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The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria

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From the thermoacidophilic archaebacterium, *Thermoplasma acidophilum*, a proteolytically active particle has been isolated which is almost identical in size and shape with the multicatalytic proteinase (prosome) from rat. This result indicates that prosomes have been developed early in evolution and that they possibly serve functions common to all living cells.

Multicatalytic proteinase; Prosome; Archaebacteria; Electron microscopy

1. INTRODUCTION

During the past several years 19 S cylinder-shaped particles [1] or 'prosomes' [2] have been identified in various animal and plant cells [3]. Their physiological function remained enigmatic, although it was demonstrated recently [4–6] that they possess intrinsic proteolytic activity and that, by morphological, immunological and biochemical criteria, prosomes are identical with the high-molecular-mass 'multicatalytic proteinase', which is ubiquitous in eukaryotic cells [7]. Size, shape and subunit composition of this cylinder-shaped molecule appear to be highly conserved over large evolutionary distances. However, earlier attempts to demonstrate the occurrence of this particle in bacteria have failed [3,8,9]. Investigations of the molecular biology of archaebacteria provided evidence that these prokaryotic organisms have some properties reminiscent of eukaryotic cells [10,11]. We, therefore, began to search for these particles in archaebacteria and in this report we show that proteolytically active particles almost identical in shape with the rat enzyme but simpler in their subunit composition are present in the thermoacidophilic archaebacterium, *Thermoplasma acidophilum*.

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2. MATERIALS AND METHODS

For identification and isolation of proteolytic activity in *Thermoplasma acidophilum* we used essentially the same chromatographic procedure as for the multicatalytic proteinase from rat skeletal muscle [12,13]. In brief, cells were suspended in a five-fold volume (w/v) of 20 mM Tris-HCl/1 mM EDTA/1 mM NaN_3/1 mM DTT, pH 7.5 (TEAD buffer). This buffer was also used in all subsequent purification steps. Cells were broken by sonification (12 × 20 s at 0°C, using a Branson sonifier model B 12 at a power of factor 4). Particulate material was pelleted (20 min, 16000 × *g*, 4°C) and the supernatant was recentrifuged (100000 × *g*, 90 min, 6°C). To separate low-molecular-mass proteins, the 100000 × *g* supernatant was chromatographed on a column of Sepharose 6B (3 × 100 cm). Fractions (2.5 ml) were assayed with the fluorigenic substrates Bz-Val-Gly-Arg-NMec and Suc-Ala-Ala-Phe-NMec [13]. The fractions containing high-molecular-mass (500–700 kDa), proteolytically active proteins were pooled and chromatographed on DEAE-Sephacel. After washing the column with TEAD buffer, a linear salt gradient (0–500 mM NaCl) in TEAD buffer was applied. Two peaks of proteolytic activity were eluted. The first peak, eluting at 80 mM NaCl, hydrolyzed the substrate Suc-Ala-Ala-Phe-NMec, followed by a second peak, eluting at

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170 mM NaCl, with activity against both Suc-Ala-Ala-Phe-NMec and Bz-Val-Gly-Arg-NMec. The fractions of each peak were pooled separately and were further processed by fast protein liquid chromatography on Mono Q and gel filtration on Sepharose 6B as described elsewhere [13].

Two-dimensional polyacrylamide gel electrophoresis was done as follows: in the first dimension, proteins were electrophoresed at pH 8.3 [14] in a non-denaturing polyacrylamide slab-gel containing a continuous gradient (5–10%, w/v) of acrylamide. The gel was run at 13 mA for 16 h at 10°C. Thereupon, one part of the gel was stained with 0.1% (w/v) Coomassie R-250 dissolved in methanol/acetic acid/H_2O (6:1:7, v/v). For electrophoresis in the second dimension, another strip of the gel containing the *Thermoplasma* proteinase was incubated in 50 mM Tris-HCl/50 mM DTE/2% (w/v) SDS/18% (w/v) sucrose, pH 7.5, for 50 min at 37°C, before it was polymerized with stacking gel solution onto a SDS/polyacrylamide resolving slab gel with 5–18% (w/v) acrylamide continuous gradient [15]. Electrophoresis was at 8 mA for 16 h.

Electrophoretic transfer of proteins from SDS gels onto nitrocellulose membranes was carried out in a Trans-Blot cell (Bio-Rad Laboratories, München, FRG) according to the instructions of the manufacturer. After blocking non-specific binding sites with 3% (w/v) gelatine dissolved in 20 mM Tris-HCl/500 mM NaCl, pH 7.5, proteins bound to the nitrocellulose filters were incubated with an antibody to the multicatalytic proteinase from rabbit skeletal muscle, raised in sheep [16]. The antibody was dissolved in 20 mM Tris-HCl/500 mM NaCl/0.05% (w/v) Tween-20/1% (w/v) gelatine, pH 7.5, to a final concentration of 32 μg/ml and was allowed to react for 15 h at 4°C. The primary antibody was visualized with alkaline phosphatase-conjugated donkey anti-sheep IgG by the method of Blake et al. [17].

For electron microscopy, purified multicatalytic proteinase particles were stained with 2% ammonium molybdate (w/v, pH 7.5). Conditions for recording electron micrographs and image analysis were as detailed in [18].

All other experimental details were as given in legends to figures and tables.

3. RESULTS AND DISCUSSION

During chromatographic fractionation of proteolytic activity in *Thermoplasma acidophilum*, two peaks containing proteolytic activity were eluted from the DEAE-Sephacel column. Upon examination in the electron microscope, it was found that the first peak (at 80 mM NaCl) predominantly contained cylinder-shaped particles indistinguishable on 'raw' micrographs from that of the multicatalytic proteinase isolated from rat skeletal muscle (not shown). The second peak, eluting at 170 mM NaCl contained ring-shaped and other particles, all distinctly different in size and shape from the cylinder-shaped particle. Following a further purification of the proteolytic activity in the first peak by fast protein liquid chromatography and gel filtration (for details see section 2), we obtained almost exclusively cylinder-shaped particles (fig.1). We have subjected electron micrographs of this preparation to digital image analysis including image classification based on an eigenvector-eigenvalue data analysis and averaging after translational and rotational alignment [19] in order to compare the *Thermoplasma acidophilum* cylinder-shaped proteinase and the corresponding rat enzyme in greater detail. After averaging over about 400 individual molecular images we obtained side-on views almost identical in size and showing the same characteristic 'reel-shape' [18]. While the two terminal parts of the reel display precisely the same mass disposition, minor differences may be discerned in the central part of the molecule. The *Thermoplasma acidophilum* enzyme shows a clearer division into two separate rod-shaped elements, while the central part of the rat enzyme is more compact and may be described in terms of two opposing bifurcate structures. Molecules in an end-on orientation are relatively rare in the case of the enzyme preparation of *Thermoplasma*, but their proportion can be increased by adding 5–50 mM $CaCl_2$ (fig.1). Correlation averaging reveals very reproducibly (i.e. independent of the choice of the 'reference') seven centres of mass arranged on a ring at angular increments sufficiently close to seven-fold symmetry, to justify imposing this symmetry. At a first glance this seems to be opposed to the situation with the rat enzyme where, with some variability from one average to another, usually six centres of mass were revealed. However, these six mass centres never appeared equivalent nor were they related to each other by six-fold symmetry and the variability encountered with the rat enzyme may actually reflect a higher degree of heterogeneity within the molecular population under scrutiny [18]. It seems worth mentioning in this context that there is evidence for eukaryotic prosomes to be variable in their subunit composition [2,20] and that the subunit composition of the eukaryotic prosomes changes during development [21]. In spite of the subtle differences hammered out by image analysis techniques it is obvious that the vertebrate and the archaebacterial enzyme have the same basic architecture.

Upon SDS-polyacrylamide gel electrophoresis the *Thermoplasma* enzyme shows a pair of bands,

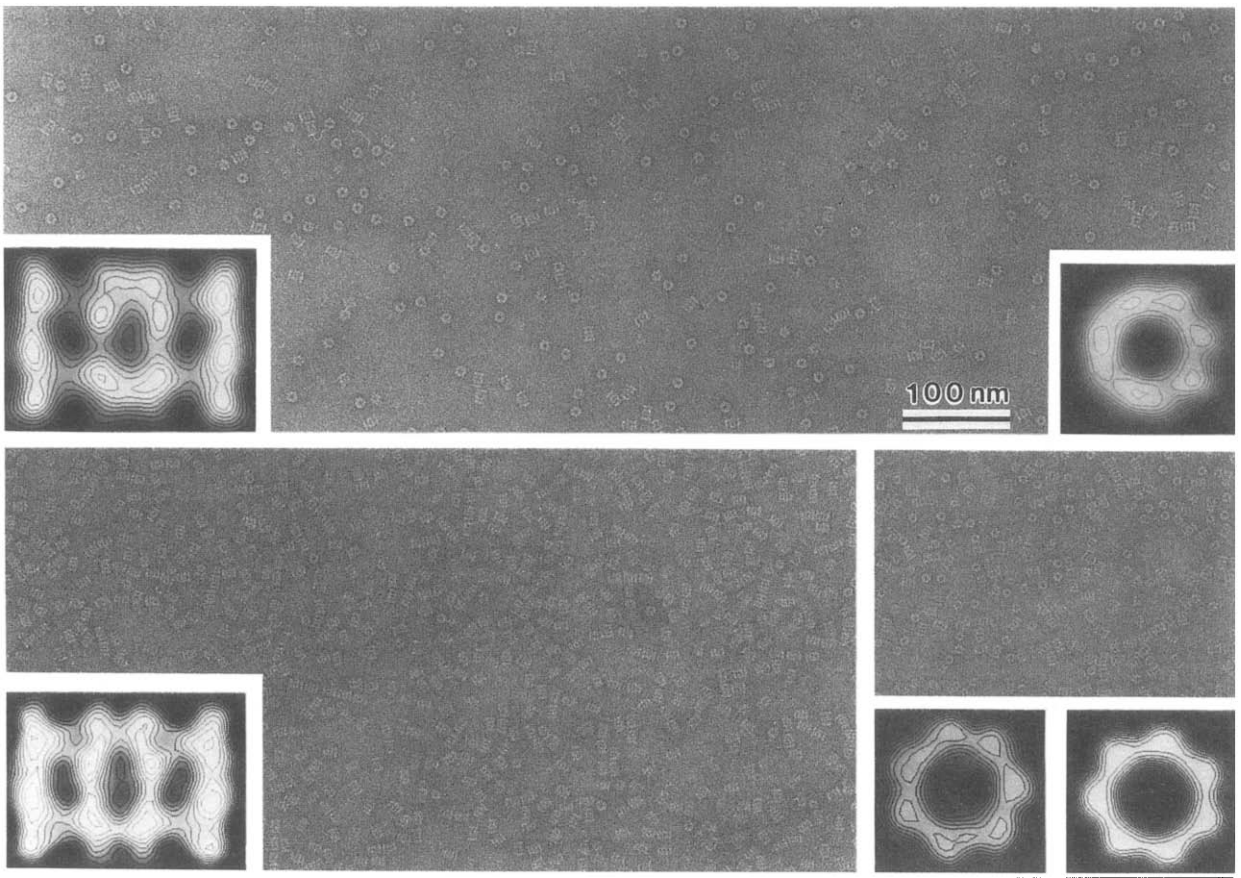

Fig.1. Electron micrographs of negatively stained (ammonium molybdate) purified multicatalytic proteinase particles seen side-on (rectangular shape) and end-on (ring-shape). (Top panel) Particles isolated from rat skeletal muscle; (insets) correlation averages of side-on views (left) and end-on views (right). (Bottom panel) Particles isolated from *Thermoplasma acidophilum*. In the absence of Ca^{2+} (left) most particles occur side-on, in the presence of Ca^{2+} (50 mM; right) more end-on views are observed. (Insets) Correlation averages of side-on and end-on orientations; the end-on view is shown without imposing any symmetry as well as after 7-fold symmetrization. Particles seen side-on have a length of approx. 15 nm and a width of approx. 11 nm. The diameter of the end-on views is close to 11 nm. For experimental details, see section 2.

while for the eukaryotic enzyme a pattern of 8–10 bands is characteristic (fig.2). Since the two subunits, designated α and β, of the archaebacterial particle assemble into a quarternary structure very similar to that of the eukaryotic enzyme, the subunits of either enzyme can be expected to share some similarity in size and shape, as well. In fact, in SDS-polyacrylamide gel electrophoresis the molecular masses of the subunits are 27 and 25 kDa for α and β, respectively, which is within the range of 25–31 kDa found for the eukaryotic enzyme.

Further investigations revealed that the subunits of the two enzymes are indeed structurally related. When purified *Thermoplasma* enzyme was subjected to SDS-polyacrylamide gel electrophoresis, transblotted onto nitrocellulose and reacted with a polyclonal antibody to the multicatalytic proteinase from rabbit skeletal muscle, the β-subunit of the archaebacterial enzyme was shown to bind this antibody, whereas no immunological reaction was detected with the α-subunit (fig.2). Limited cleavage of the two subunits with cyanogen bromide and trypsin (as well as other proteinases, not shown) resulted for each of the two subunits in a different pattern of peptide fragments (fig.2). The archaebacterial molecule thus appears to be composed of two genuinely different types of

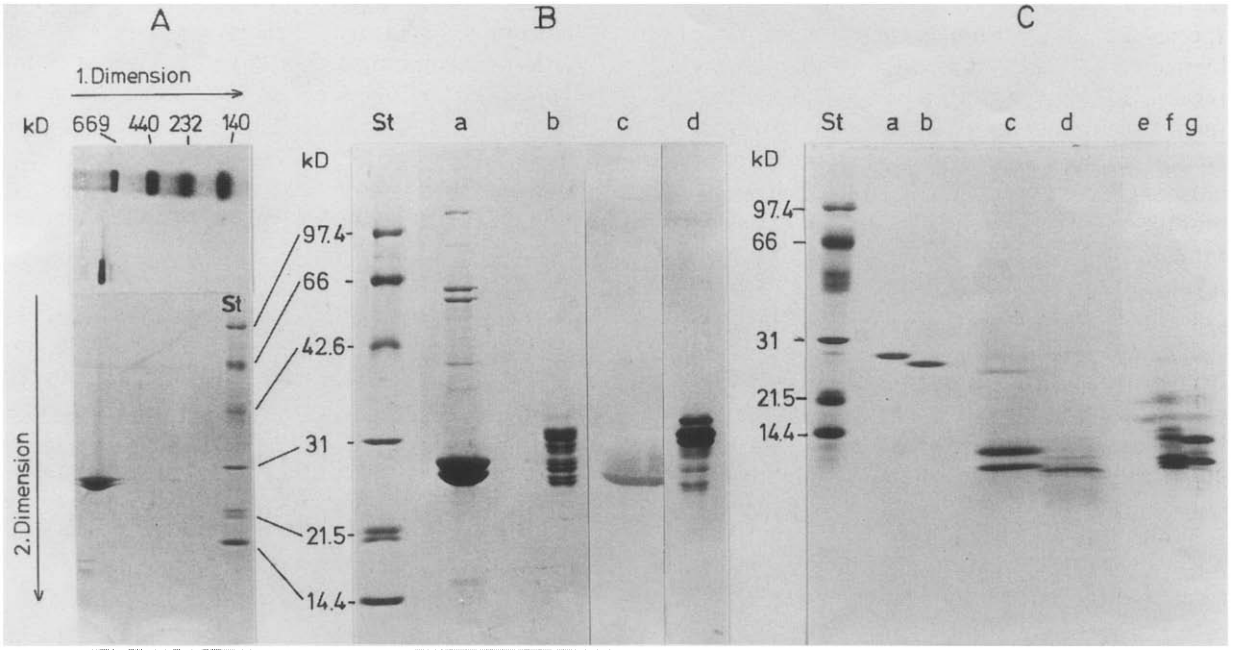

Fig.2. Two-dimensional polyacrylamide gel electrophoresis of the cylindrically shaped proteinase from *Thermoplasma* (A), its immunological reactivity with antibody to the multicatalytic proteinase from rabbit skeletal muscle (B) and peptide mapping of the isolated two subunits (α and β) of the enzyme (C). (A) Purified *Thermoplasma* proteinase (20 μg) and molecular mass standards (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa) were subjected, in the first dimension to electrophoresis in a non-denaturing polyacrylamide slab gel as described in section 2. One part of the gel was stained with 0.1% (w/v) Coomassie R-250. Another strip of the gel containing *Thermoplasma* proteinase was polymerized with stacking gel solution and resolved on SDS-polyacrylamide gradient gel as described in section 2. Electrophoresis of the proteinase as well as of molecular mass standards (St) (phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42.6 kDa; carbonic anhydrase, 31 kDa; soya bean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) in the second dimension and staining of the gel was as described in section 2. (B) 20 μg *Thermoplasma* proteinase (lanes a,c) (still containing some high molecular mass contaminants), 12 μg purified multicatalytic proteinase from rabbit skeletal muscle (lanes b,d) and molecular mass standards (St) were subjected to SDS-polyacrylamide gel electrophoresis as described above. Subsequently, the gel was cut into two parts, one of which was stained with Coomassie R-250 (lanes a,b). From the other part of the gel, proteins were transblotted onto nitrocellulose, reacted with primary antibody to multicatalytic proteinase from rabbit skeletal muscle. Primary antibody was visualized with alkaline phosphatase-conjugated second antibody as described in section 2 (lanes c,d). (C) Separation of the subunits was carried out by subjecting 86 μg of the purified proteinase to SDS-polyacrylamide gel electrophoresis on a (10–20%, w/v) acrylamide continuous gradient. The proteins were stained by Coomassie R-250 during the electrophoretic run [34]. The protein bands containing the separated subunits were excised, electrophoretically eluted from the gel and lyophilized. The dry material was dissolved in 0.1 N acetic acid and the protein precipitated by addition of ice-cold acetone, yielding 45 and 41 μg of α- and β-subunit, respectively. For cleavage of methionyl bonds 8 μg of α- and β-subunit, respectively, were treated with 10% (w/v) cyanogen bromide in 0.5 ml of 70% (v/v) formic acid at room temperature for 24 h. After addition of 5 ml H_2O the reaction mixture was lyophilized and the remaining material dissolved in 50 μl of 1 M NaOH. 7 μg of α- and β-subunit, respectively, were incubated with 1 μg of TPCK-treated bovine trypsin for 60 min at 37°C. The tryptic cleavage was stopped by boiling the mixture for 5 min. For analysis of the cleavage fragments BrCN as well as trypsin treated samples were subjected to SDS-polyacrylamide gel electrophoresis; (St) standard proteins; (lanes a,b) purified *Thermoplasma* protein subunits, α and β, respectively; (lanes c,d) α- and β-subunits after cleavage with BrCN; (lanes f,g) α- and β-subunits after cleavage with trypsin; (lane e) 1 μg bovine trypsin alone.

subunits, one of these sharing immunological epitopes with the vertebrate enzyme. Thus, the subunit composition of the archaebacterial enzyme appears to be less complex than that of the vertebrate proteinase. This can be expected to greatly facilitate further structural and functional studies.

In parallel to a reduced structural complexity the enzyme from *Thermoplasma* also shows a reduced spectrum of proteolytic activities. Different from

the multicatalytic proteinase from rat [13] or other species [22] the *Thermoplasma* proteinase does not hydrolyse peptide substrates with an arginyl residue adjacent to the leaving group. Of the different substrates tested those with an aromatic amino acid residue adjacent to the leaving group are readily hydrolysed (table 1) and at rates comparable to the hydrolysis of these substrates by the rat enzyme [4,13]. Low hydrolytic activity was also measurable with Z-Leu-Leu-Glu-2-naphthylamide and methyl-casein as substrates (data not shown). Although the assay temperature of 37°C may have been suboptimal for the activity of the archaebacterial enzyme, as has also been found for the eukaryotic enzyme [23], this temperature was chosen for comparison with the properties of the eukaryotic enzyme published previously [4,13].

While low concentrations of Ca^{2+} (5–10 mM) have a small stimulatory effect on the multicatalytic proteinase from rat skeletal muscle [24], the effect of this ion on the *Thermoplasma* proteinase is dramatic: 500 mM $CaCl_2$ leads to a 16-fold and the very high concentration of 5 M $CaCl_2$ to a 6-fold stimulation as compared to the assay without $CaCl_2$ (table 2). It should be stressed that the various Ca^{2+} concentrations have no detectable effect on the structure of the enzyme as evidenced by electron microscopic investigations. The Ca-chelating compounds, EDTA and EGTA, at 5 and 6 mM, respectively, strongly inhibit the activity of the enzyme. However, as *o*-phenanthroline, a heavy-metal chelator, has no effect on the activity of the *Thermoplasma* enzyme, we conclude that the activity of the proteinase is not dependent on a heavy-metal ion but only on Ca^{2+}.

On the other hand, the enzyme is sensitive to the action of diisopropylfluorophosphate and is partly inhibited by phenylmethylsulphonyl fluoride, indicating that a serine residue may participate in the catalysis of peptide bond hydrolysis. This property is another distinction between the archaebacterial and the eukaryotic proteinase, although some investigators have reported an inhibition of the

Table 1

Hydrolysis of synthetic substrates by purified *Thermoplasma* proteinase

Substrate	nmol/mg per min
Arg-NMec	0
Bz-Arg-NMec	0
Z-Arg-Arg-NMec	0
Z-Ala-Arg-Arg-NMec	0
Glt-Gly-Arg-NMec	0
Z-Gly-Gly-Arg-NMec	0
Bz-Val-Gly-Arg-NMec	0
Tos-Pro-Arg-NMec	0
Z-Phe-Arg-NMec	0
Bz-Phe-Val-Arg-NMec	0
Ala-Ala-Phe-NMec	0.51
Suc-Ala-Ala-Phe-NMec	0.67
Glu-Gly-Gly-Phe-NMec	0.98
Suc-Leu-Leu-Val-Tyr-NMec	1.16

For measurement of activity 0.1 ml enzyme solution (4.8 μg protein) in 20 mM Tris-HCl/1 mM EDTA/1 mM NaN_3/1 mM DTT, pH 7.5, was incubated with 100 μl of 20 μM substrate solution for 60 min at 37°C. Fluorimetric measurements of enzymatically released 4-methyl-7-coumarylamide (NMec) was performed as described elsewhere [13]

Table 2

Effect of various compounds on the activity of the *Thermoplasma* proteinase

Compound	Final concn (mM)	Activity (% of control)
Diisopropylfluorophosphate[a]	0.05	33
	0.10	0
Phenylmethylsulphonylfluoride[b]	1	73
p-Hydroxymercuri-benzene-sulphonic acid	1	97
Dithiothreitol	1	111
Cysteine	5	97
Pepstatin	0.036	100
Leupeptin	0.248	76
Chymostatin[b]	0.248	92
Antipain	0.248	99
EDTA	5	3
EGTA	6	0
o-Phenanthroline	10	126
$CaCl_2$	10	760
	50	939
	500	1686
	1000	1008
	5000	663

[a] Compound dissolved in 1.0% propanol-1 that considerably inhibits the activity. Therefore, the influence of DFP was tested in the presence of 500 mM $CaCl_2$

[b] Compound dissolved in 10% dimethylsulphoxide

Purified enzyme (2.4 μg) dissolved in 50 μl of 20 mM Tris-HCl/1 mM NaN_3/5 mM $CaCl_2$, pH 7.5, was incubated with various compounds dissolved in 50 μl of the same buffer for 10 min at 21°C. Subsequently, the activity of the enzyme was measured with the substrate Suc-Ala-Ala-Phe-NMec as described in legend to table 1

eukaryotic multicatalytic proteinase by high concentrations of diisopropylfluorophosphate [25–28]. As *p*-hydroxymercuribenzene-sulphonic acid, dithiothreitol, cysteine and pepstatin are without effect on the proteolytic activity the bacterial enzyme seems to be neither a cysteine nor an aspartic proteinase. Among the microbial proteinase inhibitors tested, only leupeptin partially inhibited the proteinase activity (table 2).

The ubiquitous occurrence of this cylindrical particle in eukaryotic cells had already prompted other investigators to search for similar molecules in prokaryotic organisms, e.g. in *Escherichia coli*, but such attempts have been unsuccessful [3,8,9]. The bacteriophage assembly protein groE, isolated from *Escherichia coli* [29,30], is another molecule which in end-on views appeared ring-shaped with a 7-fold symmetry; however, the dimensions of GroE as well as the subunit composition are clearly different from the cylinder-shaped molecule [18,31]. Only the 22 S protective antigen from the eubacterium *Bordetella pertussis* is reminiscent on electron micrographs of the structure of the multicatalytic proteinase [32]. Unfortunately, no detailed structural or biochemical analysis is available for this particle, and it is unknown whether this particle is able to catalyse peptide bond hydrolysis.

This is the first unequivocal demonstration of the cylinder-shaped, multicatalytic proteinase in prokaryotic cells. Although some features, e.g. the inability to split peptide bonds at the carboxyl side of basic amino acid residues or the enhancement of activity by Ca^{2+}, clearly distinguishes the archaebacterial from the eukaryotic enzyme, the structural similarity is striking. Further investigations covering the major ramifications of the evolutionary tree will show whether this molecule is common to all archaebacteria. Preliminary experiments with *Thermococcus celer*, an organism in a pivotal phylogenetic position amongst archaebacteria [33], have shown, that in fact the cylinder-shaped particles are also present in this organism (data not shown). In the light of our findings with archaebacteria it also needs to be reinvestigated whether the multicatalytic proteinase occurs in eubacteria.

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