standaert, R. R., Lane, W. S., Chai, S., Carey, E. J., and Schreiber S. L. (1995) Science 268, 726–731
22. Dick, L. R., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L., and Stein, R. L. (1996) J. Biol. Chem. 271, 7275–7276
23. Zweck, P., Lottspeich, F., and Baumeister, W. (1992) FEBS Lett. 312, 157–160
24. Orlowski, M., and Michaud, C. (1989) Biochemistry 28, 9270–9278

3 T. N. Akopian, A. F. Kisseliev, and A. L. Goldberg, unpublished observations.
25. Yu, B., Pereira, M. E., and Wilk, S. (1993) J. Biol. Chem. 268, 2029–2036
26. Figueiredo-Pereira, M., Chen, W.-E., Yuan, H.-M., and Wilk, S. (1995) Arch. Biochem. Biophys. 317, 69–78
27. Djaballah, H., Rowe, A., Harding, S., and Rivett, J. (1993) Eur. J. Biochem. 292, 857–862
28. Wenzel, T., Eckerskorn, G., Lottspeich, F., and Baumeister, W. (1994) FEBS Lett. 349, 205–209
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Henderson, P. (1972) Biochem. J. 127, 321–328
31. Rypniewski, W. R., Holden, H. M., and Raymant, I. (1993) Biochemistry 32, 9851–9858
32. Udenfriend, S., Stein, S., Bohlen, I., Dairman, W., Leimgruber, W., and Weigle, M. (1972) Science 178, 871–872
33. Harper, W., Hemmi, K., and Powers, J. (1985) Biochemistry 24, 1831–1840
34. Lee, D. H., and Goldberg, A. L. (1996) J. Biol. Chem. 271, 27280–27284
35. Dick, L., Moonaw, C., DeMartino, G., and Slaughter, C. (1991) Biochemistry 30, 2725–2734
36. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
37. Heemels, M., and Ploegh, H. (1995) Annu. Rev. Biochem. 64, 463–491
38. Rock, K., Gram, C., Rothstein, L., Clerk, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771
39. Grant, E., Michalek, M., Goldberg, A. L., and Rock, K. (1995) J. Immunol. 155, 3750–3758
40. Goldberg, A. L. (1992) Eur. J. Biochem. 203, 9–23
41. Maurizi, M. (1992) Experientia (Basel) 48, 178–201
42. Thompson, M. W., Singh, S. K., and Maurizi, M. R. (1994) J. Biol. Chem. 269, 18209–18215

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