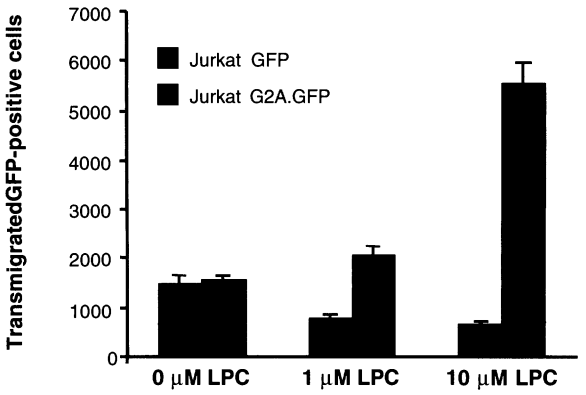

Fig. 4. LPC stimulates migration in G2A-expressing Jurkat T cells. A total of 10^5 Jurkat GFP or Jurkat G2A.GFP cells (both populations 20% GFP-positive) were allowed to transmigrate through 5-μm pore-size membranes toward the indicated concentrations of LPC for 1 hour. GFP-positive fractions (%) and cell numbers of transmigrated populations were measured by flow cytometry. Results are presented as numbers of transmigrated GFP-positive cells. Assays were performed in triplicate and results shown are representative of three independent experiments.

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19. Calcium assays were performed as described (*12*). MCF10A cells were loaded with Fura-2/AM (Molecular Probes) and $[Ca^{2+}]_i$ increases were measured in single transfected (GFP-positive) cells with a dual-wavelength spectrofluorometer (RFK-6002, Photon Technology, Brunswick, NJ) coupled to an inverted fluorescence microscope (Olympus, IX-70, Lake Success, NY).
20. HEK 293 GFP or HEK 293 G2A.GFP cells were serum-starved for 20 hours and collected in phosphate-buffered saline (PBS)/EDTA. Pelleted cells were stored at −80°C until use. Frozen cells were homogenized in "binding buffer" (10^6 cells/ml) (*13*). Assays were performed in 96-well plates in triplicate with 100-μl cell homogenate. [^{3}H]–16:0 LPC or [^{3}H]SPC were added to cell homogenates in 50 μl of binding buffer in the presence or absence of cold 16:0 LPC or SPC, or other competitors. Plates were incubated at 4°C for 2 hours, or for the indicated times. Cell-bound [^{3}H]LPC or [^{3}H]SPC was collected onto a filter (Printed Filtermat A, Wallac, Gaithersburg, MD) with an automated cell harvester (Harvester 96, Tomtec, Orange, CT). Specific binding was calculated by subtraction of nonspecific binding (in the presence of 100-fold excess unlabeled lipid) from total binding. [^{3}H]–18:0 LPC and [^{3}H]SPC were from Amersham Pharmacia Biotech (Buckinghamshire, England) (102 Ci/mmol, 1 mCi/ml for [^{3}H]–18:0 LPC, and 68 Ci/mmol, 1 mCi/ml for [^{3}H]SPC). [^{3}H] 16:0–LPC (60 Ci/mmol) was from American Radiolabelled Chemicals (St Louis, MO). LPCs (14:0, 16:0, 18:0, and 18:1), LPI, LPA, C16-PAF, and C16-lysoPAF were from Avanti Polar Lipids, (Alabaster, AL). Sphingomyelins (16:0 and 18:0), S1P, and SPC were from Toronto Research Chemicals (Toronto, ON) or Matreya (Pleasant Gap, PA).
21. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/293/5530/702/DC1.
22. HEK 293 G2A.GFP cells seeded onto glass cover slips were serum-starved for 18 hours before treatment with agonists for 2 hours. Cover slips were washed with PBS and fixed with PBS–4% paraformaldehyde. Subcellular localization of G2A.GFP was visualized under a confocal fluorescence microscope with an oil immersion lens (magnification, ×60).
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24. CHO GFP or CHO G2A.GFP cells were serum-starved for 18 hours before treatment with agonist for 10 min at 37°C. Western blotting was performed to detect total ERK MAP kinase with a polyclonal antibody to ERK2, and activated p44/42 ERK MAP kinase with a specific antibody to phospho-ERK (Santa Cruz Biotechnology).
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26. Although G2A is expressed in Jurkat cells, our experimental strategy was based on the hypothesis that increased expression in a physiologically relevant cell type may elicit a biological response. For transmigration assays, Jurkat GFP and Jurkat G2A.GFP cells were derived by retroviral infection and assayed 48 hours later. GFP-positive fractions of Jurkat GFP and Jurkat G2A.GFP populations were adjusted to 20% by the addition of appropriate numbers of parental Jurkat cells. Cells were washed three times in RPMI containing 0.25% bovine serum albumin (BSA) and finally resuspended in RPMI–0.25% BSA at 2×10^6 cells/ml. One hundred microliters of this cell suspension (10^5 cells) was applied to the upper chamber of a 6.5-mm diameter transwell cell culture insert comprising a 5-μm pore-size polycarbonate membrane (Corning Costar Corporation, Cambridge, MA) and containing 600 μl RPMI–0.25% BSA with or without agonist in the lower chamber. After incubation at 37°C for 1 hour, transmigrated cells were collected and analyzed by flow cytometry for GFP expression and cell number.
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Role of Inorganic Polyphosphate in Promoting Ribosomal Protein Degradation by the Lon Protease in *E. coli*

Akio Kuroda,[1]* Kazutaka Nomura,[1] Ryo Ohtomo,[2] Junichi Kato,[1] Tsukasa Ikeda,[1] Noboru Takiguchi,[1] Hisao Ohtake,[1] Arthur Kornberg[2]

Inorganic polyphosphate (polyP), a polymer of hundreds of phosphate (P_i) residues, accumulates in *Escherichia coli* in response to stresses, including amino acid starvation. Here we show that the adenosine 5′-triphosphate–dependent protease Lon formed a complex with polyP and degraded most of the ribosomal proteins, including S2, L9, and L13. Purified S2 also bound to polyP and formed a complex with Lon in the presence of polyP. Thus, polyP may promote ribosomal protein degradation by the Lon protease, thereby supplying the amino acids needed to respond to starvation.

PolyP is found in all microbes, fungi, plants, and animals (*1*). In *Escherichia coli*, levels of polyP are low in the exponential phase of growth, but increase more than 100-fold in response to acute stresses such as amino acid starvation and the multiple stresses in stationary phase (*2*, *3*). An *E. coli* mutant deficient in polyphosphate kinase (PPK), the principal enzyme for the synthesis of polyP, shows an extended lag in growth when shifted from a rich to a minimal medium (downshift); addition of amino acids abolishes the growth lag (*4*). Degradation of intracellular proteins is important in providing amino acids for use in the synthesis of the enzymes required for adaptations to starvation (*4*, *5*). The mutant fails to increase protein turnover after the downshift and thus extends the lag (*4*). In yeast and animal cells, the bulk degradation of proteins in response to starvation and cellular differentiation occurs by a ubiquitin-style conjugation system (*6*). However, in bacteria, the mechanisms underlying the regulation of intracellular protein degradation during amino acid starvation remain unknown (*5*).

In *E. coli*, more than 90% of cytoplasmic

[1]Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-4-1 Kagamiyama, Hiroshima 739-8527, Japan. [2]Department of Biochemistry, Stanford University, Stanford, CA 94305–5307, USA.

*To whom correspondence should be addressed. E-mail: akuroda@hiroshima-u.ac.jp

protein degradation is energy dependent (*7*). The ATP-dependent protease Lon, conserved from bacteria to humans (*8*, *9*), is responsible for the rapid turnover of both abnormal and naturally unstable proteins (*7*, *10*). Lon and two other proteases (ClpAP and ClpXP) are responsible for 70 to 80% of the energy-dependent protein degradation in *E. coli* (*7*). Here, mutants deficient in either the Lon or Clp proteases (but not the HslVU protease) showed an extended growth lag after the downshift, which was more severe in the double mutant (*lon*, *clp*) (Fig. 1A). The double mutant was also defective in protein turnover after the downshift (Fig. 1B), as is the *ppk* mutant (*4*). Addition of amino acids (50 mg/liter) abolished the growth lag of the protease mutants; once adapted to minimal medium, the mutants grew well on it. Both the Lon and Clp proteases were therefore involved in the increase in protein turnover after the downshift. Because the concentrations of adenosine 5′-triphosphate (ATP) after the downshift were similar in the wild type and the *ppk* mutant, polyP is not likely to serve as an energy source. The rate of protein turnover of a triple mutant (*lon*, *clp*, Δ*ppk-ppx*) was virtually identical to that of the *lon*, *clp* double mutant (Fig. 1B), consistent with polyP operating in the same pathway for protein degradation as the Lon and Clp proteases.

A linear chain of ^{32}P-labeled polyP [~700 inorganic phosphate (P_i) residues] was synthesized with *E. coli* PPK and [γ-^{32}P]ATP (*2*, *3*). Purified Lon protease (*8*) bound to the ^{32}P-labeled polyP even in 10 mM P_i (Fig. 2A). Presumed complexes of Lon with polyP increased with the concentration of Lon; two bands appeared shifted from the position corresponding to polyP (Fig. 2A). When DNA (pUC119), which binds Lon (*8*), was added with equimolar amounts (equivalent to 26 times the mass of polyP), there was no effect on the binding of polyP to Lon, nor was there any detectable complex of bovine serum albumin (BSA) with polyP even at 100 times the molar amounts of Lon (Fig. 2A). Previous studies (*7*, *8*), as well as our assay by gel filtration chromatography, indicated that Lon formed a tetramer under these conditions. A filter-binding assay showed that Lon bound to polyP with a dissociation constant (K_d) of about 0.5 nM irrespective of the presence of ATP, and that one tetramer of Lon bound to one molecule of polyP (Fig. 2B).

An *E. coli* lysate was fractionated by phospho-cellulose or DEAE-cellulose, and then each fraction was subjected to proteolysis by MBP-Lon (*11*) in the presence and absence of polyP. Among many proteins tested, a few were degraded only in the presence of polyP (Fig. 3A). NH_2-terminal sequences of those proteins corresponded to those of ribosomal proteins S2, L9, and L13, respectively (*12*). PolyP at 1 μM (0.7 mM as P_i residues) was effective for degradation of the S2 and L13 proteins (Fig. 3B). In response to amino acid starvation, levels of polyP increase even at concentrations >14 μM (10 mM as P_i residues) (*2*, *3*) and far exceed those required for the effective degradation of these proteins. The S2 protein remained stable even after 100 min in the absence of polyP, but was degraded by Lon in the presence of 1.4 μM polyP with a half-life of about 50 min (Fig. 3C); ATP was also required for the rapid S2 degradation by Lon. A shorter chain length of polyP (P_{65}) was less active in stimulating Lon-dependent degradation of S2 as well as of other ribosomal proteins, whereas P_{15} was inactive (Fig. 3D). PolyP did not affect casein hydrolysis by the Lon protease. To monitor the in vivo degradation of the S2 protein after the downshift, we expressed a S2–V5-epitope fusion (*13*) in the wild type and the *ppk* mutant. The S2-V5 fusion was stable without the downshift but was degraded rapidly after the downshift, and only in the wild type (Fig. 3E); the S2-V5 fusion was stable in either the *lon* or *ppk* mutants after the downshift (Fig. 3E). Thus, the S2 protein was subject to Lon-dependent degradation in the presence of polyP in vivo as well as in vitro.

The S2 protein binds late in the assembly process of the ribosome, is localized on the surface of the 30*S* subunit (*14*), and is essential for ribosomal function. Purified S2 bound

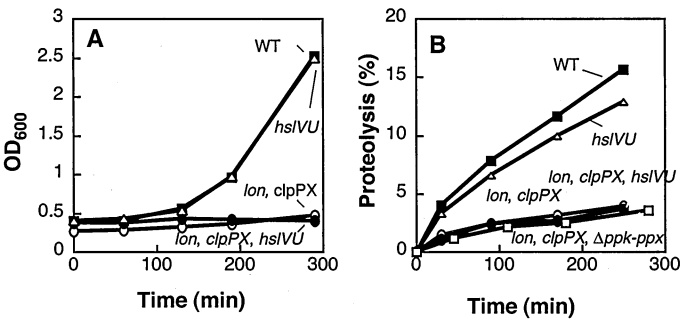

Fig. 1. Growth lag of *E. coli* protease mutants after downshift and rates of protein turnover. (**A**) *Escherichia coli* MG1655 and its derivatives KY2966 (*hslVU*), KY2347 (*lon*, *clpPX*), and KY2350 (*lon*, *clpPX*, *hslVU*) (*20*) were grown to mid-log phase in an amino acid–rich medium and downshifted to a minimal medium (*4*). Growth was measured as the optical density at 600 nm (OD_{600}) after the downshift. (**B**) Rates of intracellular protein degradation were measured (*4*); proteolysis was expressed as the percentage increase of the trichloroacetic acid–soluble radioactivity relative to the total incorporation (^{14}C-leucine).

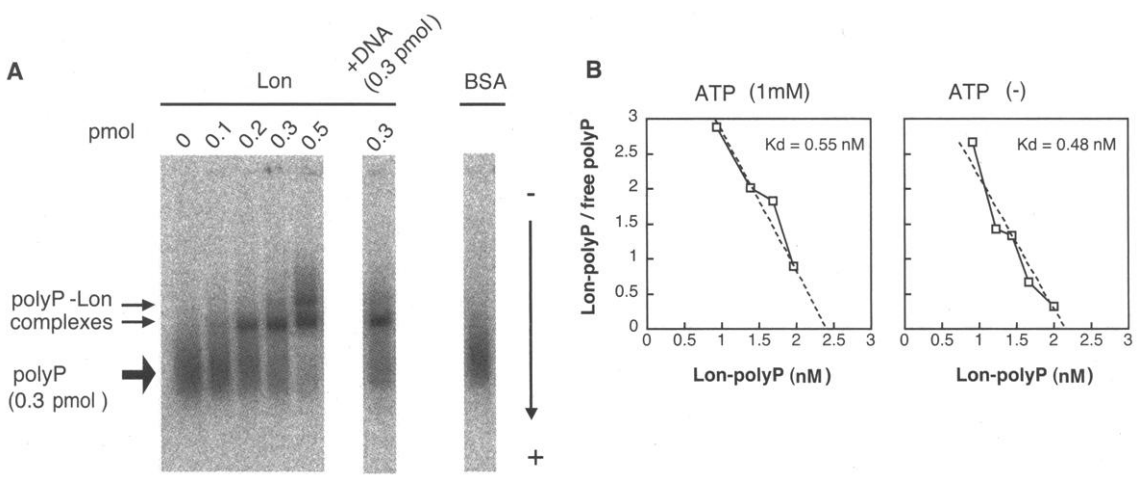

Fig. 2. Binding of polyP by purified Lon. (**A**) ^{32}P-labeled polyP (*3*) was incubated with purified Lon (*8*) at room temperature in the presence of 50 mM tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, and 10 mM P_i and then subjected to 1% agarose gel electrophoresis (TAE buffer). PolyP used in the experiments was the long-chain polyP (700 P_i residues), unless indicated otherwise. (**B**) A polyP-binding assay of Lon (2.5 nM as a tetramer) was performed with a nitrocellulose filter (*21*). ^{32}P-polyP was used at concentrations of 0.86 to 8.6 nM. A Scatchard plot yielded a straight line with a slope of $-1/K_d$ and intercept on the *x* axis corresponding to the maximum concentration of ^{32}P–polyP-Lon.

to polyP, as determined by the filter-binding assay, with a K_d of ~12 nM; the binding to polyP was observed at a level of 55% even in the presence of a 10 times the mass of pUC119 DNA and at a level of 30% in the presence of a 10 times the mass of *E. coli* total RNA (Fig. 4A). The complex formation of Lon with substrate may be necessary for efficient degradation (*15*). Lon formed a

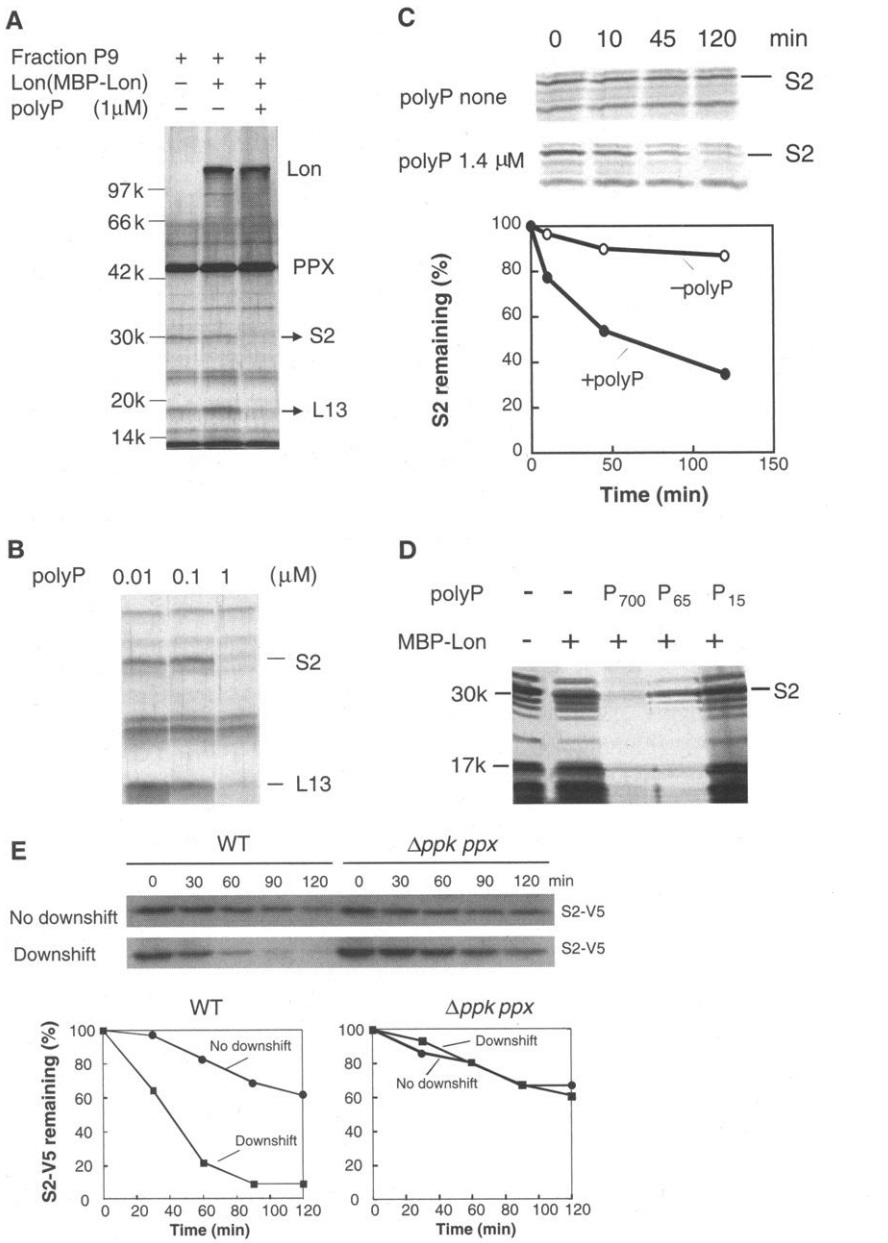

Fig. 3. Degradation of ribosomal proteins by MBP-Lon or Lon in the presence of polyP in vitro and in vivo after the downshift. (**A**) A phospho-cellulose fraction (Fraction P9) obtained from the *E. coli* lysate (*22*) was incubated with 0.6 μg of MBP-Lon (*11*) in the presence of polyP (0.7 mM as P_i; 1 μM as polymer). After incubation at 37°C for 60 min, exopolyphosphatase (yeast PPX, 3×10^4 U) was added to degrade the polyP; after 5 min, the mixture was subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE) and then visualized by silver staining (Di-ichi). (**B**) Fraction P9 was incubated with MBP-Lon (0.6 μg) in the presence of polyP (0.01, 0.1, or 1 μM) for 60 min and analyzed as described in (A). (**C**) Fraction P9 was incubated with Lon (1 μg) in the presence of polyP (1.4 μM). Samples were removed from the reaction mixture at the indicated times and then analyzed as in (A). Yeast PPX was not used. (**D**) PolyP with chain lengths of 65 and 15 residues (Sigma) was used for degradation of ribosomal proteins (*23*). The ribosomal proteins (2 μg) were incubated with MBP-Lon (0.1 μg) in the presence of polyP (0.64 mM as P_i) for 60 min at 37°C and then analyzed as in (A). (**E**) S2–V5-epitope fusion (*13*) was expressed in the wild type and in the *ppk* mutant on 2× YT medium for 2 hours in the presence of 0.2% L-arabinose. Cells were collected by centrifugation (10 min, 3000*g*) and resuspended in the 2× YT medium without L-arabinose (No downshift) or in the MOPS minimal medium without L-arabinose (Downshift). At the indicated times, total proteins (100 μl of the culture) were subjected to SDS-PAGE and Western analysis with an antibody to V5 epitope (Invitrogen).

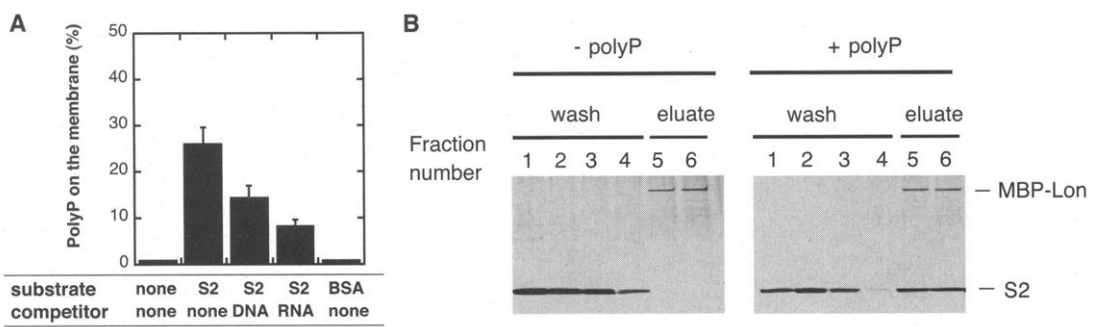

Fig. 4. A presumed complex formation with Lon and S2 in the presence of polyP. (**A**) A polyP-binding assay of the purified S2 protein (*24*) was performed with a nitrocellulose filter (*21*). The purified S2 (0.45 μg) or BSA (2 μg) was subjected to binding with 0.04 μg of ^{32}P-polyP (11 nM) in the presence of 0.45 μg of DNA (pUC119) or 0.48 μg of RNA (total *E. coli* RNA). (**B**) Purified MBP-Lon (12 μg) and S2 (50 μg) were incubated in 20 mM tris-HCl (pH 7.4) and 5 mM $MgCl_2$ in the presence or absence of 0.2 μM polyP for 10 min at 37°C without ATP. The mixture (500 μl) was applied onto a 1-ml column embedded with amylose resin. After washing the column with 50 mM tris-HCl (pH 7.4) and 200 mM NaCl (fractions 1 to 4), MBP-Lon was eluted with the same buffer containing 10 mM maltose (fractions 5 and 6). Fractions were analyzed by SDS-PAGE.

complex with S2 in the presence of polyP (Fig. 4B), suggesting that binding of S2 to polyP helped in the formation of a complex with Lon. Addition of polyP (without substrates) did not stimulate the adenosine triphosphatase activity of Lon. Thus, the stimulation of S2 degradation can be ascribed mainly to the formation of a complex between Lon and S2 in the presence of polyP.

Escherichia coli contains substantial amounts of ribosomal proteins, including S2, as disassembled (free) forms during exponential growth on a rich medium, but little on a minimal medium (*16*). About 17% of total S2 protein exists as the free form (*16*), and such ribosomal proteins may be subjected to Lon-dependent degradation (*17*). Thus, it is likely that the S2 protein found in fraction P9 was free from ribosomes, and that polyP was involved in the degradation of free ribosomal proteins after the downshift. Degradation of ribosomal proteins should release amino acids for synthesis of the key enzymes required for adaptations to starvation, as well as reduce translational activity during starvation. Most substrates for polyP-dependent degradation were basic ribosomal proteins that could bind to polyP [see supplementary material (*18*)]. In assays of polyP binding to proteins in *E. coli* lysates, most proteins were contained within the ribosome fractions, but also included Lon as well as ribosome-associated proteins. Thus, it is likely that the ribosomal proteins are the major substrates (in terms of mass) for this system. When we used intact ribosomes as substrates for Lon, Lon with polyP was ineffective in degrading intact ribosomes, but did act on ribosomes treated with ribonuclease (RNase) (*18*). Thus, polyP alone did not disassemble the ribosome, but polyP and Lon together degraded free ribosomal proteins, as well as those in the RNase-distorted ribosome. Our findings may provide insights into the regulation of protein degradation when cells are stressed or enter the stationary phase.

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11. Lon protease fused with maltose-binding protein (MBP-Lon) was purified from *E. coli* (pMAL-Lon) as described (*19*).
12. NH_2-terminal sequences of the proteins tested were ATVSMRDMLKAGVHF, MQVILLDKVAN, and MKTFTAKPETV, which matched precisely the amino acid sequences of S2 (residues 2 to 16), L9 (residues 1 to 11), and L13 (residues 1 to 11), respectively.
13. A *rpsB* (S2) gene was amplified by polymerase chain reaction with primers 5′-ATGGCAACTGTTTCCATGCGCG-3′ and 5′-CTCAGCTTCTACGAAGCTTTCT-3′, and then inserted into pBAD-TOPO plasmid (Invitrogen) to construct a S2–V5-epitope fusion gene. S2–V5-epitope fusion protein was expressed only in the presence of 0.2% L-arabinose.
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21. Purified protein was diluted at various concentrations of ^{32}P-$polyP_{700}$ (295 cpm/pmol) in 50 μl of 20 mM tris-HCl buffer (pH 7.4) and 5 mM $MgCl_2$. After incubation at 37°C for 5 min, the mixture was transferred to a nitrocellulose filter (0.45 μm), and then free ^{32}P-polyP was washed off with the same buffer containing 100 mM NaCl. The radioactivity remaining on the filter, corresponding to the amount of polyP-protein complex, was measured by scintillation counting.
22. *E. coli* MG1655 (in late exponential phase) was subjected to lysis with lysozyme and centrifuged (30 min, 30,000*g*) and then the supernatant was applied onto a phospho-cellulose column (P11, Whatman) equilibrated with 0.1 M potassium P_i. Fractions were eluted with a linear gradient of 0.1 to 0.4 M potassium P_i. Fraction P9 eluted near 0.4 M potassium P_i.
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24. Fraction P9 (*22*) containing the S2 protein was applied onto a MiniQ column (Smart, Amersham-Pharmacia-Biotech) equilibrated with 20 mM tris-HCl (pH 7.4), 0.5 mM dithiothreitol, and 1 mM EDTA. The S2 protein was eluted with a linear gradient from 20 to 500 mM NaCl. The S2 protein was also purified from 70*S* ribosome as described (*23*).

25. We thank T. Yura and M. Kanemori for providing the protease mutants. Some of the protease mutants are originally from S. Gottesman (NIH). We thank Y. Ishii for providing plasmid pMAL-Lon, and I. R. Lehman, D. Kaiser, N. Rao, C. D. Fraley, L. Bertsch, and K. Mizuta for helpful suggestions. Supported by grants from the Ministry of Education, Science and Culture of Japan and the National Institutes of Health.

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The Crystal Structure of Uncomplexed Actin in the ADP State

Ludovic R. Otterbein, Philip Graceffa, Roberto Dominguez*

The dynamics and polarity of actin filaments are controlled by a conformational change coupled to the hydrolysis of adenosine 5′-triphosphate (ATP) by a mechanism that remains to be elucidated. Actin modified to block polymerization was crystallized in the adenosine 5′-diphosphate (ADP) state, and the structure was solved to 1.54 angstrom resolution. Compared with previous ATP-actin structures from complexes with deoxyribonuclease I, profilin, and gelsolin, monomeric ADP-actin is characterized by a marked conformational change in subdomain 2. The successful crystallization of monomeric actin opens the way to future structure determinations of actin complexes with actin-binding proteins such as myosin.

Actin is the major component of the thin filaments of muscle cells and of the cytoskeletal system of nonmuscle cells, taking part in a multitude of biological functions (*1*, *2*). Monomeric actin (G-actin) assembles under physiological salt concentrations to form polymers (F-actin), a property that has so far prevented crystallization of the uncomplexed monomer. Structures have been determined, however, for complexes of actin with deoxyribonuclease I (DNase I) (*3*), gelsolin (*4*, *5*), and profilin (*6*), all proteins that prevent polymerization.

Actin and most members of its structural class, which includes the hsp70 molecular chaperones, hexokinase, and the sugar kinases (*7*, *8*), appear to undergo a conformational change coupled to ATP hydrolysis. In actin, this conformational change may play a critical role in filament dynamics (*1*, *9*). ADP-actin filaments exhibit a higher susceptibility to depolymerizing proteins (*2*). The addition of inorganic phosphate (P_i) or P_i analogs helps stabilize the ADP-actin filaments (*10*, *11*), presumably by mimicking an ADP·P_i state. The different properties of ADP·P_i- and ADP-actin filaments have been correlated with differences in their tertiary structures (*12*, *13*). Actin subdomain 2 and, in particular, its DNase I binding loop, which is involved in intermonomer interactions within the filament (*14*), have been directly linked with the conformational change by studies with electron microscopy (*12*, *15*), proteolysis (*16–18*), and fluorescence spectroscopy (*19–21*).

Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, USA.

*To whom correspondence should be addressed. E-mail: dominguez@bbri.org