The proteasome from *Thermoplasma acidophilum* is neither a cysteine nor a serine protease

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Abstract **The 20 S proteasome, found in eukaryotes and in the archaebacterium *Thermoplasma acidophilum*, forms the proteolytic core of the 26 S proteasome which is the central protease of the non-lysosomal protein degradation pathway. Inhibitor studies have indicated that the 20 S proteasome may be an unusual type of cysteine or serine protease and a recent study of the *Thermoplasma* β subunit has indicated that it carries the proteolytic activity. We have attempted to obtain information on the nature of the active site by mutating the only cysteine, both histidines and two completely conserved aspartates in the archaebacterial complex as well as all serines of the β subunit, without decreasing the catalytic activity of the enzyme to any significant extent. Indeed, mutation of the conserved aspartate in the β subunit increased the activity of the proteasome threefold. We conclude that the proteasome is not a cysteine or serine protease.**

Key words: Proteasome; Proteolysis; Archaebacterium; *Thermoplasma acidophilum*

1. Introduction

The 26 S proteasome is the central protease of the ubiquitin-dependent pathway of protein degradation and has recently been shown to play an important role in MHC class I-linked antigen display. The core of the elongated 45 nm molecule is formed by the multicatalytic proteinase or 20 S proteasome, a barrel-shaped complex consisting of four seven-membered rings (reviewed in [1–3]). The rings are formed by 14 related but different subunits that fall into two families, α-type subunits forming the outer and β-type subunits the inner rings of the complex. 20 S proteasomes, ubiquitous in eukaryotes, have also been found in the archaebacterium *Thermoplasma acidophilum*, where the complex is made up of only two proteins, α and β, that have given their names to the eukaryotic subunit families.

The role of the 26 S proteasome as the central enzyme of the non-lysosomal protein degradation pathway has generated considerable interest in the activity and regulation of the 20 S core complex. The latter was initially characterized from bovine pituitary as a multicatalytic protease with chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolase activities [4], and has more recently been proposed to contain up to five different proteolytic components on the basis of inhibitor studies [5]. Yet, despite intensive biochemical efforts, the nature of the proteasome active-site(s) remains unknown. The pH optimum of the proteasome tends to be slightly basic, making it unlikely that the enzyme is an aspartic protease [6]. Similarly, no inhibition is observable by up to 10 mM EDTA, by other chelators, or by phosphoramidon, making it unlikely that the proteasome is a metal protease [6,7]. Inhibition experiments with various cysteine and serine protease inhibitors have yielded ambiguous results and have mostly been successful at high or very high inhibitor concentrations [5–9], generally inhibiting only one of the different hydrolytic activities and sometimes concurrently activating others [5]. On the basis of these experiments, the proteasome has been variously described as a serine or cysteine protease, with most recent opinions favoring an unusual type of serine protease [5–8].

The proteasome of *Thermoplasma* is much simpler than the eukaryotic proteasomes and has only a single, chymotrypsin-like activity, although experiments with denatured haemoglobin and insulin β-chain show that the enzyme will hydrolyze virtually any peptide bond [10]. Both subunits of the *Thermoplasma* proteasome have been cloned and overexpressed in *Escherichia coli* [11], opening the system up to easy genetic manipulation. In addition, since the *Thermoplasma* proteasome contains only one type of active site, mutations are considerably simpler to interpret than in eukaryotic proteasomes. We have therefore focused on this system in order to elucidate the nature of the proteasome active site. Although we have mutated residues in both α and β subunits, we have focused on the β subunits because these carry the active site: when expressed separately, α subunits assemble into 7-membered rings but remain inactive, while β subunits have a low but significant proteolytic activity even though they only form disordered aggregates [12].

In this communication we show that no histidine or cysteine residues are involved in the catalytic mechanism of the *Thermoplasma* proteasome. In addition we also exclude all serine residues of the β subunit as well as several conserved serines of the α subunit and the universally conserved aspartate (αD84, βD51). Indeed, mutation of the latter in the β subunit increases the activity of the complex at least threefold.

2. Materials and methods

2.1. Materials

Enzymes for DNA restriction and modification were obtained from New England Biolabs, Stratagene and USB. Oligonucleotides for inverse PCR mutagenesis were synthesized on an Applied Biosystems 380A DNA synthesizer. The synthetic fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC (7-amido-4-methylcoumarin) was obtained from Bachem, Heidelberg. For screening and propagation of plasmids, we used the *E. coli* strain XL1-Blue [13]; for expression of wild-type and mutant proteasomes *E. coli* BL21(DE3) [14]. All transformations were performed by electroporation using an *E. coli* Pulser from Bio-Rad Laboratories.

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2.2. Construction of mutants

Mutations were introduced by inverse PCR mutagenesis. Two plasmids, pT7-5-β-α and pT7-5-$\beta\Delta$-α [11], that contain the genes encoding the α and β subunits of the *T. acidophilum* proteasome, were used as templates. $\beta\Delta$ stands for a deletion of 21 5′ nucleotides coding for the β pro-region. The PCR reactions and ligations were performed as described [15], but *Pfu* polymerase (Stratagene) was used in place of *Taq* polymerase, since this enzyme generates blunt ends which can be directly ligated. Incorporation of nucleotide exchanges was confirmed by DNA sequencing.

2.3. Preparation of cell extracts for the primary screen

Cultures of transformed BL21(DE3) cells were grown in SOB medium (100 μg/ml ampicillin) to an OD_{600} of 0.8 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 5 h after induction, washed with 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA,

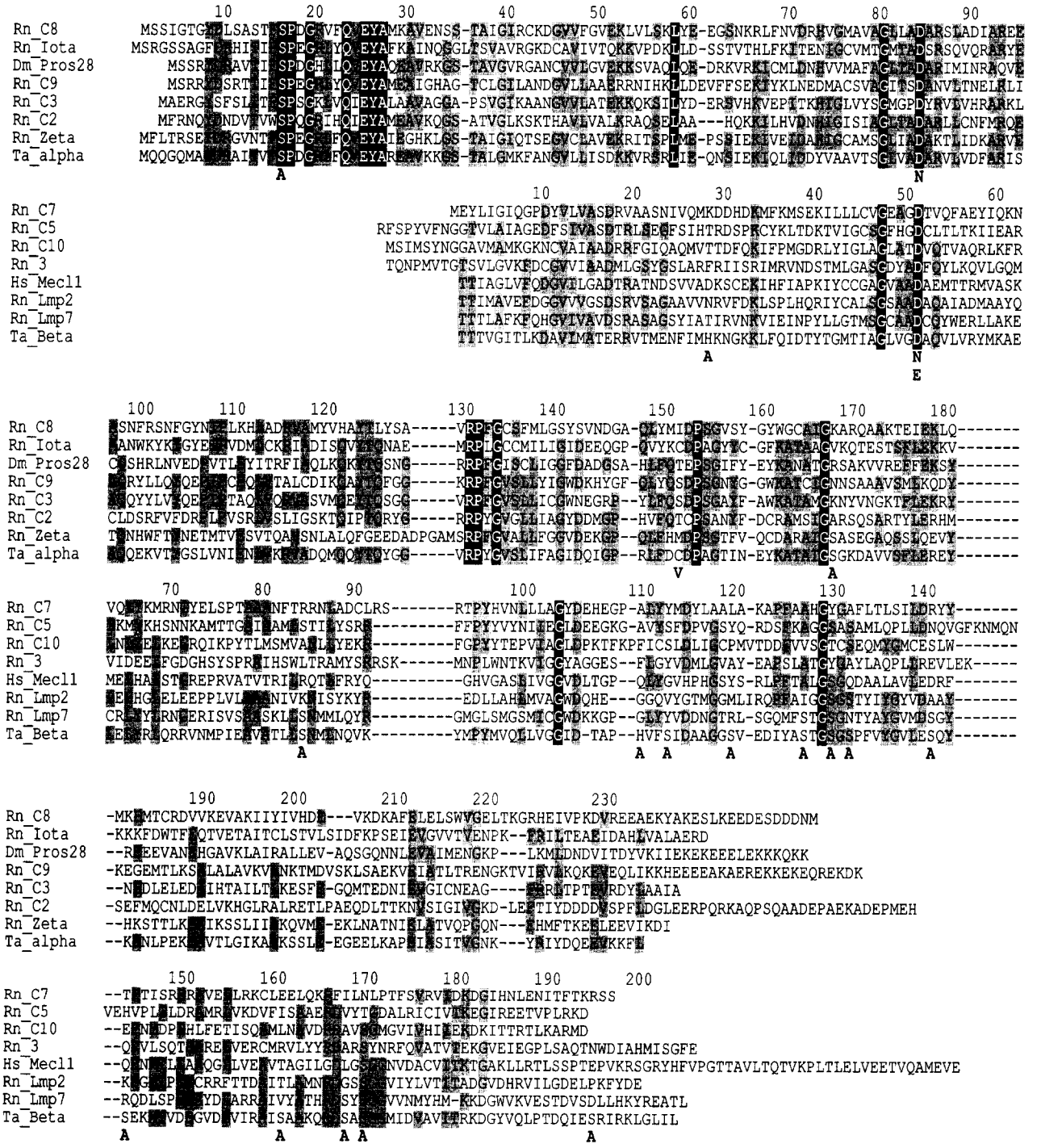

Fig. 1. Alignment of proteasome subunit sequences showing the sites of mutation. The alignment is adapted from [21]; the sequences from rat (Rn), human (Hs) and *Drosophila* (Dm) represent the seven branches of α and β subunits in eukaryotes. The numbering follows the *Thermoplasma* (Ta) sequences. Mutants are written in bold beneath the alignment. Except for subunits C7 and C10, where the cleavage site of the pro-peptide is not known, β subunits are shown in their mature form.

pH 8.0, suspended in 300 μl 20 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5 , resuspended, sonicated, heated to 85°C for 25 min and centrifuged at 12,000 × g for 10 min. Supernatants were used for assays of the proteolytic activity.

2.4. Purification of proteasomes

The isolation and purification of recombinant wild-type proteasomes and selected mutant proteasomes were carried out as described previously [16]. Protein concentrations were determined by HPLC-amino acid analysis, and samples were diluted to a final concentration of 10 μg/ml in 20 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5.

2.5. Determination of proteolytic activity and kinetic parameters

In a primary screen, the proteolytic activities of mutant proteins were compared with wild-type and with a negative control (expression strain BL21(DE3)) using crude cell extracts and Suc-Leu-Leu-Val-Tyr-AMC in 20 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5, as a substrate. 50 μl of a 400 μM substrate solution and 50 μl of crude cell extract were incubated at 60°C for 60 min. The reaction was terminated with 1 ml of 100 mM monochloroacetate and 100 mM sodium acetate, pH 4.3. Cleavage of the peptide substrate was monitored by measuring the release of AMC fluorometrically. For determination of specific activities, 50 μl of purified protein solution (10 μg/ml) and 50 μl of nine different substrate concentrations (0.01–0.7 mM) were incubated for 15 or 30 min at 60°C. K_m and V_{max} were determined graphically by plotting V vs. $V/[S]$ [17] and by direct linear plot [18], and a good agreement between the values was obtained.

2.6. Gel-electrophoresis and Western blots

Proteasomal subunits were seperated by Tricine-SDS-PAGE (16% polyacrylamide) [19], blotted onto nitrocellulose, and probed with anti-proteasome antibodies [20]. Assembly of mutant proteasomes was examined on 5% polyacrylamide gels under non-denaturing conditions followed by Western blot analysis.

3. Results

3.1. Selection of mutation sites

The active site residues in enzyme families are typically surrounded by blocks of conserved sequences, allowing the assignment of newly discovered sequences to an enzyme family and the identification of active site residues in families of enzymes not yet characterized biochemically. Such conserved regions containing amino acids with polar groups are not seen in proteasome subunits, a surprising feature of proteasome sequences being their strong divergence. The α-type subunits are distinguished from β-type subunits by a highly conserved N-terminal extension of approximately 35 residues (Fig. 1) and by a completely conserved RPxG motif. Otherwise, their sequence identity is very low and limited to only five additional positions, L58, G80, D84, P153 and G166 (all numbers refer to the *T. acidophilum* subunits). β-type subunits are even more divergent and have only four invariant residues: G47, D51, G103 and G128, of which G47, D51 and G128 are also present in α-type subunits. The hallmark of β-type subunits is a highly variable pro-sequence that may be anywhere from 4 to over 70 residues long and which is cleaved off during proteasome assembly.

In the absence of clear regions of sequence conservation, we selected residues that allowed us to address the hypothesis that the proteasome is an unusual serine or cysteine protease, and focussed in particular on the β subunit. Thus, we mutated the single cysteine, αC151, the two histidines, βH28 and βH109, all serine residues in β (βS84, βS112, βS119, βS126, βS129, βS131, βS140, βS143, βS160, βS167, βS169 and βS194), and two conserved serines in α (αS16 and αS167). In addition, we mutated the only entirely conserved potential nucleophile, aspartate αD84/βD51, in both subunits separately and jointly. All residues were mutated to alanine, except for αC151 which was mutated to valine because it was located in a predicted β-strand [21], and for the two aspartates αD84 and βD51 that were mutated to asparagine and glutamate to preserve some of the structural and electrostatic properties.

Table 1
Proteolytic assay of crude cell extracts

Mutation	Transformed plasmid	% activity
Controls	–	2
	β-α (wild-type)	100
	βΔ-α	66
β subunit mutants		
Serine → alanine	βΔS84A-α	61
	βΔS112A-α	63
	βΔS119A-α	83
	βΔS126A-α	102
	βΔS129A-α	14[a]
	βΔS131A-α	80
	βΔS140A-α	123
	βΔS143A-α	81
	βΔS160A-α	52
	βΔS167A-α	110
	βS169A-α	30[a]
	βΔS194A-α	76
	βΔS129A/S169A-α	3[a]
Histidine → alanine	βΔH28A-α	105
	βΔH109A-α	134[a]
Aspartate → asparagine	βD51N-α	295[a]
→ glutamate	βD51E-α	185[a]
α subunit mutants		
Serine → alanine	βΔ-αS16A	56
	βΔ-αS167A	55
Cysteine → valine	β-αC151V	76
Aspartate → asparagine	βΔ-αD84N	9[a]
Double (α + β) mutants		
Aspartate → asparagine	βD51N-αD84N	58[a]
Serine → alanine	βΔS129A-αS167A	35

Activities were measured by incubating 50 μl of crude cell extracts with 50 μl of substrate solution (200 μM Suc-Leu-Leu-Val-Tyr-AMC) for 60 min at 60°C. Values were not standardized for the concentration of assembled proteasomes in the extracts. Measurements were made in triplicate and contain a standard error of up to 45%. Mutants are referred to by the wild-type amino acid, followed by the residue number and followed by the mutant amino acid. β and α stand for the proteasomal genes according to their arrangement in the plasmid construct, and βΔ designates the gene deleted for the 21 nucleotides that code for the pro-region.

[a] These mutants were selected for detailed analysis (Table 2).

3.2. Construction of βΔ-α mutants

During initial screens we found that some mutations decreased the post-translational processing of the β pro-region and reduced the amount of assembled proteasomes that could be recovered. This was particularly conspicuous in the case of βS129A, where the amount of processed β subunit was significantly reduced and of proteasomes recovered was less than 10% of that recovered from a clone containing a β gene deleted for the 21 5′ nucleotides that code for the pro-region (βΔS129A-α). Only processed β subunits appear as components of intact proteasomes and the efficient removal of the pro-region might be a basic requirement for proteasome assembly. We did not investigate the effects of mutation on post-translational processing in more detail but constructed the mutants in a βΔ-α plasmid in order to overcome problems due to insufficient yield.

3.3. Screening for mutants with decreased activity

Based on earlier publications which had classified the *Thermoplasma* proteasome as chymotryptic [6] we screened mutant proteins for proteolytic activity using the chymotryptic substrate Suc-Leu-Leu-Val-Tyr-AMC. Crude cell extracts of untransformed *E. coli* BL21(DE3) served as a negative control. Table 1 summarizes activity data obtained with crude cell extracts expressed as a percentage of wild-type proteasome activity. In this initial screen, protein concentrations were estimated using Western blot analyses (data not shown), and the expression of mutant proteasomal subunits was found to be very similar to that of wild-type subunits, but the amount of assembled proteasomes was conspicuously low in some mutants despite the use of $\beta\Delta$ constructs. The four mutants with the lowest amounts of assembled proteasomes were, in order of increasing yield: $\beta\Delta$-αD84N < $\beta\Delta$S129A/S169A-α < $\beta\Delta$S129A-α < βD51N-αD84N, consistent with their low activity in proteolytic assays of crude cell extracts (Table 1). Surprisingly, mutation of the residue homologous to βS129 in α, αS167, relieved the phenotype of $\beta\Delta$S129A, yielding an increased activity for the double mutant. A similar effect was found for the mutant $\beta\Delta$-αD84N whose phenotype was relieved by mutation of the homologous residue in β, βD51.

Most other mutants characterized in the initial screen displayed activities equal to or approaching that of the wild-type enzyme within the error of the assay. Eight mutants, including the seven which showed less than 30% or more than 130% of wild-type activity (Table 1) were subjected to protein purification and detailed kinetic analysis.

3.4. Specific activities of selected mutants

Specific activity measurements of purified proteins revealed much less dramatic deviations from wild-type values (Table 2) than anticipated from the results in Table 1. Thus, the four mutants with the lowest activity in Table 1, $\beta\Delta$S129A-α, βS169A-α, $\beta\Delta$S129A/S169A-α, and $\beta\Delta$-αD84N, had 75, 55, 35 and 70% of wild-type activity, respectively. These results are consistent with our observation that unusally low concentrations of proteasomes were present in the cell extracts of these mutants. Since the mutation of residues involved in the catalytic mechanism should result in a loss of proteolytic activity, or at least in a severe decrease, we conclude that none of the residues mutated in this study participate in catalysis. Thus, we conclude that the proteasome is not a cysteine or serine protease.

Table 2
Kinetic parameters of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis

	Specific activities (%)	Kinetic parameters		
		K_m (μM)	k_{cat} ($10^{-3} \cdot s^{-1}$)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
β-α wild-type	100	85 ± 14	30 ± 2	353
Mutants				
$\beta\Delta$S129A-α	75	125 ± 41	26 ± 7	208
βS169A-α	55	75 ± 17	17 ± 3	227
$\beta\Delta$S129A/S169A-α	35	80 ± 20	10 ± 2	125
βD51N-α	330	100 ± 32	99 ± 13	990
βD51E-α	140	220 ± 60	97 ± 16	441
βD51N-αD84N	190	130 ± 21	65 ± 12	500
$\beta\Delta$-αD84N	70	70 ± 16	20 ± 2	286
$\beta\Delta$H109A-α	140	65 ± 10	37 ± 3	569

Specific activities were obtained by incubating 0.5 μg of purified protein with different substrate concentrations, as described in section 2.5. Values contain a standard error of 20–25%. Kinetic parameters were determined by direct linear plot [18]. For calculation of k_{cat}, we assumed 14 active sites per particle.

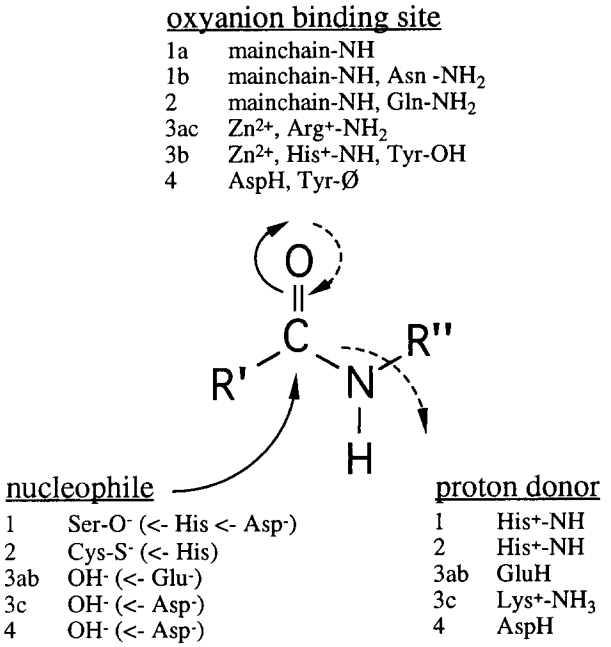

Fig. 2. General reaction mechanism of proteases. 1. Serine proteases: (a) chymotrypsin family, (b) subtilisin family. 2. Cysteine proteases. 3. Metallo-proteases: (a) carboxypeptidase A, (b) thermolysin, (c) leucine aminopeptidase. 4. Aspartic proteases.

Mutations in two residues, βS129 and βD51, appeared to cause an increase in K_m, suggesting an effect of the mutation on substrate binding or accessibility. The effect of βS129A was not observed in the double mutant $\beta\Delta$S129A/S169A-α, the reduced specific activity of which was mainly due to a decrease in V_{max}. Decreased V_{max} values were also found for mutants βS169A-α and $\beta\Delta$-αD84N. Surprisingly, some mutations appeared to cause an increase of V_{max}. Mutation of βD51, both to asparagine and to glutamate, resulted in a 3-fold increase of V_{max}. Simultaneous mutation of the homologous aspartate in α was found to reduce this increase by about 30%, consistent with its effect in mutant $\beta\Delta$-αD84N where the V_{max} is reduced by 30% relative to wild-type. A minor increase in V_{max} was also observed in the histidine mutant $\beta\Delta$H109A-α. In all four mutants with elevated V_{max}, the specific activity was higher than that of wild-type, even though in at least two mutants the K_m values had also increased.

The kinetic parameters that we measured in our proteasome preparations are significantly different from the ones reported by Dahlmann et al. [6] even when we raised the temperature of the assay to 91°C as in the other study. The reasons for this discrepancy are unclear but we note that proteasomes purified by the method of Dahlmann et al. also differ from the ones purified by our procedure in other important properties, such as their dissociation and reconstitution [22].

4. Discussion

Proteases have been classified into four families by the nature of the nucleophile attacking the peptide bond (Fig. 2) (reviewed

in [23]). Best studied are the serine proteases (trypsin, chymotrypsin, thrombin, subtilisin) in which the nucleophilic attack is made by the hydroxyl group of an active site serine, the proton of which is stripped by a charge-relay system involving a histidine and an aspartate residue. Since the structures of the chymotrypsin-like proteases are unrelated to the structure of subtilisin, it is believed that this charge-relay system has evolved at least twice independently. In a related group of enzymes, the β-lactamases, the role of the histidine is performed by a triad of serine, lysine and glutamate, while the role of the aspartate is assumed by an asparagine [24]. In cysteine proteases (papain, calpain), the nucleophilic attack is made by the thiol group of an active site cysteine, the proton of which is stripped by a histidine in what appears to be an analogue of the serine protease catalytic triad. It is not clear which residue assumes the role of the aspartate in the triad but an asparagine (papain) or a carboxyl group (viral endopeptidases) have been proposed. In metallo-proteases, the nucleophilic attack is made by the carboxyl group of a glutamate (carboxypeptidase, thermolysin, elastase) or aspartate (leucine aminopeptidase) via a bound water molecule, while the metal ion(s), typically Zn^{2+}, form the oxyanion binding site. In aspartic proteases (pepsin, renin, HIV protease), finally, the nucleophilic attack is also made by the carboxyl group of an aspartate via a bound water molecule, while a second aspartate with a shifted pK functions as the general acid. The aspartic proteases are generally monomers built of two domains with approximate twofold symmetry in which the catalytic aspartates occupy equivalent positions, but homodimeric aspartic proteases are also found in retroviruses and are believed to represent an ancestral form of the enzyme.

Because proteasomes could not be assigned to any of these four families on the basis of sequence similarity or patterns of conserved residues [25,26], a classification was attempted on the basis of inhibitor studies [5–8]. From its basic pH optimum [6] and its insensitivity to pepstatin [7], it was concluded that the proteasome is not an aspartic protease. From its insensitivity towards chelators and phosphoramidon [6,7] it was also concluded that it is not a metal protease. The inhibitors found to be effective were mainly serine or cysteine protease inhibitors although the results were generally inconclusive, as the inhibitors were required in high concentrations [5–9]. The interpretation of their effect was further obscured by the fact that the eukaryotic proteasome contains multiple proteolytic activities and inhibitors often inhibited only one of these [5]. The two inhibitor classes that proved to be of some promise were coumarins [5–8], which are serine protease inhibitors, and peptide aldehydes [5,9], which act against cysteine proteases. On the basis of these results it was proposed that the proteasome is an unusual form of serine or cysteine protease.

We have addressed this hypothesis by generating a large number of proteasome mutants in *Thermoplasma*, because in this organism the proteasome contains only one type of active site and results can therefore be interpreted more easily. We have mutated the only cysteine and both histidines in the complex and have found that these mutants had activities comparable to wild-type. We can thus rigourously exclude the possibility that the proteasome is a cysteine protease. Furthermore, we have mutated individually all serine residues in the β subunit without obtaining significant inactivation. Since the *Thermoplasma* β subunit retains residual proteolytic activity when expressed separately, while the α subunit assembles into rings but remains completely inactive [12], we can essentially exclude the possibility that the proteasome is a serine protease. Certainly we can exclude the possibility that it is a serine protease of the type containing a Ser-His-Asp catalytic triad on the basis of the histidine mutants. As a final hypothesis, we explored the possibility that the proteasome is related to aspartic proteases and that the α and β subunits are analogous to the two domains of these enzymes. In this hypothesis, the universally conserved aspartates αD84 and βD51 would correspond to the catalytic residues of the aspartic proteases. To our surprise, mutation of D84 in α subunits did not significantly affect activity, while mutation of D51 in β subunits enhanced activity threefold, clearly showing that the proteasome is also not related to aspartic proteases. While we are unable to rationalize the phenotype of the βD51 mutants at this point, the fact that two of fifteen sites mutated in β resulted in an increased V_{max} suggests that the proteasome has evolved to operate at a lower catalytic efficiency than is achievable with this type of active site.

In conclusion, we have shown that the proteasome is not a serine or cysteine protease and that it is not related mechanistically to the aspartic proteases. Combined with the complete lack of sequence relatedness between proteasomes and other proteases and with the inconclusive nature of most inhibitor studies, the results presented here indicate that the proteasome is a novel type of protease.

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