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The ATP-dependent CodWX (HslVU) protease in *Bacillus subtilis* is an N-terminal serine protease

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HslVU is a two-component ATP-dependent protease, consisting of HslV peptidase and HslU ATPase. CodW and CodX, encoded by the *cod* operon in *Bacillus subtilis*, display 52% identity in their amino acid sequences to HslV and HslU in *Escherichia coli*, respectively. Here we show that CodW and CodX can function together as a new type of two-component ATP-dependent protease. Remarkably, CodW uses its N-terminal serine hydroxyl group as the catalytic nucleophile, unlike HslV and certain β-type subunits of the proteasomes, which have N-terminal threonine functioning as an active site residue. The ATP-dependent proteolytic activity of CodWX is strongly inhibited by serine protease inhibitors, unlike that of HslVU. Replacement of the N-terminal serine of CodW by alanine or even threonine completely abolishes the enzyme activity. These results indicate that CodWX in *B.subtilis* represents the first N-terminal serine protease among all known proteolytic enzymes.

Keywords: ATP-dependent protease/*cod* operon/CodWX/HslVU/N-terminal serine protease

Introduction

A fundamental feature of intracellular protein degradation is its requirement for metabolic energy (Goldberg, 1992; Gottesman *et al*., 1997). In eukaryotic cells, ATP is required for ubiquitin conjugation and for function of the 26S proteasome, which is responsible for the degradation of most cell proteins (Seufert and Jentsch, 1992; Baumeister *et al.*, 1998; Ciechanover and Schwartz, 1998). This enzyme complex consists of the 20S proteasome, which forms a proteolytic core, and the 19S regulatory complex harboring multiple ATPase activities (Ciechanover, 1994; Coux *et al.*, 1996). The barrel-shaped 20S proteasome is comprised of α- and β-type subunits. The α-type subunits, which are proteolytically inactive, form the outer ring, and the β-type subunits, which contain the active sites, form inner rings of the complex.

Although *Escherichia coli* and other bacteria lack the ubiquitin–proteasome pathway, they contain at least three multimeric ATP-dependent proteases (Goldberg, 1992; Chung, 1993; Gottesman, 1996; Chung *et al*., 1997). Rapid degradation of many abnormal polypeptides and various regulatory proteins requires protease La (Lon), a heat shock protein composed of multiple but identical 87 kDa subunits. This homopolymer has both serine active sites for proteolysis and ATP-cleaving sites in the same polypeptide (Waxman and Goldberg, 1982; Menon and Goldberg, 1987). Unlike protease La, protease Ti (ClpAP) consists of two different multimeric components, both of which are required for proteolysis (Hwang *et al*., 1988). ClpA, which is composed of 84 kDa subunits, contains ATP-hydrolyzing sites, while ClpP, which is composed of 21 kDa subunits, is a serine protease. ClpA is a member of a family of highly conserved polypeptides, present in both prokaryotic and eukaryotic organisms (Gottesman *et al*., 1990). This family contains one or two regions of particularly high homology, each of which has a consensus sequence for ATP binding (Walker *et al*., 1982). Among these family members, the ClpX ATPase can function together with ClpP in the degradation of certain regulatory proteins (Gottesman *et al*., 1993; Wojtkowiak *et al*., 1993; Mhammedi-Alaoui *et al*., 1994; Lehnherr and Yarmolinsky, 1995; Grimaud *et al*., 1998).

Another type of ATP-dependent protease, HslVU (also called ClpYQ), is the product of the *hslVU* operon in *E.coli*. This operon encodes two heat shock proteins: a 19 kDa HslV and a 50 kDa HslU (Chuang *et al*., 1993). HslV harbors the peptidase activity, while HslU provides an essential ATPase activity, both of which can function together as a two-component protease (Missiakas *et al*., 1996; Rohrwild *et al*., 1996; Yoo *et al*., 1996). HslU itself has ATPase activity, while HslV by itself is a weak protease that slowly degrades certain hydrophobic peptides, such as *N*-carbobenzoxy (Cbz)-Gly-Gly-Leu-7-amido-4-methylcoumarin (AMC), and polypeptides including insulin B-chain and casein (Yoo *et al*., 1996; Seol *et al*., 1997). In the reconstituted enzyme, HslU markedly stimulates the proteolytic activity of HslV (>20-fold), while HslV increases the rate of ATP hydrolysis by HslU several-fold (Rohrwild *et al*., 1996; Yoo *et al*., 1996).

The primary sequence of HslV is similar to that of certain β-type subunits of the 20S proteasomes from eukaryotes, certain eubacteria and the archaebacterium *Thermoplasma acidophilum* (Seemüller *et al*., 1996; DeMot *et al*., 1999). Most of the β-subunits are synthesized as inactive precursors, and processed during their incorporation into the 20S proteasome. Moreover, the removal of propeptides exposes the N-terminal Thr residues of certain mature β-type subunits, which serve as the catalytic sites for proteolysis (Fenteany *et al.*, 1995;

Seemüller *et al*., 1996). Like the β-type subunits of the enzyme, HslV has two conserved Thr residues in its N-terminus (Seemüller *et al*., 1996). Furthermore, deletion of the N-terminal threonine or its replacement by valine completely abolishes the proteolytic activity of the HslVU protease (Yoo *et al.*, 1997b). In addition, it is inhibited by lactacystin, a *Streptomyces* metabolite that covalently modifies the N-terminal Thr residue on certain β-type subunits of the 20S proteasome (Fenteany *et al.*, 1995; Rohrwild *et al*., 1996; Yoo *et al*., 1996; Seol *et al*., 1997). Therefore, the HslVU protease appears to function through a similar proteolytic mechanism, using the N-terminal Thr catalytic residue, to the mammalian and archaebacterial proteasomes, even though HslVU does not contain α-type proteasome subunits.

Slack *et al*. (1995) have reported the sequence of the *codVWXY* operon in *Bacillus subtilis*. Among the deduced products of the operon, CodW and CodX show 52% identity in their amino acid sequences to HslV and HslU in *E.coli*, respectively. Interestingly, CodW has an extension of five amino acids preceding two Thr residues, unlike HslV but similar to certain β-type subunits of the 20S proteasomes. Furthermore, the N-terminal extension of CodW begins with two consecutive Ser residues, assuming that its N-terminal methionine is removed during or after translation, as is that of HslV (Rohrwild *et al*., 1996). Therefore, we examined whether the N-terminal extension of CodW could be processed for the exposure of the putative Thr active site, or whether the N-terminal Ser residue might replace the catalytic function of the internal Thr residue. In the present studies, we show that the extension is not processed but, instead, the N-terminal serine acts as an active site residue for proteolysis. To our knowledge, CodWX is the first protease that has N-terminal serine as an active site residue in its CodW subunits.

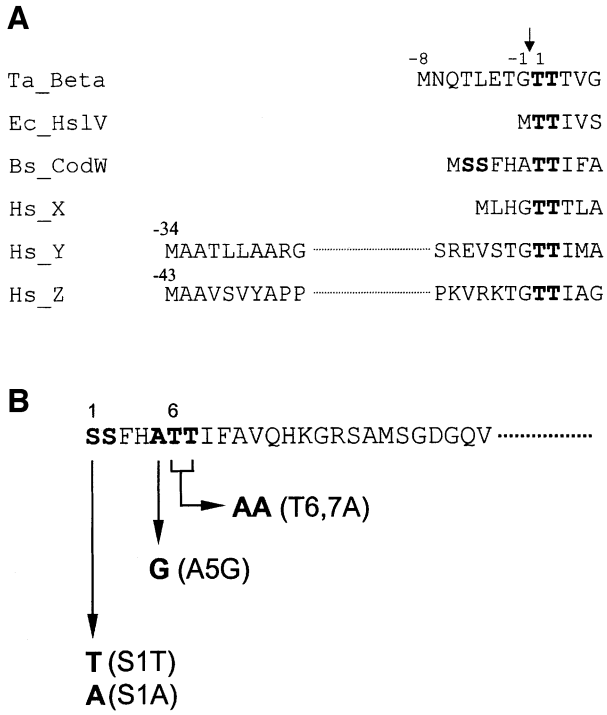

Fig. 1. Sequence alignment of CodW with various β-type subunits of the proteasomes. (**A**) The amino acid sequence of CodW was compared with that of β-type subunits of the proteasomes, which are known to be active in proteolysis. The arrow indicates the position at which processing occurs. The first of the two internal Thr residues is numbered as 1, and the others are indicated relative to the number. (**B**) Site-directed mutagenesis was performed to replace the N-terminal Ser by Thr or Ala, or to replace two internal Thr residues by two Ala. Mutagenesis was also performed to substitute Ala5 with Gly. The first of the two Ser residues is numbered as 1, and the others are indicated relative to the number.

Results

N-terminal regions of CodW, HslV and β-type subunits of proteasomes

The deduced N-terminal amino acid sequence of CodW from the nucleotide sequence of the *codVWXY* operon (Slack *et al*., 1995) was compared with that of HslV and various β-type subunits of the proteasomes. Figure 1A shows that CodW has a short extension of five amino acids, in addition to the N-terminal Met, preceding two Thr residues, unlike HslV but similar to the *Thermoplasma* proteasome and the human X, Y and Z subunits. Therefore, it appeared possible that CodW could be processed to remove the putative propeptide for exposure of the Thr residue at its N-terminus. Remarkably, CodW has two consecutive Ser residues in its N-terminus, unlike any other β-type subunits known to have catalytic activity. This finding raised an additional possibility that CodW might use the N-terminal Ser residue as the catalytic nucleophile for its proteolytic function in the presence of CodX and ATP.

CodW has an N-terminal Ser residue

In order to identify the N-terminal amino acid residue of the CodW protein expressed in *E.coli*, we cloned *codW* from the *codVWXY* operon from genomic DNA of *B.subtilis* by PCR. We also cloned *codX* from the operon by PCR to determine whether CodX forms an ATP-dependent protease with CodW (see below). To determine whether CodW might use the N-terminal Ser as an active site residue (see below), we also generated a number of CodW mutants in which the N-terminal Ser was replaced by either Thr or Ala (referred to as S1T and S1A, respectively) and the two internal Thr residues were substituted with two Ala residues (T6,7A) (Figure 1B). Each of these clones was then transformed into *E.coli* cells, in which the *hslVU* operon was disrupted. All of the proteins were then purified to apparent homogeneity from the transformed cells (Figure 2A).

It is of note that all of the CodW proteins show much higher mobility than HslV in the same gel, in spite of the fact that the calculated molecular weight of CodW (19 478 Da) (Slack *et al*., 1995) is slightly higher than that of HslV (18 961 Da) (Chuang *et al*., 1993). Thus, it appeared as if CodW might have been processed in its N-terminal region and/or elsewhere. However, the molecular weight of CodW determined by mass spectrometry was identical to that deduced from its nucleotide sequence without the codon sequence for N-terminal Met (Figure 2B). Furthermore, the amino acid sequence analysis by Edman degradation revealed that each of the purified CodW proteins began with the expected

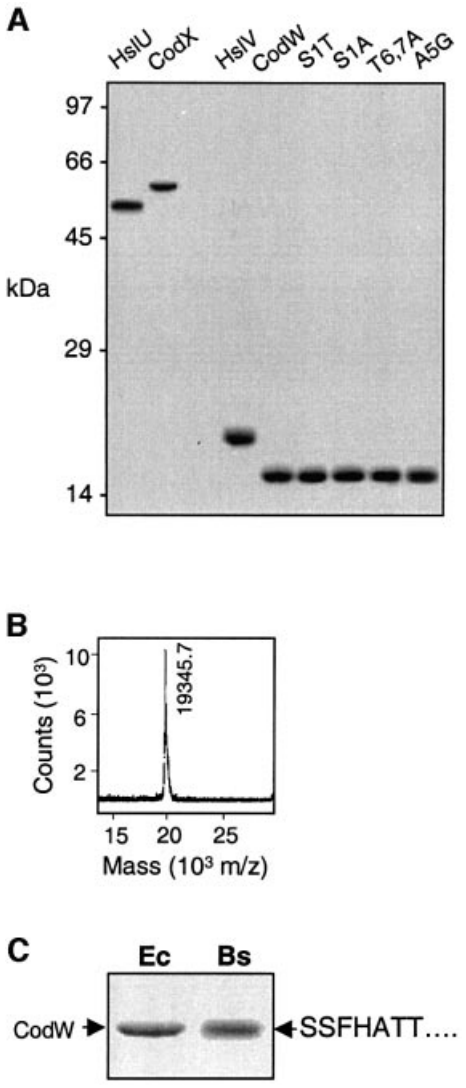

Fig. 2. SDS–PAGE, mass spectrometry and N-terminal sequence determination. (**A**) CodX and CodW and its mutant forms, which had been expressed in *E.coli*, were purified and subjected to SDS–PAGE on 13% slab gels, followed by staining with Coomassie Blue R-250. The purified HslV and HslU proteins were also run on the gel to compare their mobility with that of CodW and CodX. Each lane contained 5 μg of protein. (**B**) The purified CodW protein (expressed in *E.coli*) was subjected to mass spectrometry for determination of its molecular weight. (**C**) The CodW protein was purified from the extract of *B.subtilis* using affinity beads, which were prepared by cross-linking anti-CodW anti-IgGs to protein A–Sepharose. The purified protein was subjected to SDS–PAGE, followed by staining with Coomassie Blue R-250 (lane Bs). As a control, the CodW protein expressed and purified from *E.coli* was also subjected to SDS–PAGE (lane Ec). The proteins in the gel were then transferred onto PVDF membranes and subjected to Edman degradation for determination of their N-terminal sequences.

N-terminal amino acids (e.g. SSFHATT for the wild-type CodW; Figure 2C, lane Ec). These results indicate that the CodW protein expressed in *E.coli* is not processed and has the Ser residue at its N-terminus.

In order to determine whether the CodW protein translated in *B.subtilis* indeed has the Ser residue at its N-terminus, it was affinity purified from the cell extracts using anti-CodW IgG-conjugated protein A–Sepharose as described in Materials and methods. The purified CodW protein was then subjected to N-terminal amino acid

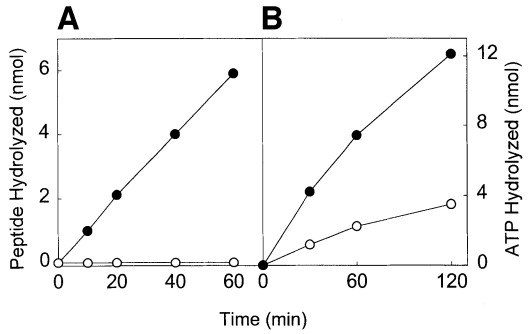

Fig. 3. ATP-dependent degradation of Cbz-Gly-Gly-Leu-AMC and hydrolysis of ATP by CodWX. (**A**) Degradation of Cbz-Gly-Gly-Leu-AMC was assayed by incubating 2 μg of CodX and 1 μg of CodW for various periods at 37°C in the absence (white circles) and presence of 1 mM ATP (black circles) as described in Materials and methods. (**B**) ATP hydrolysis was assayed by incubating 2 μg of CodX and 1 mM ATP for various periods at 37°C in the absence (white circles) and presence of 1 μg of CodW (black circles) as described in Materials and methods.

sequence analysis by Edman degradation. The sequence was also found to begin with SSFHATT (Figure 2C, lane Bs). Thus, it is clear that the CodW protein has the N-terminal Ser residue whether it is expressed in *B.subtilis* or *E.coli*.

CodWX is an ATP-dependent protease

To determine whether the purified CodW and CodX proteins could form an active ATP-dependent protease *in vitro*, like HslV and HslU, they were incubated together for various periods at 37°C in the presence and absence of ATP. As shown in Figure 3A, the incubation mixture could hydrolyze Cbz-Gly-Gly-Leu-AMC, which is an excellent substrate for HslVU (Yoo *et al*., 1996), in the presence of ATP but not in its absence. In addition, CodW alone was not able to degrade the peptide whether or not ATP was present (data not shown). These results indicate that CodW and CodX form a two-component ATP-dependent CodWX protease, homologous to the HslVU protease in *E.coli*.

Like HslU and other ClpA family members, CodX has a consensus Walker A-type ATP-binding motif (Walker *et al*., 1982). Therefore, we examined whether CodX has ATPase activity. Figure 3B shows that CodX alone has relatively weak ATPase activity; however, the ATPase activity of CodX could markedly be stimulated by CodW, similar to that of the HslU ATPase, which is activated by the HslV peptidase (Yoo *et al*., 1996). In addition, the stimulatory effect of CodW was dependent on its concentration, and this effect was maximal at an ~1:2 molar ratio of CodW to CodX, assuming that CodW and CodX are a dodecamer and hexamer like HslV and HslU, respectively (data not shown). These results indicate that CodW and CodX interact with each other and mutually activate the catalytic function of each protein, most likely in the CodWX complex.

CodWX is an N-terminal Ser protease

In order to determine whether CodW indeed uses its N-terminal Ser as an active site residue, we first examined the effects of various inhibitors of Ser proteases on the hydrolysis of Cbz-Gly-Gly-Leu-AMC by CodWX.

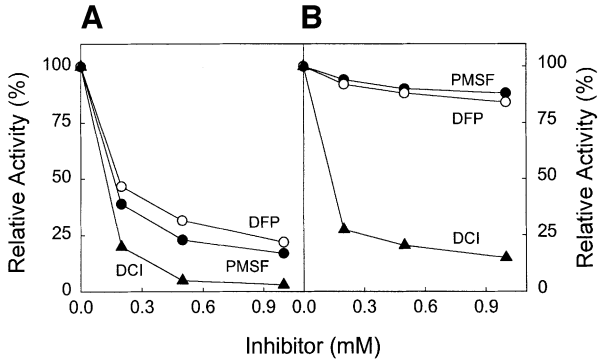

Fig. 4. Effects of various inhibitors of Ser proteases on the hydrolysis of Cbz-Gly-Gly-Leu-AMC by CodWX and HslVU. (**A**) CodW (1 μg) was incubated for 30 min at 37°C with increasing concentrations of PMSF (black circles), DFP (white circles) or DCI (black triangles). After incubation, 2 μg of CodX, 1 mM ATP and 0.1 mM Cbz-Gly-Gly-Leu-AMC were added to the samples, which were then incubated for a further 30 min at 37°C. The release of AMC was then assayed as described in Materials and methods. (**B**) HslV (0.1 μg) was incubated with increasing amounts of the inhibitors as above. After incubation, 0.4 μg of HslU, 1 mM ATP and 0.1 mM Cbz-Gly-Gly-Leu-AMC were added to the samples, which were then incubated for a further 10 min at 37°C. The activity was expressed relative to that without the inhibitors, which was expressed as 100%.

As shown in Figure 4A, both di-isopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluorophosphate (PMSF) inhibited the peptide-degrading activity in a dose-dependent fashion, showing ~55–65% inhibition at 0.2 mM. On the other hand, HslVU having the Thr active site was much less sensitive to inhibition by either of the reagents at the same concentrations tested (Figure 4B). These results strongly suggest that the N-terminal Ser of CodW plays an essential role in the catalytic function of CodWX. We also examined the effects of the reagents that are known to inhibit the multiple peptidase activities of the 26S proteasomes. Peptide aldehyde inhibitors, including Cbz-Leu-Leu-leucinal (MG132), Cbz-Leu-Leu-norvalinal (MG115) and 3,4-dichloroisocoumarin (DCI), strongly inhibited the hydrolysis of Cbz-Gly-Gly-Leu-AMC by both CodWX and HslVU (Table I). Moreover, lactacystin, which binds specifically and covalently to the N-terminal Thr catalytic site in β-type subunits of the proteasomes (Fenteany and Schreiber, 1998), showed an even higher inhibitory effect on CodWX than on HslVU. Thus, it appears that the hydroxyl group of Ser at the N-terminus of CodW functions as an active nucleophile, like that of the N-terminal Thr catalytic site in HslV or β-type subunits of the proteasomes.

In order to clarify further the involvement of the N-terminal Ser residue in proteolysis, the purified mutant forms of CodW (S1T, S1A and T6,7A) (see Figures 1B and 2A) were incubated with Cbz-Gly-Gly-Leu-AMC in the presence of CodX and ATP. Figure 5A shows that S1A is not at all capable of cleaving the peptide, indicating that the N-terminal Ser of CodW is essential for the proteolytic function of the CodWX protease. Furthermore, S1T did not show any activity on the peptide either, indicating that the Ser residue could not be substituted even with Thr. Although it is unclear at present why Thr could not replace Ser, these results strongly suggest that the N-terminal Ser of CodW serves as an active site residue.

Table I. Effects of various protease inhibitors on the hydrolysis of Cbz-Gly-Gly-Leu-AMC by CodWX and HslVU

Inhibitors	Concentrations (μM)	% inhibition of	
		CodWX	HslVU
MG132	5	78	77
MG115	5	79	78
Lactacystin	100	90	25
N-ethylmaleimide	100	68	65

Peptide hydrolysis was assayed as described in Figure 4 but in the absence and presence of the indicated concentrations of the inhibitors. Similar data were obtained in at least three independent experiments.

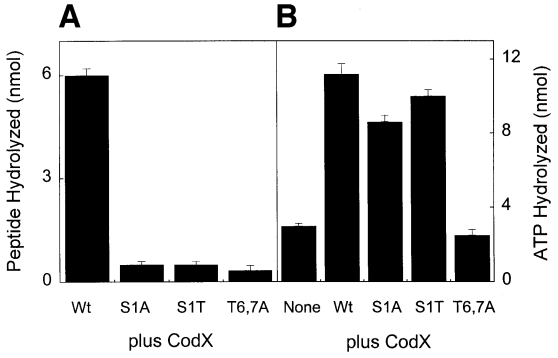

Fig. 5. Effects of the S1A, S1T and T6,7A mutations in CodW on the hydrolysis of Cbz-Gly-Gly-Leu-AMC and ATP by CodWX. (**A**) Degradation of the peptide was assayed by incubation of 2 μg of CodX and 1 μg of the wild-type CodW (Wt) or its mutant forms, at 37°C for 1 h. (**B**) ATP hydrolysis was also assayed by incubation of 2 μg of CodX in the absence (None) and presence of 1 μg of the wild-type CodW (Wt) or its mutant forms, at 37°C for 1 h.

Of note was the finding that T6,7A, in which two internal Thr residues were replaced by two Ala, could not degrade Cbz-Gly-Gly-Leu-AMC at all (Figure 5A), despite the fact that the mutant protein has the intact N-terminal Ser residue. Therefore, we examined whether T6,7A as well as other mutant CodW proteins could interact with CodX by determining its ability to stimulate the ATPase activity of CodX. Figure 5B shows that both S1A and S1T can stimulate ATP hydrolysis by CodX to a similar extent to that seen with the wild-type CodW, suggesting that the mutant forms of CodW are capable of interacting with the CodX ATPase, although neither of them could degrade the peptide in the presence of CodX. On the other hand, T6,7A was not at all capable of stimulating ATP hydrolysis by CodX, indicating that T6,7A cannot interact with CodX.

We have previously shown that oligomerization of HslV is essential for interaction with HslU and thus for its proteolytic activity (Yoo *et al.*, 1997a). To determine whether the replacement of two internal Thr residues by two Ala might cause a defect in the oligomerization of CodW, the purified T6,7A protein was subjected to gel filtration analysis on a Superose-6 column. As shown in Figure 6, the peak of the wild-type CodW, as well as of S1A or S1T, was eluted in the fractions with a size of ~240 kDa, which corresponds to the size of a dodecamer.

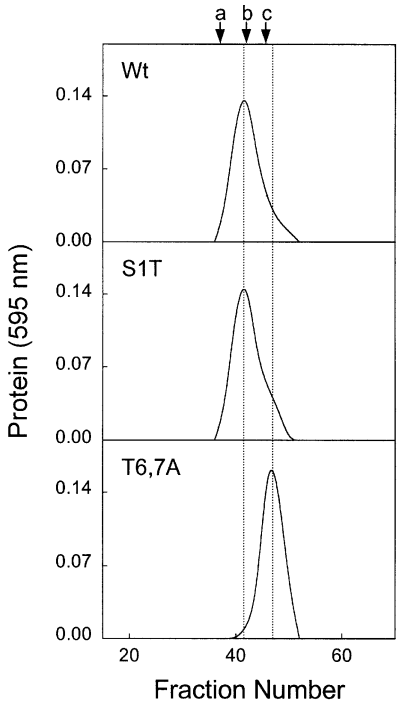

Fig. 6. Effects of the S1A, S1T and T6,7A mutations on the oligomerization of CodW. The wild-type CodW and its mutant forms (200 μg) were incubated in the presence of 0.1 M NaCl for 30 min at 37°C. After incubation, each sample was subjected to gel filtration on a Superose-6 column (1 × 30 cm) that had been equilibrated with buffer A containing 0.1 M NaCl. The proteins were eluted with the same buffer, and aliquots of the fractions were assayed for protein using the methods of Bradford (1976). Note that the elution pattern of S1A is nearly identical to that of S1T (data not shown). The size markers used were: a, thyroglobulin (669 kDa); b, catalase (232 kDa); c, ovalbumin (45 kDa).

In contrast, T6,7A was eluted as a single peak with a monomeric size (~20 kDa), indicating that the mutant protein can not form an oligomer. Thus, it appears that the inability of T6,7A to cleave the peptide substrate is due to its inability to form an oligomer that can interact with the CodX ATPase.

Previously, it has been demonstrated that the processing of β-type subunits of the proteasomes requires the presence of Gly at position –1, and that the substitution of Gly by other amino acids, such as Ala, completely prevents the processing (Seemüller *et al*., 1996; Ditzel *et al*., 1998). Unlike the proteasome subunits, CodW has Ala preceding two internal Thr residues (see Figure 1). To determine whether the inability of CodW in auto-processing is due to the presence of Ala in place of Gly, we substituted the Ala residue with Gly. The mutant form of the CodW protein (A5G) was purified to apparent homogeneity (the last lane in Figure 2A) and subjected to Edman degradation for determination of the amino acid sequence of its N-terminal region as above. Its sequence was found to begin with SSFHGTT (data not shown), indicating that A5G is not processed. Furthermore, A5G hydrolyzed Cbz-Gly-Gly-Leu-AMC nearly as well as the wild-type CodW in the presence of CodX and ATP, and stimulated the ATPase activity of CodX to the same extent seen with wild-type CodW (data not shown). These results confirm that CodW is an N-terminal Ser protease that requires no internal processing for activity.

SulA degradation by a CodW–HslU hybrid protease

SulA is a cell division inhibitor protein in *E.coli* encoded by the SOS-inducible *sulA* gene, which is also called *sfiA* (Bi *et al*., 1991; De Boer *et al*., 1992; Mukherjee *et al*., 1993). We have previously shown that HslVU degrades the inhibitor protein, although less efficiently than does protease La (Lon) (Seong *et al*., 1999). We have also recently demonstrated that HslU alone functions as a molecular chaperone in the prevention of SulA aggregation (Seong *et al*., 2000). Thus, HslVU, in addition to Lon, has been suggested to play an important role in the regulation of cell division. In an attempt to elucidate the physiological role of CodWX, we examined whether CodWX could degrade SulA. Since SulA has a high tendency to aggregate *in vivo* as well as *in vitro*, a maltose-binding protein–SulA fusion protein (MBP–SulA) was used as the substrate. In addition, this fusion protein has been shown to retain the inhibitory function *in vivo* and to behave as a substrate for both HslVU and Lon *in vitro* (Sonezaki *et al*., 1995). As shown in Figure 7A, CodWX could not degrade MBP–SulA, unlike HslVU. However, when MBP–SulA was incubated with CodW and HslU, it was hydrolyzed to an extent similar to that seen with HslV and HslU. On the other hand, the mixture of CodX and HslV did not show any activity on the substrate, despite our finding that CodW/HslU degrades Cbz-Gly-Gly-Leu-AMC nearly as well as does CodX/HslV (data not shown). Thus, it appears that HslU, but not CodX, recognizes MBP–SulA for degradation by either HslV or CodW. These results suggest that the ATPase subunits confer the specificity of protein substrates for degradation by the proteolytic subunits in their complex.

When *E.coli* cells are under SOS conditions (e.g. UV irradiation or treatment with UV mimicking agents, such as nitrofurantoin) SulA accumulates and blocks the cell growth by inhibiting cell division (Bi and Lutkenhaus, 1990). In order to determine whether the hybrid forms of the proteases (specifically CodW–HslU) could function *in vivo*, the cDNAs for CodW and HslU or for CodX and HslV were transformed into *hsl/lon* mutant cells and grown on an agar plate containing nitrofurantoin. As a control, the cDNAs for CodW and CodX or for HslU and HslV were also transformed as above. Figure 7B shows that the mutant cells transformed with the cDNAs for CodW and HslU survive as well as those for HslV and HslU, unlike those transformed with the cDNAs for CodW and CodX or CodX and HslV. In addition, the *hsl/lon* mutant cells without transformation could not survive under the same growth conditions, while the wild-type cells grew normally as expected. These results are consistent with our finding that CodW–HslU, but not CodX–HslV, is capable of hydrolyzing MBP–SulA. Thus, CodW could complement HslV in SulA degradation in *E.coli*, implicating an overlapping function in the regulation of cell division in *B.subtilis* by degrading an unknown SulA-like protein(s) in the cells.

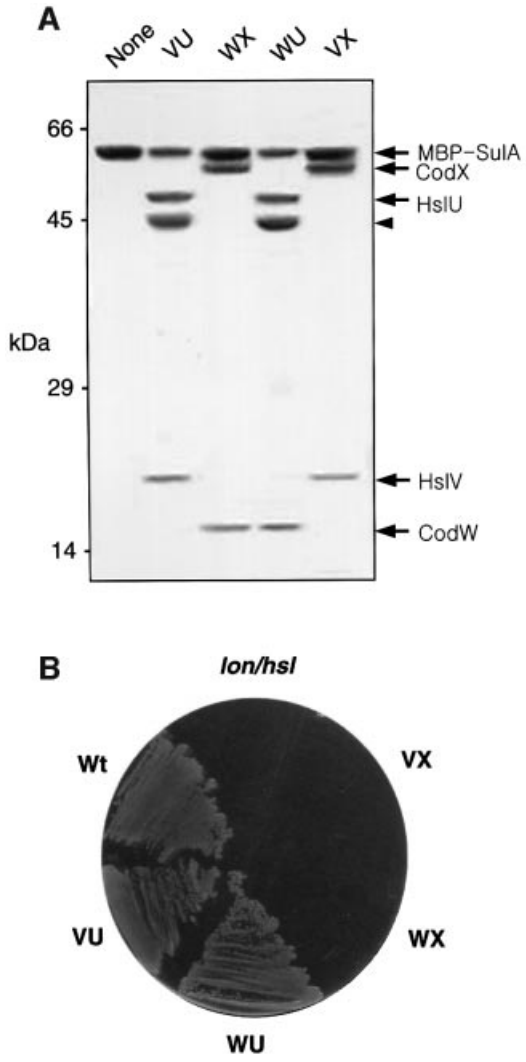

Fig. 7. CodW complements HslV in degradation of SulA and survival of *E.coli* cells under SOS conditions. (**A**) Hydrolysis of MBP–SulA was assayed by incubation of 5 μg of MBP–SulA, 1 mM ATP and 1 μg of CodW (W) or HslV (V), and 2 μg of CodX (X) or HslU (U) in combinations as indicated. After incubation for 1 h at 37°C, the reaction was stopped by adding 30 μl of 0.75 M Tris–HCl pH 6.8 containing 7.5% SDS and 10% 2-mercaptoethanol. They were then subjected to SDS–PAGE on 13% slab gels, followed by staining with Coomassie Blue R-250. The arrowhead indicates the major degradation product that contains MBP. (**B**) The cDNAs for CodW, CodX, HslV and HslU were transformed into MC1000LH (*lon::tet, hslVU::kan*) in combinations as indicated, and grown overnight on an agar plate containing nitrofurantoin (2 μg/ml). As a control, MC1000LH (*hsl/lon*) and its wild-type MC1000 (Wt) were also plated and grown as above.

Discussion

Lines of evidence presented in this study indicate that CodWX is an ATP-dependent protease that has N-terminal Ser as an active site residue. First, mass spectrometric analysis and N-terminal amino acid sequencing data of CodW, which was expressed and purified from *E.coli* or *B.subtilis*, revealed that the N-terminal extension of five amino acids preceding two Thr residues was not processed, but that the N-terminal Met was removed, hence exposing the two consecutive Ser residues in its N-terminus. Furthermore, a mutant form of CodW in which Ala preceding two internal Thr residues was replaced by Gly is not processed, unlike certain β-type subunits of the proteasomes that require the presence of Gly at position –1 for processing and, thus, for exposure of the Thr catalytic site at their N-termini (Seemüller *et al*., 1996; Ditzel *et al*., 1998). Thus, CodW in *B.subtilis* may represent an ancient form of β-type proteasome subunits, but be more evolved than HslV in *E.coli*.

Secondly, the substitution of the N-terminal Ser by Ala (S1A) abolished the proteolytic activity of the CodWX protease. Surprisingly, the replacement of the N-terminal Ser even by Thr (S1T) also completely blocked the activity of CodWX. In contrast, it has recently been reported that the Thr to Ser substitution in the β-subunits of *Thermoplasma* proteasomes results in a several-fold reduction in their proteolytic activity (Kisselev *et al*., 2000). In the hydrolysis of small peptides, however, the same mutation in HslV or the archaeal proteasomes shows relatively little influence on catalytic activity (Seemüller *et al*., 1995; Yoo *et al*., 1997b; Kisselev *et al*., 2000). Apparently, this new N-terminal Ser protease has some interesting structural adaptation, allowing protein as well as peptide degradation that is lacking in classical proteasomes. Thus, in the case of S1T, introduction of the methylene group might freeze the micro-environment in the active site region of CodW by interaction with hydrophobic side chains of nearby amino acids, and may prevent the access of the scissile bond to the hydroxyl group acting as a nucleophile for proteolysis. X-ray crystallographic analysis of CodWX should clarify the structural and functional role of the Ser residue in CodW.

Finally, CodWX showed a much higher sensitivity to the inhibitors specific to Ser proteases, such as DFP and PMSF, than HslVU. Of interest was the finding that lactacystin, which binds specifically and covalently to the N-terminal Thr catalytic site in β-type subunits of the proteasomes, showed a much higher inhibitory effect on CodWX than on HslVU, although the peptide aldehyde inhibitors, such as MG132 and MG115, which are also known to inhibit the proteolytic activity of the proteasomes, blocked the activity of both CodWX and HslVU to a similar extent. It has been reported that lactacystin interacts with structural elements unique to the β-type catalytic subunits of the proteasomes, and the electrophilic carbonyl at the C-4 position of *clasto*-lactacystin β-lactone, which is an active intermediate of lactacystin, could also react with the hydroxyl group of Ser (Fenteany and Schreiber, 1998). It has also been demonstrated that the T1S mutant form of *Thermoplasma* proteasome is several-fold more sensitive to inhibition by the *clasto*-lactacystin β-lactone than the wild-type enzyme (Kisselev *et al*., 2000). Thus, it appears that the structure at the catalytic site of CodW is more similar to that of β-type subunits of the proteasome than that of HslV. Taken together, we conclude that CodWX in *B.subtilis* is an ATP-dependent protease that uses the N-terminal Ser as an active site residue. There are other N-terminal nucleophile hydrolases, such as penicillin acylase (Choi *et al*., 1992; Duggleby *et al*., 1995) and glycosylasparaginase (Oinonen *et al*., 1995; Tikkanen *et al*., 1996), which have Ser and Thr in

their N-termini, respectively. To our knowledge, however, CodWX is the first protease that has an N-terminal Ser active site residue in its CodW subunits.

The two internal Thr residues in CodW appear to play a structural role, such as in the interaction between the CodW subunits and thus in their oligomerization. Upon gel filtration analysis, T6,7A was found to behave as a monomer (~20 kDa), unlike the wild-type CodW or the S1A or S1T mutant, which ran as a protein with a size of a dodecamer (i.e. 240 kDa). Furthermore, nearly identical results were obtained when the two internal Thr residues were deleted, i.e. the mutant protein is unable to form a dodecamer, stimulate the ATPase activity of CodX or degrade peptides, whether or not CodX and ATP are present (data not shown). We have previously shown that treatment of HslV with *N*-ethylmaleimide, a sulfhydryl blocking agent, dissociates the dodecameric protein into monomers and abolishes its interaction with HslU, hence resulting in the complete loss of proteolytic activity (Yoo *et al*., 1998). Thus, it appears that formation of the CodW oligomer is essential for its interaction with CodX and that the two internal Thr residues may play an important role in oligomerization of the CodW subunits.

Of interest is the finding that CodW–HslU, but not CodWX or HslV–CodX, could degrade SulA, a cell division inhibitor protein in *E.coli*, as well as HslVU under *in vitro* conditions. Furthermore, CodW could complement HslV in 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 in cell survival implicates a possible role of CodWX in the regulation of cell division in *B.subtilis*, like HslVU in *E.coli* (Khattar, 1997; Seong *et al*., 2000). However, CodWX could not degrade SulA, and *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 recently been reported to function in cell division of *B.subtilis* in a similar fashion to SulA (Levin *et al*., 1999). The possibility of CodWX being involved in the regulation of cell division through degradation of EzrA is currently under investigation.

Materials and methods

Materials

Polyclonal antibodies against CodW were prepared by subcutaneous injection of each of the purified proteins into albino rabbits. All reagents for the PCR, including *Taq* polymerase and restriction endonucleases, were purchased from Promega. Peptide substrates were obtained from Bachem Feinchmikalien AG (Bubendorf, Switzerland). All other reagents were purchased from Sigma, unless otherwise indicated.

For overproduction of MBP–SulA, pMAL-p2-SulA (Higashitani *et al*., 1997) was transformed into an *E.coli* strain MC1000 [*araD139 Δ(ara-leu)7679 galU galK Δ(lac)174 rpsL thi-1*]. MBP–SulA was then purified from the cells using amylose resin as described (Seong *et al*., 1999). MC1000LH strain (*lon::tet, hslVU::kan*) was obtained by two sequential P1 transductions of the disrupted *lon* and *hsl* DNAs into MC1000 as described previously (Seong *et al*., 1999). For co-expression of CodW and HslU, pCodW and pAC1/HslU plasmids were constructed as described (see below; Seong *et al*., 2000). pAC1/CodX and pBS/HslV were also constructed for co-expression of CodX and HslV as described (see below; Yoo *et al*., 1998).

Cloning of codW and codX

The PCR amplification method was used for cloning *codW* and *codX*. Nucleotide sequences of the primers used for amplification were based on the sequence reported by Slack *et al*. (1995). The primers used for CodW were 5′-ATTGGCAGTATGGCACATTATA-3′, which is located 244 bp upstream of the translational start site of *codV* and hence includes the promoter region of the *codVWXY* operon, and GGCATTAATCCG-CTCAGCGC, which is located 712 bp downstream of the translational termination site of *codW*. Thus, this clone encodes not only CodW but also CodV. The primers used for CodX were GGACTTGGGATCCAT-GGAAAAAAAACC, which was designed to create a *Bam*HI site just in front of the translation start site of *codX*, and AATCCTCCCGGG-CATTCCTCATAT, which is located 35 bp downstream of the translational termination site of *codX*. The PCR products for CodW and CodX were ligated into pGEM-T vectors, and the resulting recombinant plasmids were referred to as pCodW and pGEM/CodX, respectively. For overexpression of CodX, we used the promoter of the *htrA* gene in pBluescript (Seol *et al*., 1991). PCR was performed using a mutagenic oligonucleotide (AAGGATCCCTCAGCCGCCGTTGCAGAG) containing a *Bam*HI site and T7 primer, and its product was ligated into pGEM-T vector. The 1.4 kb DNA fragment carrying the *codX* sequence was cut out by digestion of pGEM/CodX with *Bam*HI and *Apa*I, and ligated into the latter pGEM-T vector. The resulting plasmid containing the *codX* sequence under the control of the *htrA* promoter was designated pCodX. Both pCodW and pCodX plasmids were transformed into MC1000H carring *hslVU::kan* (Seong *et al*., 1999). For co-expression of CodX with HslV, the coding region for CodX in pCodX was cut out and ligated into pAC1 (Seong *et al*., 2000) and the resulting plasmid was referred to as pAC1/CodX.

Mutagenesis

Site-directed mutagenesis was carried out by two sequential PCRs using pCodW as the template. The primary PCR was carried out using mutagenic primers, which were designed for replacing Ser1 of CodW by Thr or Ala, or replacing both Thr6 and Thr7 by two Ala residues (see Figure 1B). Mutagenesis was also performed to replace Ala5 by Gly. Prior to the secondary PCRs, the same amount of primary PCR fragments was mixed and subjected to annealing between the fragments by denaturation–renaturation reactions. The DNAs with recessed 3′-OH ends were extended by Klenow and used as the templates for secondary PCRs. After the secondary PCR, the mutated fragments were ligated into pGEM-T vector. The resulting plasmids were transformed into MC1000H carring *hslVU::kan* (Seong *et al*., 1999). Deletion or substitution of the nucleotides by mutagenesis was confirmed by DNA sequencing.

Purification of CodW and CodX

To purify CodW and its mutant forms, extracts were prepared from the *E.coli* cells harboring pCodW and its mutants as described previously (Yoo *et al*., 1996). They were then loaded on a Q-Sepharose column (2.5 × 8 cm) equilibrated with buffer A [20 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol]. After washing with buffer A, the bound proteins were eluted with a linear gradient of 0–400 mM NaCl. The fractions containing the 19 kDa CodW were pooled, dialyzed against buffer B (50 mM potassium phosphate pH 6.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 10% glycerol), and applied to a hydroxyapatite column (1.5 × 6 cm) equilibrated with buffer B. After washing with the same buffer, proteins were eluted with a linear gradient of 50–500 mM phosphate. Aliquots of the resulting fractions were subjected to SDS–PAGE under denaturing conditions. The fractions containing CodW or its mutant forms with apparent homogeneity were pooled and kept frozen at –70°C.

For purification of CodX, extracts were prepared from the cells carrying pCodX and loaded onto a phosphocellulose column (2.5 × 20 cm) equilibrated with buffer C (25 mM potassium phosphate pH 6.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 10% glycerol). After washing the column with the same buffer, proteins were eluted with a linear gradient of 25–300 mM phosphate. The fractions containing the 52 kDa CodX were pooled, dialyzed against buffer A containing 0.1 M NaCl, and applied to a Q-Sepharose column (2 × 10 cm) equilibrated with the dialysis buffer. The bound proteins were eluted using a linear gradient of 100–400 mM NaCl. The fractions containing CodX were pooled, dialyzed in buffer A containing 0.1 M NaCl, and applied to a heparin–Sepharose CL-6B column (1.5 × 7 cm) equilibrated with the dialysis buffer. Proteins bound to the column were eluted with a linear gradient of 0.1–0.5 M NaCl. Aliquots of the resulting fractions were subjected to SDS–PAGE, and the fractions containing CodX with apparent homogeneity were pooled and kept frozen at –70°C.

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, reaction mixtures (0.1 ml) containing appropriate amounts of the purified CodW and CodX proteins in 0.1 M Tris–HCl pH 8, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 1 mM EDTA were incubated at 37°C for 30 min. After incubation, the samples were added with the peptide to 0.1 mM and incubated further for various periods. 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 the similar reaction mixtures at 37°C but in the absence of the peptide substrate. After incubation, 0.2 ml of 1% SDS were added to the samples, and the phosphate released was determined as described (Ames, 1966). Proteins were assayed by their absorbance at 280 nm or by the method of Bradford (1976) using bovine albumin as a standard.

Gel filtration analysis

CodW and its mutant forms (200 µg) were incubated in the presence of 0.1 M NaCl for 30 min at 37°C. After incubation, each sample was subjected to gel filtration using a Superose-6 HR 10/30 column (Pharmacia, Sweden) that had been equilibrated with buffer A containing 0.1 M NaCl at 4°C. Proteins were eluted with the same buffer, and aliquots (200 µl) were assayed for protein concentration by the method of Bradford (1976).

N-terminal sequence analysis of affinity-purified CodW from B.subtilis extracts

Exponentially growing *B.subtilis* cells were pelleted and resuspended in a lysis buffer containing 0.5 mg/ml lysozyme, 3 µg/ml DNase I, 3 µg/ml RNase A, 5 mM EDTA and 1 mM PMSF. After incubation for 1 h at 37°C, extracts were prepared by sonication of the cell suspension, followed by centrifugation for 1 h at 100 000 *g*. To prepare anti-CodW antibody affinity beads, IgGs were purified from anti-CodW antiserum and cross-linked to protein A–Sepharose CL-4B (Pharmacia, Sweden) as described (Gersten and Marchalonis, 1978; Schneider *et al*., 1982). The extracts were then mixed with the affinity beads and incubated for 2 h at 4°C with gentle rocking. After incubation, the beads were washed with 50 mM Tris–HCl pH 8.0 containing 0.5 M NaCl, and the proteins bound to the beads were eluted with the Tris buffer containing 0.2% SDS. Purified proteins were subjected to SDS–PAGE and blotted onto PVDF membranes. After briefly staining the membranes with Coomassie Blue R-250, the bands that migrated to the same position as the purified CodW protein were cut out and subjected to N-terminal amino acid sequence analysis by Edman degradation.

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