BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 613–616 (1997)
ARTICLE NO. RC977735

BREAKTHROUGHS AND VIEWS

Characterization of Energy-Dependent Proteases in Bacteria

Chin Ha Chung, Soon Ji Yoo, Jae Hong Seol, and Man Sik Kang
Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Received October 25, 1997

Protein breakdown in both bacterial and eukaryotic cells requires ATP (1). In eukaryotes, ATP is required for both ubiquitination of target proteins and degradation of the ubiquitinated proteins. The 26S proteasome, that is responsible for the ATP-dependent proteolysis, consists of two multimeric components: the 20S proteasome that is the catalytic core containing multiple peptidase activities and the 19S regulatory complex that has multiple ATPase activities. ATP hydrolysis is required for assembly of these components into the 26S proteasome.

Bacterial cells lack ubiquitin, but contain several proteolytic enzymes that require ATP for their function (1-4). For example, the soluble extract of *Escherichia coli* contains 3 different ATP-dependent proteases: protease La (Lon), protease Ti (Clp), and HslVU protease. Protease La rapidly hydrolyzes abnormal proteins and many regulatory proteins, such as SulA, RcsA, the λN protein, and CcdA (5). Protease Ti (ClpAP) and its alternative form (ClpXP) degrade abnormal proteins and some other regulatory proteins, including the λO protein, MazE, and RpoS (5). Thus, these proteases contribute to the control of many important cellular functions. However, *in vivo* substrates for HslVU protease have not yet been identified, although it has been implicated that this enzyme degrades a cell division inhibitor, SulA (6). In addition, all of these proteases can hydrolyze some model substrates, such as casein and insulin B-chain. Here, we summarize the recent findings in biochemical characteristics of the ATP-dependent proteases in *E. coli.*

PROTEASE LA (Lon)

Protease La, the *lon* gene product, is a heat shock protein (1-4). This enzyme has been characterized as a tetramer (400-450 kDa) of 87-kDa subunits, each of which has an ATP-binding site and a Ser active site for proteolysis (7). However, recent studies indicate a much higher molecular mass of 800-900 kDa, more consistent with an octameric structure (7). In addition, ATP has no effect on the oligomeric nature of the enzyme. Thus, protease La seems to have a tendency to dimerize under certain conditions.

Protease La shows protein-activated ATPase activity that is essential for protein degradation. However, ATP binding, but not its hydrolysis, is required for cleavage of small peptides (7). Furthermore, mutation of the active site Ser residue prevents proteolysis but has no effect on the ATPase activity or its stimulation by protein substrates (8). In addition, recent work has shown that protein degradation by protease La does not require ATP hydrolysis if the substrate lacks a stable secondary structure (9). Thus, it appears that ATP hydrolysis is required for unfolding of protein substrates to expose scissile bonds.

An interesting property of protease La is that it binds to DNA with a high affinity. In fact, protease La has initially been isolated as a DNA-binding protein, assuming that it may be a regulator of gene expression (10). Hydrolysis of both ATP and protein substrates is activated by DNA, especially by single stranded DNA, but not by RNA or tRNA (11). In addition, protein substrates promote the dissociation of the enzyme from DNA. The physiological significance of the DNA effect is uncertain, since there is no apparent sequence specificity for activation by DNA. However, it has recently been demonstrated that protease La binds specifically to a TG-rich sequence found in the HIV-2 enhancer (12). Although it is obvious that the bacterial protease has no role in regulating HIV-2 gene expression, this finding suggests that protease La can target similar regulatory sites in bacterial promoters and hence influence gene expression.

PROTEASE TI (Clp)

Unlike protease La, protease Ti (Clp) consists of two different components, both of which are required for

0006-291X/97 $25.00

proteolysis (1-4). While ClpA, which belongs to the Hsp100/Clp family (13), has two conserved ATP-binding motifs in the 84-kDa subunit, ClpP, which is a heat shock protein, has a Ser active site for proteolysis in the 21-kDa subunit. ClpP consists of two super-imposed rings, each having 7 subunits, while ClpA is a trimer in the absence of ATP and forms a single ring of 6 subunits in its presence (14). Mutational analysis has revealed that the first ATP-binding site is responsible for hexamer formation of ClpA and the second site is essential for its ATPase activity (15).

Both ATP-binding sites in ClpA appear to be essential for formation of the ClpA/ClpP complex (16). A ClpA mutant, which carries a Lys to Thr substitution in the first ATP-binding site and hence is unable to form a hexamer, cannot be assembled to the ClpA/ClpP complex. Another ClpA mutant, which has a Lys to Thr substitution in the second ATP-binding site and hence is unable to hydrolyze ATP but allows oligomerization, can neither interact with ClpP. Thus, both ATP-binding sites in ClpA seem to play a role in assembly of the ClpA/ClpP complex.

In the presence of ADP, the trimeric ClpA can form a hexamer but is unable to be assembled into the ClpA/ClpP complex (16). Thus, the conformations of the ADP- and ATP-bound ClpA hexamers appear to differ at least in their ability to interact with ClpP. Furthermore, ADP prevents the ClpA/ClpP complex formation even in the presence of ATP. The K_m for ATP of protease Ti for both ATP cleavage and proteolysis is about 0.2 mM, and the K_i for ADP for the hydrolytic processes is less than one-fifth of the K_m value. Thus, ADP, which can be generated during ATP hydrolysis by protease Ti and has much higher affinity than ATP, is likely to play a role in the disassembly of the enzyme complex.

Like protease La, protease Ti is a protein-activated ATPase, which is essential for degradation of large proteins (1-4). However, short peptides can be hydrolyzed by ClpP alone (17), while degradation of larger peptides requires ATP binding only (18). Thus, ATP binding seems to induce a conformational change in ClpA, which allows the large peptide and protein substrates access to the active site of ClpP, and its hydrolysis may be involved in unfolding of large proteins to expose scissile bonds. In addition, rapid assembly and disassembly of the ClpA/ClpP complex at the expense of ATP may help recycling of the enzyme in the process of protein breakdown or prevention of inappropriate or excessive degradation of cell proteins.

Protein-activated ATPase activity is a characteristic of molecular chaperones. Like DnaK and DnaJ, ClpA also functions as a chaperone in the *in vitro* activation of the plasmid P1 RepA replication initiator protein (19). ClpX, which also belongs to the Hsp100/Clp family and has protein-activated ATPase activity, also functions as a chaperone in the protection of λO protein from heat inactivation (20). ClpX is a heat shock protein encoded together with ClpP from the *clpPX* operon (21,22). This ATPase has only one ATP-binding site, unlike ClpA, but can combine with ClpP to form an alternative type of protease Ti that is capable of degrading λO protein and the bacteriophage Mu vir repressor (21-23).

The *clpA* gene has two translational start sites and hence encodes two polypeptides of 84 and 65 kDa (24). The 65-kDa ClpA lacks the ATPase activity and is unable to support the proteolysis by ClpP, despite the fact that it retains both ATP-binding sites. The N-terminal region (168 amino acids) that is absent in the 65-kDa ClpA contains a Cys residue (Cys^{47}) that is involved in the interaction with protein substrates (25). The 65-kDa protein, however, is capable of inhibiting the protein-activated ATPase activity of the 84-kDa ClpA by forming a mixed oligomeric complex, although has no effect on its intrinsic ATPase activity, and hence blocks the proteolytic activity of protease Ti. In addition, the 65-kDa protein prevents the ClpP-mediated degradation of the 84-kDa ClpA in the absence of protein substrates (26). Thus, the 65-kDa protein may play a role in the regulation of the ATP-dependent proteolysis by protease Ti by direct interaction with the 84-kDa ClpA as well as by controlling its level, when degradable proteins are not available.

HslVU PROTEASE

HslVU protease is composed of two heat shock proteins, 19-kDa HslV and 50-kDa HslU, that are encoded by the *hslVU* operon (27-29). The primary sequence of HslV is related to that of certain β-subunits of the 20S proteasomes from eukaryotes, the archaebacterium *Thermoplasma acidophilum*, and certain bacteria (30), and that of HslU is about 50% identical to ClpX (13). HslU, like ClpX, has one ATP-binding motif, and behaves as a monomer or dimer in the absence of ATP and as a hexamer or heptamer in its presence. Replacement of the Lys residue in the ATP-binding motif by Thr prevents ATP binding and hence the oligomerization of HslU (31). On the other hand, HslV behaves as a dodecamer whether or not ATP is present. With β,γ-imido-ATP present, HslV and HslU form cylindrical four-ring structures in which the HslV dodecamer is flanked at each end by a HslU ring (32).

HslV by itself is a weak protease that can degrade certain hydrophobic peptides and proteins, and its activity can be activated at least 20-fold by HslU and ATP (27-29, 33). HslU has an intrinsic ATPase activity, which can be stimulated 2- to 3-fold by HslV. Thus, these HslV and HslU must alter each other's conformation. Like certain β-type subunits of the 20S proteasome, HslV has two conserved Thr residues at its N-terminus. Lactacystin that specifically interacts with the N-terminal Thr of HslV as well as of the 20S proteasome subunits inhibits the proteolytic activity of

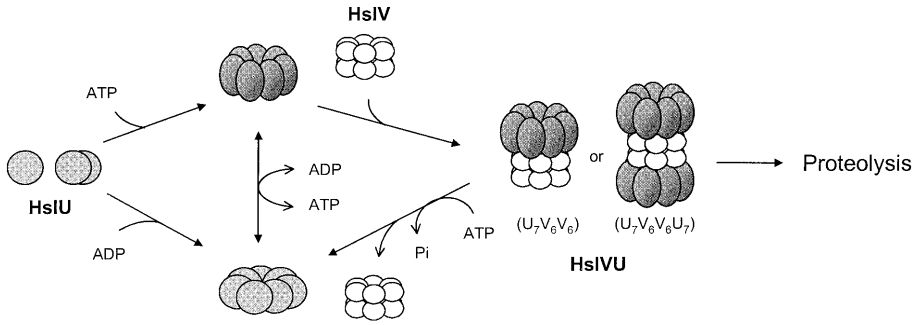

FIG. 1. A schematic model for assembly and disassembly of HslVU protease.

HslVU protease (33, 34). Moreover, substitution of the Thr residue with Val or its deletion completely abolish the enzyme activity (35). Thus, the N-terminal Thr in HslV must act as the active site nucleophile as in the 20S proteasome. However, the inactive HslV protein lacking the N-terminal Thr interacts more tightly with HslU than the wild-type HslV, and stimulates the ATPase activity of HslU nearly 10-fold. In contrast, the HslV lacking both Thr residues at its N-terminus cannot interact with HslU or stimulate the ATPase activity of HslU. Thus, the N-terminal Thr of HslV may provide a steric constraint on the interaction of the second Thr with HslU in addition to its role in the catalytic function (35).

In addition to the N-terminal Thr, a Ser residue in HslV appears to also be involved in the catalytic function. Replacement of Ser^{124} by Ala completely blocks the proteolytic activity but shows no effect on either the ability of HslV in stimulating the ATPase activity of HslU or forming the HslV/HslU complex (35). Moreover, structural analysis has revealed that Ser^{124} is located closely to the catalytic Thr residue (36). The Ser^{172} to Ala substitution also abolishes the proteolytic activity. However, this mutation located distantly from the N-terminal Thr prevents the ability of HslV to interact with HslU and hence to stimulate the ATPase activity of HslU. Thus, it appears that Ser^{124} somehow participates in the catalytic function of HslV and Ser^{172} is involved in the HslV/HslU complex formation.

HslVU protease also is a protein-activated ATPase (33). However, ATPγS which is a nonhydrolyzable ATP analog can support the hydrolysis of both peptides and model protein substrates by HslVU protease much better than ATP (37). Similar observation has been made with β,γ-imido-ATP but only in the presence of KCl (38). Furthermore, the HslV/HslU complex formation as well as the HslU oligomerization can be achieved at much lower concentrations of ATPγS than ATP. In addition, the K_m for ATP (less than 30 μM) in supporting the proteolytic activity of HslVU protease is different from that for ATP hydrolysis (0.25 mM) (33, 37). Thus, it seems clear that the assembly of HslV/HslU complex and its proteolytic activity requires ATP binding but not its hydrolysis. However, it remains to be determined whether degradation of *in vivo* substrates also requires ATP-binding only.

ADP can support oligomerization of HslU (37). However, ADP-bound HslU cannot be assembled to the HslV/HslU complex. Thus, the conformations of ADP-bound and ATP- or ATPγS-bound form of HslU must be different, like those of ClpA. ADP inhibits the assembly of the HslV/HslU complex in a concentration-dependent fashion, and a much higher concentration of ADP is required for preventing the formation of the ATPγS-bound complex than the ATP-bound complex (37). Furthermore, even in the absence of ADP, the HslV/HslU complex formed in the presence of ATP, but not the ATPγS-bound complex, spontaneously dissociates into individual components upon prolonged incubation. Thus, ADP generated upon ATP hydrolysis must be responsible for dissociation of the HslV/HslU complex. Similar to protease Ti, the association-and-dissociation of HslV and HslU at the expense of ATP may be involved in rapid recycling of HslVU protease in the process of proteolysis or blocking inappropriate or excessive degradation of cell proteins. A model for assembly and disassembly of HslVU protease is shown in Fig. 1.

ACKNOWLEDGMENT

This work was supported by grants from Korea Science and Engineering Foundation through RCCD and the Korea Ministry of Education to C.H.C.

REFERENCES

1. Goldberg, A. L. (1992) *Eur. J. Biochem.* **203,** 9–23.
2. Maurizi, M. R. (1992) *Experientia* **48,** 178–201.
3. Chung, C. H. (1993) *Science* **262,** 372–374.
4. Chung, C. H., Seol, J. H., and Kang, M. S. (1996) *Biol. Chem.* **377,** 549–554.
5. Gottesman, S. (1996) *Annu. Rev. Genet.* **30,** 465–506.
6. Khattar, M. M. (1997) *FEBS Lett.* **414,** 402–404.
7. Goldberg, A. L., Moerschell, R. P., Chung, C. H., and Maurizi, M. R. (1994) *Methods Enzymol.* **244,** 350–375.

8. Fischer, H., and Glockshuber, R. (1993) *J. Biol. Chem.* **268,** 22502–22507.
9. Van Melderen, L., Thi, M. H. D., Lecchi, P., Gottesman, S., Couturier, M., and Maurizi, M. R. (1996) *J. Biol. Chem.* **271,** 27730–27738.
10. Zehnbauer, B. A., Foley, E. C., Herderson, G. W., and Markovitz, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2043–2047.
11. Chung, C. H., and Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 795–799.
12. Fu, G. K., Smith, M. J., and Markovitz, D. M. (1997) *J. Biol. Chem.* **272,** 534–538.
13. Schirmer, E. C., Golver, J. R., Singer, M. A., and Lindquist, S. (1996) *Trends Biochem. Sci.* **21,** 289–296.
14. Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K., and Steven, A. C. (1995) *J. Mol. Biol.* **250,** 587–594.
15. Seol, J. H., Baek, S. H., Kang, M. S., Ha, D. B., and Chung, C. H. (1995) *J. Biol. Chem.* **270,** 8087–8092.
16. Seol, J. H., Woo, K. M., Kang, M. S., Ha, D. B., and Chung, C. H. (1995) *Biochem. Biophys. Res. Commun.* **217,** 41–51.
17. Woo, K. M., Chung, W. J., Ha, D. B., Goldberg, A. L., and Chung, C. H. (1989) *J. Biol. Chem.* **264,** 2088–2091.
18. Thompson, M. W., and Maurizi, M. R. (1994) *J. Biol. Chem.* **269,** 18201–18208.
19. Wickner, S., Gottesman, S., Skowyra, D., Hoskins, J., McKenny, K., and Maurizi, M. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12218–12222.
20. Wawzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C., and Zylicz, M. (1995) *EMBO J.* **14,** 1867–1877.
21. Wojtkowiak, D., Georgopoulos, C., and Zylicz, M. (1993) *J. Biol. Chem.* **268,** 22609–22617.
22. Gottesman, S., Clark, W. P., de Crecy-Lagard, V., and Maurizi, M. R. (1993) *J. Biol. Chem.* **268,** 22618–22626.
23. Mhammedi-Alaoui, A., Pato, M., Gama, M. J., and Toussaint, A. (1994) *Mol. Microbiol.* **11,** 1109–1116.
24. Seol, J. H., Yoo, S. J., Kim, K. I., Kang, M. S., Ha, D. B., and Chung, C. H. (1994) *J. Biol. Chem.* **269,** 29468–29473.
25. Seol, J. H., Kwon, J., Yoo, S. J., Kim, H. S., Kang, M. S., and Chung, C. H. (1997) *Biol. Chem.* in press.
26. Seol, J. H., Yoo, S. J., Kang, M. S., Ha, D. B., and Chung, C. H. (1995) *FEBS Lett.* **377,** 41–43.
27. Rohrwild, M., Coux, O., Hunag, H. -C., Moerschell, R. P., Yoo, S. J., Seol, J. H., Chung, C. H., and Goldberg, A. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5808–5813.
28. Yoo, S. J., Seol, J. H., Shin D. H., Rohrwild, M., Kang, M. S., Tanaka, K., Goldberg, A. L., and Chung, C. H. (1996) *J. Biol. Chem.* **271,** 14035–14040.
29. Missiakas, D., Schwager, F., Betton, J. -M., Georgopoulos, C., and Rains, S. (1996) *EMBO J.* **15,** 6899–6909.
30. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R., and Baumeister, W. (1995) *Science* **268,** 579–582.
31. Shin, D. H., Yoo, S. J., Shim, Y. K., Seol, J. H., Kang, M. S., and Chung, C. H. (1996) *FEBS Lett.* **398,** 151–154.
32. Rohrwild, M., Pfeifer, G., Santarius, U., Muller, S. A., Huang, H. -C., Enger, A., Baumeister, W., and Goldberg, A. L. (1997) *Nature Struct. Biol.* **4,** 133–139.
33. Seol, J. H., Yoo, S. J., Shin, D. H., Shim, Y. K., Kang, M. S., Goldberg, A. L., and Chung, C. H. (1997) *Eur. J. Biochem.* **247,** 1143–1150.
34. Fenteany, G., Stanaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268,** 726–731.
35. Yoo, S. J., Shim, Y. K., Seong, I. S., Seol, J. H., Kang, M. S., and Chung, C. H. (1997) *FEBS Lett.* **412,** 57–60.
36. Bochehtler, M., Ditzer, L., Groll, M., and Huber, R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6070–6074.
37. Yoo, S. J., Seol, J. H., Seong, I. K., Kang, M. S., and Chung, C. H. (1997) *Biochem. Biophys. Res. Commun.* **238,** 581–586.
38. Huang, H. -C., and Goldberg, A. L. (1997) *J. Biol. Chem.* **272,** 21364–21372.