Molecular architecture of the ATP-dependent CodWX protease having an N-terminal serine active site

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CodWX in *Bacillus subtilis* is an ATP-dependent, N-terminal serine protease, consisting of CodW peptidase and CodX ATPase. Here we show that CodWX is an alkaline protease and has a distinct molecular architecture. ATP hydrolysis is required for the formation of the CodWX complex and thus for its proteolytic function. Remarkably, CodX has a 'spool-like' structure that is formed by interaction of the intermediate domains of two hexameric or heptameric rings. In the CodWX complex, CodW consisting of two stacked hexameric rings (WW) binds to either or both ends of a CodX double ring (XX), forming asymmetric (WWXX) or symmetric cylindrical particles (WWXXWW). CodWX can also form an elongated particle, in which an additional CodX double ring is bound to the symmetric particle (WWXXWWXX). In addition, CodWX is capable of degrading EzrA, an inhibitor of FtsZ ring formation, implicating it in the regulation of cell division. Thus, CodWX appears to constitute a new type of protease that is distinct from other ATP-dependent proteases in its structure and proteolytic mechanism.

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Introduction

ATP-dependent proteases are responsible for the degradation of the majority of proteins in cells, including misfolded proteins and short-lived regulatory proteins, thus playing essential roles in protein quality control and regulation of a variety of cellular processes (Goldberg, 1992; Gottesman, 1996). In eukaryotes, the 26S proteasome, which consists of the 20S proteasome forming a proteolytic core and the 19S regulatory complex harboring multiple ATPases, is responsible for the degradation of most of the cell proteins that are ubiquitinated (Seufert and Jentsch, 1992; Coux *et al.*, 1996; Hochstrasser, 1996; Baumeister *et al.*, 1998). In this process, ATP is utilized in

the ubiquitylation of target proteins, unfolding of the protein substrates by the 19S complex, and translocating them into the inner proteolytic chamber of the 20S proteasome (Benaroudj and Goldberg, 2000; Benaroudj *et al.*, 2003).

Although bacteria lack the ubiquitin–proteasome pathway, they contain a number of architecturally related ATP-dependent proteases that also function in the elimination of abnormal proteins and the rapid degradation of regulatory proteins. These enzymes include the ClpAP, ClpXP and HslVU (ClpQY) proteases (Goldberg, 1992; Chung, 1993; Gottesman, 1996; Chung *et al.*, 1997). Of these, HslVU is a homolog of the eukaryotic 26S proteasome (Seemüller *et al.*, 1995; Missiakas *et al.*, 1996; Rohrwild *et al.*, 1996; Yoo *et al.*, 1996). HslU, a member of the Clp/Hsp100 family of proteins, is an ATPase and has a chaperone activity (Seol *et al.*, 1997; Neuwald *et al.*, 1999; Seong *et al.*, 2000). It interacts with and activates the proteolytic activity of HslV, which alone is a weak peptidase. Other members of the Clp/Hsp100 family include the ClpA and ClpX ATPases, which activate ClpP, their common protease (Gottesman *et al.*, 1997, 1998). These multi-component ATP-dependent proteases require ATP hydrolysis for the degradation of native, folded proteins, but not for the cleavage of small peptides and some unfolded polypeptides. Therefore, it has generally been known that the ATPase activity of the Clp/Hsp100 family members functions in unfolding of protein substrates and channeling them into the inner proteolytic chamber of their cognate proteases.

Electron micrographs of HslV have revealed that the peptidase forms a double ring of two stacked hexamers (Rohrwild *et al.*, 1997). On the other hand, HslU was shown to exist as both hexameric and heptameric rings. In the HslVU complex, HslV and HslU form a cylindrical four-ring structure, in which the HslV dodecamer is flanked at each end by a HslU ring (Kessel *et al.*, 1996; Rohrwild *et al.*, 1997; Ishikawa *et al.*, 2000). Recently, X-ray crystallographic studies have revealed that HslU is folded into three distinct domains: the N-terminal (N), intermediate (I) and C-terminal (C) domains (Bochtler *et al.*, 2000), and that in the HslVU complex, two HslU hexamers bind to both ends of the HslV dodecamer in a U₆V₆V₆U₆ configuration (Sousa *et al.*, 2000; Wang *et al.*, 2001a). These results eliminate the possibility that HslU might also exist as a heptameric form. Electron microscopic and X-ray crystallographic studies have also revealed that two ClpA or ClpX hexamers bind to both ends of the ClpP tetradecamer in a similar configuration, thus leading to a symmetric mismatch in their complex (Wang *et al.*, 1997; Beuron *et al.*, 1998; Grimaud *et al.*, 1998).

The crystal structures of the HslVU complex with respect to the orientation of the HslU ATPase recently

have been a matter of debate. In the earlier structure of HslVU (Bochtler *et al.*, 2000), the ATP-binding rings of HslU are located distal to the HslV peptidase, with the I domains bridging the interstitial space between them. In contrast, two hexameric ATP-binding rings of HslU bind intimately to opposite sides of the HslV peptidase and the HslU I-domains extend outward from the complex in the structure obtained by others (Sousa *et al.*, 2000; Wang *et al.*, 2001b). Recent biochemical evidence showing that the activation of HslV requires its binding to the C-terminal tail region of HslU located opposite the I domain and that the deletion of seven amino acids from the C-terminus prevents the formation of the HslVU complex indicates that the HslVU structure, in which HslV is contacted by HslU through the N- and C-terminal domains, is relevant (Seong *et al.*, 2002).

Both CodW and CodX in *Bacillus subtilis* display >50% identity in their amino acid sequences with HslV and HslU in *Escherichia coli*, respectively (Slack *et al.*, 1995). Recently, we have demonstrated that the CodW peptidase and the CodX ATPase can function together as a novel type of two-component ATP-dependent protease (Kang *et al.*, 2001). Significantly, CodW uses the N-terminal serine hydroxyl group as the catalytic nucleophile, unlike HslV in *E.coli* and the β -type proteasome subunits, which use the N-terminal threonine as a nucleophile (Fenteany *et al.*, 1995; Seemüller *et al.*, 1995; Yoo *et al.*, 1997b). In the present studies, we show that CodWX is a distinctive protease, not only in the use of the N-terminal serine active site but also in its molecular architecture and proteolytic mechanism, as compared with the other known ATP-dependent proteases, including HslVU.

Results

CodWX is an alkaline protease

When comparing the rate of peptide hydrolysis using *N*-carbobenzoxycarbonyl (Cbz)-Gly-Gly-Leu-7-amido-4-methylcoumarin (AMC) as a substrate, CodWX was found to cleave the substrate at pH 8 only ~20% as well as HslVU. CodWX and HslVU hydrolyzed the peptide at the rates of 26 and 131 pmol/min, respectively, upon incubation with the same amount of the enzymes (i.e. 0.1 μ g of the peptidases and 0.4 μ g of the ATPases). Since *Bacillus* is known to have numerous hydrolases with alkaline pH optima, the effect of pH on the peptide hydrolysis was examined. As shown in Figure 1A, CodWX was maximally active at pH 9.5 in the hydrolysis of both Cbz-Gly-Gly-Leu-AMC and ATP. On the other hand, HslVU showed the highest activities at pH between 8.0 and 8.5. These results indicate that CodWX is an alkaline protease. Thus, all the subsequent experiments were performed at the optimum pHs for the enzymes (i.e. at pH 9.5 using sodium borate buffer for CodWX and at pH 8.0 using HEPES buffer for HslVU).

Requirement for ATP cleavage for peptide hydrolysis by CodWX

HslVU hydrolyzes Cbz-Gly-Gly-Leu-AMC and insulin B-chain more efficiently in the presence of ATP γ S, a non-hydrolyzable ATP analog, than with ATP (Yoo *et al.*, 1997a). β,γ -imido-ATP, which also is a non-hydrolyzable ATP analog, can also support the proteolytic activity of

Fig. 1. Catalytic activities of CodWX and HslVU. (A) Hydrolysis of Cbz-Gly-Gly-Leu-AMC (left panel) and ATP (right panel) was assayed at various pHs by incubation of 0.1 μ g of CodW and 0.4 μ g of CodX (open circles) or 0.1 μ g of HslV and 0.4 μ g of HslU (filled circles) for 10 min at 37°C in the presence of 1 mM ATP as described in Materials and methods. The buffers used were 20 mM HEPES for pH from 7.0 to 8.5 and 20 mM sodium borate for pH from 8.5 to 10.5. (B) Hydrolysis of Cbz-Gly-Gly-Leu-AMC by CodWX (left panel) and HslVU (right panel) was assayed at pH 9.5 and pH 8.0, respectively, as above, but in the presence of ATP or its non-hydrolyzable analogs and in the absence (open bar) or presence of 0.2 M KCl (closed bar). The activities of the enzymes were expressed relative to those seen with ATP only, which were expressed as 100%.

HslVU better than ATP, but only in the presence of monovalent cations such as K⁺ (Huang and Goldberg, 1997). Moreover, a mutant form of HslU that is unable to cleave ATP supports the HslV-mediated hydrolysis of Cbz-Gly-Gly-Leu-AMC and insulin B-chain by forming the HslVU complex in the presence of ATP but not in its absence (Yoo *et al.*, 1998). Therefore, it has been suggested that ATP binding but not its cleavage is essential for the proteolytic activity of HslVU at least on peptides and protein substrates that do not have appreciable secondary structure. To determine whether the peptidase activity of CodWX could also be uncoupled from ATP hydrolysis, the enzyme activity was assayed in the presence of ATP analogs. As shown in Figure 1B, CodWX did not exhibit any activity in the presence of either ATP γ S or β,γ -imido-ATP whether it was treated with KCl or not. These results indicate that ATP hydrolysis is required for the peptidase activity of CodWX, unlike that of HslVU.

Oligomeric nature of CodW and CodX

X-ray crystallographic studies have revealed that HslU has a hexameric ring structure and HslV is a dodecamer consisting of two stacks of hexameric rings (Bochtler *et al.*, 2000; Sousa *et al.*, 2000; Wang *et al.*, 2001a). Gel filtration analysis under non-denaturing conditions has shown that

Fig. 2. Gel filtration analysis for oligomerization of CodX in the presence of NaCl, ATP or both. (A) Purified CodX or HslU (0.2 mg each) was incubated at 37°C for 1 h in the absence (open circles) or presence of 1 mM ATP (filled circles). After incubation, each sample was subjected to gel filtration on a Superose-6 HR 10/30 column that had been equilibrated with 20 mM sodium borate pH 9.5 for CodX or 20 mM HEPES pH 8.0 for HslU. These buffers also contained 0.1 M NaCl, 10 mM MgCl₂, 1 mM DTT and 1 mM EDTA. Fractions of 0.4 ml were collected, and aliquots of them were assayed for protein concentration. (B) CodX was chromatographed as above but using sodium borate buffer containing 0.1 or 0.3 M NaCl in the absence or presence of 1 mM ATP. Fractions were collected as above, and aliquots were subjected to SDS-PAGE followed by staining with Coomassie blue R-250. The size markers used were thyroglobulin (669 kDa), β -galactosidase (443 kDa), catalase (232 kDa) and bovine serum albumin (67 kDa).

the purified CodW has a molecular mass of ~250 kDa, corresponding to a size of a dodecamer of 20 kDa subunits (Kang *et al.*, 2001). These results imply that CodW, like HslV, is composed of two stacked hexameric rings.

In order to determine the oligomeric nature of CodX, the purified ATPase was also subjected to gel filtration on a Superose-6 column equilibrated with 0.1 M NaCl, which is a typical salt concentration used for gel filtration analysis under non-denaturing conditions. For comparison, HslU was also chromatographed under similar experimental conditions. As reported previously (Yoo *et al.*, 1996), HslU was recovered in the fractions corresponding to the size of a hexamer or heptamer (i.e. 300–350 kDa) in the presence of ATP, but was present as a monomer or dimer in its absence (Figure 2A). In contrast, CodX was eluted in the fractions corresponding to about twice the size of HslU (650–700 kDa) whether ATP is present or not, suggesting that the isolated CodX may behave as a dodecamer or tetradecamer of 52 kDa subunits.

To examine whether salt concentration might influence the oligomerization state of CodX, gel filtration was performed as above but in the presence of a higher salt concentration (i.e. 0.3 M NaCl). When ATP was present,

CodX persistently ran as a complex of 650–700 kDa whether the salt was added or not (Figure 2B). Without ATP, however, addition of 0.3 M NaCl resulted in complete dissociation of CodX into monomeric subunits. These results suggest that ionic interaction between the CodX subunits is primarily responsible for their oligomerization. ADP and non-hydrolyzable ATP analogs, including ATP γ S and β , γ -imido-ATP, also supported the oligomerization of CodX in the presence of 0.3 M NaCl, whereas AMP could not (data not shown). Thus, it appears that ATP binding, but not its hydrolysis, is required for the oligomerization of the CodX subunits.

Requirement for ATP hydrolysis for CodWX complex formation

To determine whether CodW and CodX can form a stable CodWX complex under *in vitro* conditions, cross-linking analysis was performed by incubation of CodW, CodX or both with glutaraldehyde in the absence or presence of various adenine nucleotides. The cross-linked products were then separated by SDS-PAGE on gradient gels followed by silver staining. For comparison, HslV and HslU were also treated as above (Figure 3A, left panel). Like HslV, CodW alone could form a dodecamer as well as a hexamer (Figure 3A, right panel). On the other hand, in the presence of ATP, CodX ran on the gel much more slowly than HslU. Moreover, CodX behaved as a protein even larger than the HslVU complex (V₁₂U₆ with a size of ~500 kDa). These results are consistent with the finding that CodX alone behaves on a gel filtration column as a protein of 650–700 kDa (see Figure 2). CodX also migrated much more slowly than HslU in the presence of ADP or ATP analogs (data not shown), again indicating that the binding of the adenine nucleotides is sufficient for CodX oligomerization. These results suggest that CodX is composed of two hexameric or heptameric rings, unlike HslU consisting of a single hexameric ring.

When cross-linking analysis was carried out after incubation of CodW and CodX in the presence of ATP, a protein complex that ran even more slowly than CodX appeared in the gels of SDS-PAGE (Figure 3A, right panel). The changes in the relative amounts of CodW or CodX did not show any influence on the formation of the protein complex in the presence of ATP. However, no such complex was formed when CodX and CodW were incubated in the presence of ADP or ATP γ S. β , γ -imido-ATP also could not support the formation of the large protein complex (data not shown). These results strongly suggest that the formation of the CodWX complex requires not only ATP binding but also its hydrolysis, unlike that of HslVU which requires ATP binding only. To determine whether the CodWX complex formed upon cross-linking in the presence of ATP might have proteolytic activity, the cross-linked sample just prior to loading onto the gels was assayed for the hydrolysis of Cbz-Gly-Gly-Leu-AMC. It cleaved the peptide ~85% as well as the mixture of CodW and CodX incubated without glutaraldehyde. Thus, it is likely that the CodWX complex generated by incubation of CodW and CodX is a catalytically active form.

In order to confirm whether ATP cleavage is essential for CodWX complex formation, CodW, CodX or both were incubated with ATP or ATP γ S. After incubation, the

Fig. 3. Cross-linking and gel filtration analyses for formation of CodWX and HslVU complexes in the presence of various adenine nucleotides. (A) In the left panel, HslV (2 µg), HslU (2 µg) or both were incubated in 20 mM HEPES pH 8.0 with 0.4% glutaraldehyde for 30 min at 37°C in the absence or presence of ATP, ADP or ATP_γS. In the right panel, CodW (2 µg), CodX (2 µg) or both were also incubated as above but in 20 mM sodium borate pH 9.5. After incubation, the samples were subjected to SDS-PAGE followed by silver staining. Note that, for unknown reasons, HslV and CodX were stained much more poorly than HslU and CodW, respectively, by silver nitrate. (B) CodW (50 µg) and CodX (150 µg) were incubated with 0.1 mM ATP (a) or 0.1 mM ATP_γS (b) for 30 min at 37°C. CodX with 0.1 mM ATP (c) or CodW alone (d) was also incubated for the same period. After incubation, each sample was chromatographed on a Superose-6 column that had been equilibrated with 20 mM sodium borate buffer pH 9.5 containing 10 mM MgCl₂, 1 mM EDTA and 0.1 M NaCl. Note that the elution of CodX does not exactly overlap with that of CodW, most probably due to dissociation of a part of the CodWX complex during gel filtration. Aliquots of the column fractions were subjected to SDS-PAGE on 13% slab gels. Proteins in the gels were then stained with Coomassie blue R-250.

samples were subjected to gel filtration on a Superose-6 column, followed by SDS-PAGE of the column fractions. CodW and CodX that had been incubated in the presence of ATP ran as much larger proteins (Figure 3B, a) than those incubated and chromatographed individually (Figure 3B, c and d). In contrast, CodW and CodX that had been incubated with ATP_γS behaved as if each of them was incubated and chromatographed separately (Figure 3B, b). Similar results were obtained when the

Fig. 4. Effect of Triton X-100 on formation of the CodWX complex and its peptidase activity. (A) The cross-linking analysis was performed as in Figure 3A but in the presence of ATP (left panel) and also with increasing concentrations of Triton X-100 (right panel). (B) Hydrolysis of Cbz-Gly-Gly-Leu-AMC (open circles) and ATP (filled circles) was assayed by incubation of 0.1 µg of CodW and 0.4 µg of CodX for 10 min at 37°C in the presence of 1 mM ATP and increasing concentrations of Triton X-100.

same experiments were performed upon incubation with β,γ -imido-ATP (data not shown). These results again indicate that ATP hydrolysis is required for CodWX complex formation. Thus, it appears that the inability of CodW and CodX to hydrolyze Cbz-Gly-Gly-Leu-AMC in the presence of ATP analogs (see Figure 1B) is due to their inability to form the CodWX complex without ATP hydrolysis.

Both the cross-linking and gel filtration analyses suggest that CodX behaves as a dimer of two hexameric or heptameric rings, unlike HslU consisting of a single hexameric ring. To determine whether the CodX double ring (XX) could be dissociated into two single rings (X), CodX was treated with increasing concentrations of Triton X-100 and then subjected to cross-linking analysis. At the detergent concentrations >0.01% (w/v), CodX ran on the SDS-polyacrylamide gel to the same position as HslU (Figure 4A). Moreover, a CodWX complex that has the same size as HslVU (i.e. UVV) was formed upon incubation of CodX with CodW in the presence of 0.01% Triton X-100, suggesting that the complex is composed of a CodX single ring and a CodW double ring. However, the CodWX complex (WWX) was gradually disassembled upon increasing the detergent concentration. These results suggest that a hydrophobic interaction may be responsible for the dimerization of two CodX single rings and for the interaction between CodX and CodW.

We then examined the effects of increasing concentrations of Triton X-100 on peptide hydrolysis. Figure 4B shows that the peptidase activity determined in the presence of the detergent up to 0.02% is nearly identical to that seen in its absence, suggesting that the CodWX

Fig. 5. Electron micrographs and correlation averages of negatively stained CodW. (A) The electron micrograph was obtained by negative staining of purified CodW with 2% uranyl acetate as described in Materials and methods. (B) The correlation average of 884 top-on views shows six centers of masses arranged on a ring, which form a stain-filled cavity. The diameters of the ring and central cavity are ~11 and 3 nm, respectively. (C) The average of 294 side-on views shows a two-layered structure with a dyad across the equatorial plane. The height and width of the particle are 10 and 11 nm, respectively.

complex containing a CodX single ring (WWX) is as active as that having a CodX double ring (WWXX) at least on the peptide substrate. However, the peptidase activity was gradually decreased upon increasing the detergent concentration >0.02%. Since CodX retained its single ring form (X) at all concentrations of Triton X-100 tested, it is likely that the decrease in the peptidase activity at high concentrations of Triton X-100 is due to disassembly of the CodWX complex (WWX) into a CodX single ring (X) and a CodW double ring (WW). Upon treatment with Triton X-100, the CodX double ring generated in the presence of ATP γ S could also be dissociated into single rings, but was unable to form any complex with CodW, unlike HslU (data not shown). Thus, ATP hydrolysis appears essential for CodWX complex formation also in the presence of the detergent.

Architecture of the CodWX complex

To determine the structure of the CodWX complex as well as its components, electron microscopic analysis was performed. The electron micrographs of the negatively stained CodW oligomer showed two basic views, ring-shaped and two-layered structures, depending on the different orientation on the grid (Figure 5A). A total of 884 top-on views of well-stained CodW particles were translationally and rotationally aligned, and analyzed for rotational symmetry on the basis of eigenvector-eigenvalue and correlation average (van Heel and Frank, 1981). The average image of 884 top-on views revealed an unequivocal 6-fold symmetry (Figure 5B). No other statistically significant symmetry, in particular no 7-fold rotational symmetry, could be detected. The average of

294 side-on views of CodW showed a two-layered structure with an equal distribution of mass across the equatorial plane of the oligomer (Figure 5C). In addition, the architecture and dimensions of CodW (11 nm diameter $\times$ 10 nm height) are similar to the average of the HslV dodecamer (Rohrwild *et al.*, 1997). These results suggest that CodW is a dodecamer consisting of two stacked hexameric rings with a dyad across the equatorial plane of the molecule.

The electron micrographs of the negatively stained CodX oligomer also showed two basic views, ring-shaped and striated structures, depending on the different orientation on the grid (Figure 6A). A total of 431 top-on views of well-stained CodX particles were translationally aligned and subjected to multivariate statistical analysis (van Heel and Frank, 1981). The eigenimages obtained from translationally, but not rotationally, aligned images revealed both 6-fold (Figure 6B, a, numbers 4, 5, and 7) and 7-fold rotational symmetry (numbers 8 and 9). Using the 10 most significant eigenvectors, 16 classes were discriminated according to the similarity of features after rotational alignment but without application of any symmetrization. The class averages revealed that 57% of the total particles have 6-fold rotational symmetry, while 38% show 7-fold symmetry (Figure 6B, b). The remaining particles (5%), particularly numbers 5, 12, 14 and 16, showed heterogeneous images, perhaps due to incomplete stain embedding or to an unintentional inclination during preparation or microscopy. The average images of the top-on views show six or seven centers of mass arranged on a ring with low density in its center (Figure 6C, a and b, respectively).

A total of 466 side-on views of well-stained CodX particles were subjected to rotational and translational alignment, and classified into three groups based on eigenvector-eigenvalue data analysis (Figure 6B, c). Remarkably, the projected image of the class average revealed a 'spool-like' structure, consisting of four rings (Figure 6C, c). The outer rings appear to have more mass and larger diameter (14 nm) than inner rings (12 nm). An axis of 2-fold symmetry passed through the center of the complex in this projection. These results suggest that CodX behaves as a dimer consisting of two hexameric or heptameric rings that most probably are in contact through the I domains in the center of the complex. These results are consistent with the findings that CodX behaves as a molecule with twice the size of hexameric HslU upon gel filtration and cross-linking analysis (see Figures 2 and 3).

We then examined the structure of the CodWX complex after incubation of CodW and CodX in the presence of ATP. The average images of side-on views of the CodWX complex showed at least three different types of cylindrical particles. A double ring of hexameric or heptameric CodX (XX) was flanked by a double ring of CodW hexamer (WW) on either or both sides, thus forming an asymmetric WWXX (Figure 7A, left panel) or symmetric WWXXWW configuration (middle panel). It was also found that an additional double ring of CodX is attached to the symmetric form of the CodWX complex, yielding an elongated, WWXXWWXX configuration (Figure 7A, right panel). We also determined the structure of the CodWX complex in the presence of both Triton X-100 and ATP. The average of the side-on views of the complex

Fig. 6. Electron micrographs and multivariate statistical analysis of CodX. (A) The purified CodX protein was incubated with 1 mM ATP and cross-linked by treatment with glutaraldehyde as described in Materials and methods. The electron micrograph of the protein was then obtained by negative staining with 2% uranyl acetate. The arrowheads indicate representative side-on views of CodX. (B) In (a), the average (Av) of translationally, but not rotationally, aligned 431 particles with end-on orientation and 10 most significant eigenimages (numbers 1–10) are shown. In (b), the non-symmetrized class averages (numbers 1–16) were derived from rotationally aligned images using the 10 most significant eigenvectors. The numerals shown below the class averages are the number of particles seen in each class. In (c), the non-symmetrized class averages were derived from the 466 negatively stained particles with side-on orientation. (C) The class averages of top-on views were grouped according to the similarity of rotational symmetry based on the graphs of the angular correlation coefficient, and then averaged. The averaged images of top-on views show six (a) and seven (b) centers of masses arranged on a ring with low density in its center. The numbers of particles used for averaging were 244 and 164 for hexameric and heptameric forms, respectively. The average of 466 side-on views is also shown after symmetrization (c). The diameter and height of CodX are ~14 and 17.5 nm, respectively.

revealed two different types of cylindrical particles. A double ring of CodW (WW) was flanked by a single ring of CodX (X) on either or both sides, thus forming an asymmetric WWX (Figure 7B, left panel) or symmetric XWWX configuration (right panel).

Figure 7C represents a schematic model for the assembly of the CodWX complex by the interaction of

Fig. 7. Correlation averages of the CodWX complex and their schematic presentations. (A) The average of side-on views of CodWX with WWXX (42 particles), WWXXWW (161 particles) and WWXXWWXX configurations (53 particles). (B) The average of side-on views of CodWX with WWX (188 particles) and XWWX configurations (108 particles). (C) A schematic model for the assembly of the CodWX complexes by interaction of CodW and CodX in the absence or presence of Triton X-100. Note that each single ring in CodX is drawn as a hexamer for the sake of simplicity. The open arrow indicates the lateral or apical side of the I domains as a possible site for substrate binding and entry.

CodW and CodX. The various configurations of the CodWX complex, which were determined in the absence of any detergent, are strikingly different from the structure of HslVU, ClpAP or ClpXP (Kessel *et al.*, 1996; Rohrwild *et al.*, 1997; Grimaud *et al.*, 1998; Ortega *et al.*, 2000; Ishikawa *et al.*, 2001). The novel structures of CodWX should be due to the distinctive property of CodX in the formation of a ‘spool-shaped’ double ring structure, in which the I domains are contacting each other. In contrast, the structure of CodWX seen with Triton X-100 resembles that of HslVU and other ATP-dependent proteases. Although it is unlikely that *B. subtilis* has any substance that acts like Triton, it remains to be investigated further whether the structures of the CodWX complex containing the dimeric form of CodX indeed represent the authentic form of the enzyme in the cell.

Degradation of EzrA by CodWX protease

Unlike HslVU, CodWX could not degrade Sula, a cell division inhibitor protein in *E. coli*, despite the fact that both proteases display >50% identity in amino acid sequences between their respective subunits (Kang *et al.*, 2001). However, CodW could complement HslV for the survival of *E. coli* cells under SOS conditions, indicating that CodW and HslU form a hybrid protease that is capable of degrading Sula *in vivo*. This ability of CodW to complement HslV for cell survival implicates a possible

Fig. 8. Degradation of *in vitro* translated EzrA by CodWX. (A) *In vitro* translated EzrA protein was incubated with CodW (1 μ g) in the absence or presence of CodX (4 μ g) for 1 h at 37°C. The EzrA protein was also incubated with HslV (1 μ g) and HslU (4 μ g) or with the mixture of CodW and HslU or HslV and CodX. Incubations of CodW and CodX were also performed in the presence of 0.01% Triton X-100. After incubation, the samples were subjected to SDS-PAGE as described in Materials and methods. The arrow indicates the *in vitro* translated EzrA protein, and the asterisk indicates a non-specific protein band. (B) The intensity of the EzrA protein bands was scanned using a densitometer, and expressed relative to that without the Hsl or Cod protein ('None'), which was expressed as 100%.

role for CodWX in the regulation of cell division in *B.subtilis*, like HslVU in *E.coli* (Khattar, 1997; Seong *et al.*, 1999). However, *B.subtilis* does not have any protein that has similarity in its amino acid sequence to Sula. Instead, the latter organism has a protein, called EzrA, which has been reported to function in the regulation of cell division of *B.subtilis* (Levin *et al.*, 1999).

In an attempt to elucidate the physiological role of CodWX, we examined whether CodWX could degrade EzrA. During the course of this study, we noticed that EzrA containing a membrane-binding domain is highly insoluble when overproduced in *E.coli* (data not shown). Therefore, EzrA was *in vitro* translated and used as a substrate. As shown in Figure 8, the *in vitro* translated EzrA protein was degraded by CodWX but not by CodW alone or by HslVU. The EzrA protein was also degraded by the mixture of HslV and CodX, although to an extent significantly less than that seen with CodWX. On the other hand, the mixture of CodW and HslU did not show any activity on the substrate. These results suggest that CodX, but not HslU, recognizes EzrA for degradation by either CodW or HslV. To determine whether CodWX containing the CodX single ring form (i.e. WWX and XWWX) can also degrade EzrA, the mixtures of CodW and CodX were incubated with the protein substrate in the presence of 0.01% Triton X-100. EzrA was degraded by the mixture in the presence of the detergent as well as in its absence (Figure 8, right panels). These results again indicate that CodWX containing the CodX single ring form is as active as that having the dimeric form of CodX.

Discussion

In its structure and catalytic mechanism, CodWX in *B.subtilis* appears to represent a distinctive protease among all known ATP-dependent proteases, including HslVU. (i) CodW uses its N-terminal serine hydroxyl

group as the catalytic nucleophile, unlike HslV and the β -type proteasome subunits in which the N-terminal threonine functions as a nucleophile (Seemüller *et al.*, 1995; Yoo *et al.*, 1997b; Fenteany *et al.*, 1995; Kang *et al.*, 2001). In fact, CodWX represents the first N-terminal serine protease among all known serine proteases, including Lon and Clp proteases. (ii) CodWX is an alkaline protease that is maximally active at pH 9.5 against both peptide and ATP, unlike the other ATP-dependent proteases which show their activities maximally at neutral or slightly alkaline pHs (e.g. pH 8 for HslVU). (iii) CodWX requires ATP hydrolysis for the cleavage of small peptide substrates, unlike the other ATP-dependent proteases that require only its binding. (iv) ATP hydrolysis is required for the formation of the CodWX complex, whereas ATP binding, but not its hydrolysis, is essential for the assembly of the other ATP-dependent protease complexes. (v) CodX ATPase forms a double ring structure, unlike the ATPase components of the other ATP-dependent proteases, which are composed of a single ring. Thus, CodWX appear to constitute a new type of ATP-dependent protease.

ATP hydrolysis is essential for the degradation of native, folded proteins by multicomponent ATP-dependent proteases in *E.coli* but not for that of small peptide substrates. For example, HslVU requires ATP hydrolysis for the degradation of Sula, a cell division inhibitor protein, but not for that of Cbz-Gly-Gly-Leu-AMC (Seong *et al.*, 1999). Therefore, it has been suggested that ATP cleavage is required for unfolding and channeling of native protein substrates into the inner chamber of the proteolytic core (Gottesman *et al.*, 1997; Larsen and Finley, 1997). However, CodWX requires ATP hydrolysis for the cleavage of the same peptide substrate. Moreover, ATP cleavage is also essential for the formation of the CodWX complex, whereas ATP binding is sufficient for the assembly of the Hsl and Clp proteases. In addition, CodW absolutely requires CodX for its peptidase activity, whereas HslV and ClpP by themselves are weak peptidases (Kang *et al.*, 2001). Thus, it appears that ATP hydrolysis by CodX is required not only for unfolding of protein substrates but also for induction of conformational change that is essential for its interaction with and activation of the CodW peptidase.

Electron microscopic analysis has revealed that the CodW peptidase shows a strictly 6-fold symmetry, similar to HslV hexamer but unlike ClpP and the 20S proteasome, both of which are composed of heptameric rings (Bochtler *et al.*, 1997; Groll *et al.*, 1997; Wang *et al.*, 1997). On the other hand, the CodX ATPase was found to have both 6- and 7-fold symmetry, suggesting that a single ring of CodX can exist as either a hexamer or heptamer. This observation raises the possibility of symmetric mismatch in axial stacking of the heptameric form of CodX with CodW hexamer in the CodWX complex. Electron microscopic analysis of HslU ATPase had also demonstrated that HslU could exist as either a hexameric or heptameric ring. However, recent X-ray crystallographic studies have unambiguously revealed that HslU forms a hexameric ring (Bochtler *et al.*, 2000; Sousa *et al.*, 2000; Wang *et al.*, 2001a). Therefore, a single ring of CodX may also be composed of hexamer, although clarification of the oligomeric nature of CodX awaits X-ray crystallographic analysis.

Remarkably, CodX was found to form a 'spool-like' structure that is composed of two hexameric or heptameric rings, which are in contact through the I domains in the center of the ATPase. In the CodWX complex, a double ring of CodX (XX) is flanked by a double ring of CodW (WW) on either or both sides, thus forming an asymmetric WWXX or symmetric WWXXWW configuration (see Figure 7). An elongated, WWXXWWXX configuration is also formed by the binding of an additional double ring of CodX to the symmetric form. The geometry of interaction between the ATPase and peptidase rings is crucial in specifying the positions of the sites to which protein substrates bind, where they are unfolded, and through which they are channeled into the digestion chamber inside the peptidase. Therefore, it has been suggested that the I domains exposed to the distal surfaces of the HslVU complex would be suitable for substrate binding (Ishikawa *et al.*, 2000). In addition, this proposition is consistent with reports showing that protein substrates bind to the distal surfaces of both ClpXP and ClpAP (Ortega *et al.*, 2000; Ishikawa *et al.*, 2001). However, the I domains of CodX proximally bind each other; hence, their apical regions are excluded from the solution, unlike the I domains of a single hexameric HslU ring that are entirely exposed to the solution. Thus, the most crucial question to be answered is how protein substrates can be accessed into the CodWX complex for their unfolding and subsequent degradation.

One possibility is that the dimeric structure of CodX may be generated by an artificial interaction between the apical regions of the I domains of CodX subunits under *in vitro* conditions. Therefore, under physiological conditions, a single ring form of CodX may be predominant and interact with CodW to form the WWX or XWWX structure. This possibility is supported by our finding that low concentrations of Triton X-100 dissociate the CodX double ring into single rings. However, we could not find any single ring form of CodX under all other experimental conditions tested (e.g. variation of pH and/or concentrations of the proteins, divalent cations or adenine nucleotides). In addition, it is unknown whether any substance that acts like Triton X-100 exists in *B. subtilis*. Another possibility is that the binding of protein substrates to the lateral side of the I domains may cause dissociation of the CodX double ring into single rings, thus leading to formation of the WWX or XWWX structure with CodW. However, insulin B-chain that is degraded by CodWX in the presence of ATP did not influence the formation of the CodX double ring upon cross-linking or electron microscopic analysis (data not shown).

An additional possibility is that in the CodWX complex, the lateral side of the I domains of dimeric CodX serves as the site for the binding and entry of substrates. The cross-linked CodWX complex, in which the CodX double ring cannot be dissociated into two single rings even in the presence of SDS (Figure 3A, right panel), could hydrolyze Cbz-Gly-Gly-Leu-AMC nearly as well as the CodWX complex without cross-linking. Furthermore, CodWX containing the dimeric form of CodX hydrolyzes both peptides and proteins (EzrA and insulin B-chain) as well as that containing the CodX single ring form, which can be generated upon treatment with Triton X-100, indicating that the substrates must access the lateral side of the I domains of the dimeric CodX. In addition, it has been

reported recently that protein substrates are accessible to the lateral PDZ domain of the HtrA (DegP) protease, a hexamer formed by staggered association of two trimeric rings, but not to its apical pore (Krojer *et al.*, 2002). Thus, it appears most likely that the lateral side of the I domains of CodX serve as the binding and entry sites for substrates (see Figure 7C, open arrow).

Materials and methods

Assays

Peptide hydrolysis by HslVU was assayed as described previously using Cbz-Gly-Gly-Leu-AMC as a substrate (Yoo *et al.*, 1996). For CodWX, the reaction mixtures (0.1 ml) containing appropriate amounts of the purified CodW and CodX proteins were incubated for 30 min at 37°C in 20 mM sodium borate buffer pH 9.5 containing 10 mM MgCl₂, 1 mM EDTA, 1 mM ATP, 1 mM dithiothreitol (DTT) and 0.1 M NaCl. After incubation, the samples were added with 0.1 mM of the peptide substrate and incubated further for 10 min. The reaction was stopped by adding 0.1 ml of 1% (w/v) SDS and 0.8 ml of 0.1 M sodium borate pH 9.1. The release of AMC was then measured. ATP hydrolysis was assayed by incubating similar reaction mixtures for 1 h at 37°C but in the absence of the peptide substrate. After incubation, 0.2 ml of 1% SDS was added to the samples, and the inorganic phosphates released were determined as described (Yoo *et al.*, 1996).

Cross-linking analysis

The purified HslU, HslV or both, or CodW, CodX or both were incubated in 20 mM HEPES buffer pH 8.0 or 20 mM sodium borate buffer pH 9.5, respectively, containing 10 mM MgCl₂, 1 mM EDTA and 0.4% (v/v) glutaraldehyde in the absence or presence of various adenine nucleotides in a total volume of 0.1 ml. After incubation for 30 min at 37°C, the samples were mixed with 30 µl of 0.75 M Tris-HCl pH 6.8 containing 7.5% SDS and 10% 2-mercaptoethanol. The samples were then subjected to SDS-PAGE on 3.5–8% (w/v) gradient slab gels (Laemmli, 1970). Proteins in the gels were then visualized by silver staining.

Electron microscopy

The purified CodX, CodW or both were incubated for 30 min at room temperature in 20 mM sodium borate buffer pH 9.5 containing 10 mM MgCl₂, 1 mM EDTA, 100 mM NaCl in the absence or presence of 1 mM ATP. After incubation, the samples were added with glutaraldehyde (final concentration 0.03%) and incubated further for 3 min at room temperature. The CodW (0.08 mg/ml), CodX (0.02 mg/ml) or both were then applied to glow-discharged carbon-coated copper grids. After allowing the proteins to adsorb for 1–2 min, the grids were rinsed on droplets of deionized water, and stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded with an FEI Technai 12 microscope at a magnification of ×51 600 (nominal magnification of ×52 000) and an acceleration voltage of 120 kV.

Image processing

Light-optical diffractograms were used to select the micrographs, to examine the defocus and to verify that no drift or astigmatism was present. Suitable areas were digitized as arrays of 1024 × 1024 pixels with Leaf Scan 45 at a pixel size of 20 µm, corresponding to 0.38 nm at the specimen level. For image processing, the SEMPER (Saxton *et al.*, 1979) and EM (Hegerl, 1996) software packages were used. From digitized micrographs, smaller frames of 64 × 64 pixels containing individual particles were extracted interactively. These images were aligned translationally and rotationally using standard correlation methods (Baumeister *et al.*, 1988; Kim *et al.*, 2000). An arbitrarily chosen reference was used for the first cycle of alignment and averaging, and the resulting average was used as a reference in the second refinement cycle. For analysis of the rotational symmetry of top-on view images, the individual images were aligned translationally but not rotationally (Marco *et al.*, 1994). These aligned images were subjected to multivariate statistical analysis (van Heel and Frank, 1981). The resulting eigenimages represent all important structural features of the original data set. If the images have different rotational symmetries in the original data set, the eigenimages would reveal the different symmetry axes. Moreover, these images can be distinguished and subsequently separated based on eigenimages. The rotationally aligned images were classified based on

eigenvector–eigenvalue data analysis, and subsequent averaging was performed for each class separately. The average was finally symmetrized based on angular correlation coefficients (Dürr, 1991).

Degradation of *in vitro* translated EzrA

For *in vitro* expression of the EzrA protein, the *ezrA* gene was cloned using a PCR amplification method. The PCR products were ligated into pGEM-T vectors, and the resulting recombinant plasmids were referred to as pGEM/EzrA. The EzrA protein was *in vitro* translated upon incubation of pGEM/EzrA with [³⁵S]methionine and using an *in vitro* transcription and translation kit (TNT T7-coupled rabbit reticulocyte lysates, Promega), according to the supplier's instructions. The purified CodW (1 µg) and CodX (4 µg) proteins were incubated in the presence of ATP for 30 min at 37°C in 20 mM sodium borate buffer pH 9.5 containing 10 mM MgCl₂ and 1 mM EDTA. After incubation, the samples were added with 10 µl of the *in vitro* translated EzrA protein in a total volume of 0.1 ml and incubated further for 2 h. The reaction was stopped by mixing with 25 µl of 0.75 M Tris–HCl pH 6.8 containing 7.5% SDS and 10% 2-mercaptoethanol. The samples were then subjected to SDS–PAGE on 12% slab gels and visualized by autoradiography.

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References

- Baumeister, W., Dahlmann, B., Hegerl, R., Kopp, F., Luehn, L. and Pfeifer, G. (1988) Electron microscopy and image analysis of the multicatalytic proteinase. *FEBS Lett.*, **241**, 239–245.
- Baumeister, W., Walz, J., Zuhl, F. and Seemüller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease. *Cell*, **92**, 367–380.
- Benaroudj, N. and Goldberg, A.L. (2000) PAN, the proteasome-activating nucleotidase from archaeobacteria, is a protein-unfolding molecular chaperone. *Nat. Cell Biol.*, **2**, 833–839.
- Benaroudj, N., Zwickl, P., Seemüller, E., Baumeister, W. and Goldberg, A.L. (2003) ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol. Cell*, **11**, 69–78.
- Beuron, F., Maurizi, M.R., Belnap, D.M., Kocsis, E., Booy, F.P., Kessel, M. and Steven, A.C. (1998) At sixes and sevens: characterization of the symmetry mismatch of the ClpAP chaperone-assisted protease. *J. Struct. Biol.*, **123**, 248–259.
- Bochtler, M., Ditzel, L., Groll, M. and Huber, R. (1997) Crystal structure of heat shock locus V (HslV) from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **94**, 6070–6074.
- Bochtler, M., Hartmann, C., Song, H.K., Bourenkov, G.P., Bartunik, H.D. and Huber, R. (2000) The structures of HslU and the ATP-dependent protease HslIU–HslIV. *Nature*, **403**, 800–805.
- Chung, C.H. (1993) Proteases in *Escherichia coli*. *Science*, **262**, 372–374.
- Chung, C.H., Yoo, S.J., Seol, J.H. and Kang, M.S. (1997) Characterization of energy-dependent proteases in bacteria. *Biochem. Biophys. Res. Commun.*, **241**, 613–616.
- Coux, O., Tanaka, K. and Goldberg, A.L. (1996) Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.*, **65**, 801–847.
- Dürr, R. (1991) Displacement field analysis: calculation of distortion measures from displacement maps. *Ultramicroscopy*, **38**, 135–141.
- Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. and Schreiber, S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*, **268**, 726–731.
- Goldberg, A.L. (1992) The mechanism and function of ATP-dependent protease in bacterial and animal cells. *Eur. J. Biochem.*, **203**, 9–23.
- Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.*, **30**, 465–506.
- Gottesman, S., Maurizi, M.R. and Wickner, S. (1997) Regulatory subunits of energy-dependent proteases. *Cell*, **91**, 435–438.
- Gottesman, S., Roche, E., Zhou, Y. and Sauer, R.T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.*, **12**, 1338–1347.
- Grimaud, R., Kessel, M., Beuron, F., Steven, A.C. and Maurizi, M.R. (1998) Enzymatic and structural similarities between the *Escherichia coli* ATP-dependent proteases, ClpXP and ClpAP. *J. Biol. Chem.*, **273**, 12476–12481.
- Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H.D. and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature*, **386**, 463–471.
- Hegerl, R. (1996) The EM program package: a platform for image processing in biological electron microscopy. *J. Struct. Biol.*, **116**, 30–34.
- Hochstrasser, M. (1996) Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.*, **30**, 405–439.
- Huang, H.C. and Goldberg, A.L. (1997) Proteolytic activity of the ATP-dependent protease HslVU can be uncoupled from ATP hydrolysis. *J. Biol. Chem.*, **272**, 21364–21372.
- Ishikawa, T., Maurizi, M.R., Belnap, D. and Steven, A.C. (2000) Docking of components in a bacterial complex. *Nature*, **408**, 667–668.
- Ishikawa, T., Beuron, F., Kessel, M., Wickner, S., Maurizi, M.R. and Steven, A.C. (2001) Translocation pathway of protein substrates in ClpAP protease. *Proc. Natl Acad. Sci. USA*, **98**, 4328–4333.
- Kang, M.S., Lim, B.K., Seong, I.S., Seol, J.H., Tanahashi, N., Tanaka, K. and Chung, C.H. (2001) The ATP-dependent CodWX (HslVU) protease in *Bacillus subtilis* is an N-terminal serine protease. *EMBO J.*, **20**, 734–742.
- Kessel, M., Wu, W., Gottesman, S., Kocsis, E., Steven, A.C. and Maurizi, M.R. (1996) Six-fold rotational symmetry of ClpQ, the *E. coli* homolog of the 20S proteasome and its ATP-dependent activator, ClpY. *FEBS Lett.*, **398**, 274–278.
- Khattar, M.M. (1997) Overexpression of the *hslVU* operon suppresses SOS-mediated inhibition of cell division in *Escherichia coli*. *FEBS Lett.*, **414**, 402–404.
- Kim, K.I., Cheong, G.W., Park, S.C., Ha, J.S., Woo, K.M., Choi, S.J. and Chung, C.H. (2000) Heptameric ring structure of the heat-shock protein ClpB, a protein-activated ATPase in *Escherichia coli*. *J. Mol. Biol.*, **303**, 655–666.
- Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M. and Clausen, T. (2002) Crystal structure of DegP (HtrA) reveals a new protease–chaperone machine. *Nature*, **416**, 455–459.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Larsen, N.C. and Finley, D. (1997) Protein translocation channels in the proteasome and other proteases. *Cell*, **91**, 431–434.
- Levin, P.A., Kurtser, I.G. and Grossman, A.D. (1999) Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA*, **96**, 9642–9647.
- Marco, S., Urena, D., Carrascosa, J.L., Waldmann, T., Peter, J., Hegerl, R., Pfeifer, G., Sack-Kongehl, H. and Baumeister, W. (1994) The molecular chaperone TF55: assessment of symmetry. *FEBS Lett.*, **341**, 152–155.
- Missiakas, D., Schwager, F., Betton, J.M., Georgopoulos, C. and Raina, S. (1996) Identification and characterization of HslV HslU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherichia coli*. *EMBO J.*, **15**, 6899–6909.
- Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA+: a class of chaperone-like ATPases associated with the assembly, operation and disassembly of protein complexes. *Genome Res.*, **9**, 27–43.
- Ortega, J., Singh, S.K., Ishikawa, T., Maurizi, M.R. and Steven, A.C. (2000) Visualization of substrate binding and translocation by the ATP-dependent protease, ClpXP. *Mol. Cell*, **6**, 1515–1521.
- Rohrwild, M., Coux, O., Huang, H.C., Moerschell, R.P., Yoo, S.J., Seol, J.H., Chung, C.H. and Goldberg, A.L. (1996) HslV–HslU: a novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc. Natl Acad. Sci. USA*, **93**, 5808–5813.
- Rohrwild, M., Pfeifer, G., Santarius, U., Müller, S.A., Huang, H.C., Engel, A., Baumeister, W. and Goldberg, A.L. (1997) The ATP-dependent HslVU protease from *Escherichia coli* is a four-ring structure resembling the proteasome. *Nat. Struct. Biol.*, **4**, 133–139.
- Saxton, W.O., Pitt, T.J. and Horner, M. (1979) Digital image processing: the Semper system. *Ultramicroscopy*, **4**, 343–354.
- Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R. and Baumeister, W. (1995) Proteasome from *Thermoplasma acidophilum*: a threonine protease. *Science*, **268**, 579–582.
- Seol, J.H., Yoo, S.J., Shin, D.H., Shim, Y.K., Kang, M.S., Goldberg, A.L. and Chung, C.H. (1997) The heat-shock protein HslVU from *Escherichia coli* is a protein-activated ATPase as well as an ATP-dependent proteinase. *Eur. J. Biochem.*, **247**, 1143–1150.
- Seong, I.S., Oh, J.Y., Yoo, S.J., Seol, J.H. and Chung, C.H. (1999)

- ATP-dependent degradation of SulA, a cell division inhibitor, by the HslVU protease in *Escherichia coli*. *FEBS Lett.*, **456**, 211–214.
- Seong,I.S., Oh,J.Y., Lee,J.W., Tanaka,K. and Chung,C.H. (2000) The HslU ATPase acts as a molecular chaperone in prevention of aggregation of SulA, an inhibitor of cell division in *Escherichia coli*. *FEBS Lett.*, **477**, 224–228.
- Seong,I.S., Kang,M.S., Choi,M.K., Lee,J.W., Koh,O.J., Wang,J., Eom,S.H. and Chung,C.H. (2002) The C-terminal tails of HslU ATPase act as a molecular switch for activation of HslV peptidase. *J. Biol. Chem.*, **277**, 25976–25982.
- Seufert,W. and Jentsch,S. (1992) *In vivo* function of the proteasome in the ubiquitin pathway. *EMBO J.*, **11**, 3077–3080.
- Slack,F.J., Serrero,P., Joyce,E. and Sonenshein,A.L. (1995) A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *Mol. Microbiol.*, **15**, 689–702.
- Sousa,M.C., Trame,C.B., Tsuruta,S., Wilbanks,S.M., Reddy,R.S. and McKay,D. (2000) Crystal and solution structures of an HslUV protease–chaperone complex. *Cell*, **103**, 633–643.
- van Heel,M. and Frank,J. (1981) Use of multivariate statistics in analyzing the images of biological macromolecules. *Ultramicroscopy*, **6**, 187–194.
- Wang,J., Hartling,J.A. and Flanagan,J.M. (1997) The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis. *Cell*, **91**, 447–456.
- Wang,J. *et al.* (2001a) Crystal structures of the HslVU peptidase–ATPase complex reveal an ATP-dependent proteolysis mechanism. *Structure*, **9**, 177–184.
- Wang,J., Song,J.J., Seong,I.S., Franklin,M.C., Kamtekar,S., Eom,S.H. and Chung,C.H. (2001b) Nucleotide-dependent conformational changes in a protease-associated ATPase HslU. *Structure*, **9**, 1107–1116.
- Yoo,S.J., Seol,J.H., Shin,D.H., Rohrwild,M., Kang,M.S., Tanaka,K., Goldberg,A.L. and Chung,C.H. (1996) Purification and characterization of the heat shock proteins HslV and HslU that form a new ATP-dependent protease in *Escherichia coli*. *J. Biol. Chem.*, **271**, 14035–14040.
- Yoo,S.J., Seol,J.H., Seong,I.S., Kang,M.S. and Chung,C.H. (1997a) ATP binding, but not its hydrolysis, is required for assembly and proteolytic activity of the HslVU protease in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **238**, 581–585.
- Yoo,S.J., Shim,Y.K., Seong,I.S., Seol,J.H., Kang,M.S. and Chung,C.H., (1997b) Mutagenesis of two N-terminal Thr and five Ser residues in HslV, the proteolytic component of the ATP-dependent HslVU protease. *FEBS Lett.*, **412**, 57–60.
- Yoo,S.J., Kim,H.H., Shin,D.H., Lee,C.S., Seong,I.S., Seol,J.H., Shimbara,N., Tanaka,K. and Chung,C.H. (1998) Effects of the Cys mutations on structure and function of the ATP-dependent HslVU protease in *Escherichia coli*. *J. Biol. Chem.*, **273**, 22929–22935.

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