

cGMP generation and subsequent activation of PKG are required for acid-induced changes in H^+/HCO_3^- fluxes. Many of the effects of the signaling molecule NO are mediated by stimulation of NO-sensitive guanylate cyclase resulting in cGMP formation and the subsequent activation of cGMP-dependent protein kinase (PKG) (7, 15). We tested the effect on HCO_3^- transport of 10 μ M LY-83583, an inhibitor of soluble guanylate cyclase, during acid incubation (Fig. 2). LY-83583 markedly attenuated the reversal of polarity of net HCO_3^- flux in response to low pH, and there was no net HCO_3^- flux (-3.76 ± 0.17 to 0.18 ± 0.09 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, $P < 0.01$; Table 1); at 3.9 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, the difference between acid-incubated and basal net flux was much smaller than vehicle or inactive agent (D-NAME or BQ-123, Table 1 and Fig. 2). There was a only a 16% increase in H^+ secretion, smaller than seen with vehicle or inactive agent (3.25 ± 0.11 to 3.79 ± 0.17 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, $P < 0.05$; Table 1 and Fig. 3), and a 49% reduction in HCO_3^- secretion (-7.01 ± 0.27 to -3.60 ± 0.23 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, $P < 0.01$), such that at 51% the ratio of acid-incubated/basal HCO_3^- secretory flux was significantly increased compared with vehicle or inactive agent (Fig. 4). The increase in acid/basal H^+ secretion and the decrease in acid/basal HCO_3^- secretion were smaller than those observed after acid incubation with vehicle ($P < 0.05$; Table 1).

The role of PKG in the acid-induced adaptation was tested by examining the effect of 2 μ M KT-5823, a specific cell-permeant inhibitor of PKG, on H^+/HCO_3^- fluxes. KT-5823 substantially prevented the reversal of polarity of HCO_3^- flux in response to low pH, so that there was no net flux after acid incubation (-4.22 ± 0.15 to 0.08 ± 0.05 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, $P < 0.01$; Table 1); at 4.3 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, the change in net flux from basal to acid incubated was significantly smaller than vehicle or inactive controls (Fig. 2). The increase in acid/basal H^+ secretion and the decrease in acid/basal HCO_3^- secretion were smaller than those observed after acid incubation with vehicle or inactive controls ($P < 0.05$; Table 1 and Figs. 3 and 4). These results suggest that inhibitors of guanylate cyclase and PKG partially prevent the adaptive increase in H^+ secretion and the decrease in HCO_3^- secretion that would be anticipated in response to metabolic acidosis.

Taken together, these data indicate that the adaptive decrease in HCO_3^- secretion in response to acidosis is mediated in large part by ET-ET_B-NO-guanylate cyclase-PKG signaling pathway. In contrast, the adaptive increase in H^+ secretion, which may be directly stimulated by ET-ET_B signaling, tends to be less affected by the NO-signaling pathway.

DISCUSSION

To investigate the role of ET and NO in intercalated cells of the CCD, we made use of our *in vitro* acidosis model in which incubation of a CCD for 3 h at pH 6.8 induces a change in polarity of net HCO_3^- flux from net secretion to net absorption (21, 26). This model recapitulates the pathophysiological condition of 3 days of NH_4Cl loading (in the drinking water) that results in metabolic acidosis *in vivo* (21, 25). The adaptation to acid treatment is accomplished by two adjustments: increased H^+ secretion, presumably by existing and perhaps recruited α -intercalated cells, and decreased HCO_3^- secretion by β -intercalated cells (23, 24, 33). The rabbit CCD contains both α - and β -intercalated cells, and both types are active under normal

conditions. Previously, we showed that luminal bafilomycin, by inhibiting the apical H^+ -ATPase of α -intercalated cells of CCDs from normal rabbits, reduces H^+ secretion and thereby makes the net HCO_3^- flux more negative (higher rate of secretion under basal conditions). When bafilomycin was used after acid incubation, the basal and adaptive increases in H^+ secretion were inhibited, such that net HCO_3^- transport was not significantly different from zero (33). The effect of inhibiting the H^+ - K^+ -ATPase was previously shown (33) to be much smaller than that of inhibiting the H^+ -ATPase in acid-adapted CCDs, indicating a limited role for the H^+ - K^+ -ATPase in adapting to metabolic acidosis. These data show that there is a substantial amount of HCO_3^- secretion in CCDs from normal rabbits, but after they are incubated at low pH, the HCO_3^- secretion rate is greatly diminished, such that when the offsetting H^+ secretion by α -intercalated cells is inhibited by bafilomycin, there is virtually no net HCO_3^- transport. These data also show that there are partially offsetting H^+ secretory fluxes in CCDs from normal rabbits but after acid incubation, the basal and adaptive increase in H^+ secretion are entirely inhibited by a vacuolar H^+ -ATPase inhibitor. In this study, we extended these observations by demonstrating that ET_B receptor signaling in response to low pH mediated the decreased HCO_3^- secretion as well as the adaptive increase in H^+ secretion. It is not surprising that the ET_B receptor antagonist entirely inhibited the adaptive increase in H^+ secretion, because Wesson's group showed inhibition of B- but A-type ET receptors blunts not only the decreased HCO_3^- secretion but also the increased H^+ secretion in the distal tubule of rats given dietary acid (42). Thus ET, via the ET_B receptor, regulates adaptive changes in H^+/HCO_3^- fluxes during metabolic acidosis.

NO has been found to play an important role in mediating salt and water transport, in addition to hemodynamics and tubuloglomerular feedback in the kidney (17). Less is known about how NO affects acid-base transport. Mice lacking nNOS have defective proximal tubule HCO_3^- reabsorption and develop metabolic acidosis (41). Mice lacking inducible NOS, but not endothelial NOS, also show reduced proximal HCO_3^- reabsorption (40). These studies show that inducible and nNOS stimulate proximal HCO_3^- reabsorption. Also, acidosis stimulates NO synthesis in rat lung (18) and canine heart (11), perhaps because the cellular acidosis increases cellular calcium levels (4, 38), and calcium activates NOS (15).

The CCD is known to express endothelial, inducible, and nNOS (1, 14, 30, 31, 37) and NO has important actions in the CCD. NO inhibits ADH-stimulated osmotic water permeability in the CCD (5, 6). Also, in M-1 cultured CCD cells, NO inhibits sodium transport at the apical membrane sodium channel (27). The present study adds modulation of β -intercalated cell HCO_3^- secretory fluxes in response to acidosis to the actions of NO on ion transport in the CCD. There are no reports of NO stimulating H^+ secretion in the CCD; and furthermore, results of this study fail to demonstrate a pivotal role for NO guanylate cyclase in upregulating H^+ secretion. However, Tojo et al. (31) showed that exogenous donors of NO inhibit bafilomycin-sensitive H^+ -ATPase activity in microdissected rat CCDs, suggesting that an inducible form of NOS downregulates H^+ secretion by α -intercalated cells. It is possible that ET-ET_B signaling to control H^+ secretion works via another pathway that predominates over the NO-guanylate cyclase system.

Activation of soluble guanylate cyclase leading to the production of cGMP is a key function of NO as a signal generator. cGMP has several targets, with the major one being PKG (7). cGMP has recently been shown to cause the membrane insertion of aquaporin-2 in renal epithelial cells (3). Results presented in the present study demonstrate that activation of guanylate cyclase and subsequent stimulation of PKG are critical for the adaptation of the β -intercalated cell to acidosis. Based on the analysis of HCO_3^- flux and cell pH changes in response to luminal Cl^- removal, we suggest that cGMP activation of PKG stimulates endocytosis of apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers, leading to a decrease in HCO_3^- secretion in response to acidosis. Identification of substrates of PKG that directly or indirectly influence endocytic pathways is an important area for further investigation.

GRANTS

This work was supported in part by a Grant-in-Aid from the American Heart Association (0455829T) New York State Affiliate (G. J. Schwartz) and by grants from the Ministry of Education, Culture, Sports, Science and Technology, and Ministry of Health, Labor and Welfare of Japan (S. Tsuruoka).

REFERENCES

- Ahn KY, Mohaupt MG, Madsen KM, and Kone BC. In situ hybridization localization of mRNA encoding inducible nitric oxide synthase in rat kidney. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F748–F757, 1994.
- Bhowmick N, Narayan P, and Puett D. The endothelin subtype A receptor undergoes agonist- and antagonist-mediated internalization in the absence of signaling. *Endocrinology* 139: 3185–3192, 1998.
- Bouley R, Breton S, Sun T, McLoughlin M, Nsumu NN, Lin HY, Ausiello DA, and Brown D. Nitric oxide and atrial natriuretic factor stimulate cGMP-dependent membrane insertion of aquaporin 2 in renal epithelial cells. *J Clin Invest* 106: 1115–1126, 2000.
- Cannon C, van Adelsberg J, Kelly S, and Al-Awqati Q. Carbon-dioxide-induced exocytotic insertion of H^+ pumps in turtle bladder luminal membrane: role of cell pH and calcium. *Nature* 314: 443–446, 1985.
- Garcia NH, Pomposiello SI, and Garvin JL. Nitric oxide inhibits ADH-stimulated osmotic water permeability in cortical collecting ducts. *Am J Physiol Renal Fluid Electrolyte Physiol* 270: F206–F210, 1996.
- Garcia NH, Stoos BA, Carretero OA, and Garvin JL. Mechanism of the nitric oxide-induced blockade of collecting duct water permeability. *Hypertension* 27: 679–683, 1996.
- Hanafy KA, Krümenacker JS, and Murad F. NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med Sci Monit* 7: 801–819, 2001.
- Hercule HC and Oyekan AO. Cytochrome P450 omega/omega-1 hydroxylase-derived eicosanoids contribute to endothelin(A) and endothelin(B) receptor-mediated vasoconstriction to endothelin-1 in the rat preglomerular arteriole. *J Pharmacol Exp Ther* 292: 1153–1160, 2000.
- Herrera M and Garvin JL. Endothelin stimulates endothelial nitric oxide synthase expression in the thick ascending limb. *Am J Physiol Renal Physiol* 287: F231–F235, 2004.
- Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, and Marumo F. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest* 91: 1367–1373, 1993.
- Kitakaze M, Node K, Takashima S, Asanuma H, Asakura M, Sanada S, Shinozaki Y, Mori H, Sató H, Kuzuya T, and Hori M. Role of cellular acidosis in production of nitric oxide in canine ischemic myocardium. *J Mol Cell Cardiol* 33: 1727–1737, 2001.
- Kohan DE. Endothelin synthesis by rabbit renal tubule cells. *Am J Physiol Renal Fluid Electrolyte Physiol* 261: F221–F226, 1991.
- Laghmani K, Preisig PA, Moe OW, Yanagisawa M, and Alpern RJ. Endothelin-1/endothelin-B receptor-mediated increases in NHE3 activity in chronic metabolic acidosis. *J Clin Invest* 107: 1563–1569, 2001.
- Mohaupt MG, Elzie JL, Ahn KY, Clapp WL, Wilcox CS, and Kone BC. Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int* 46: 653–665, 1994.
- Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002–2012, 1993.
- Ohuchi T, Yanagisawa M, and Garipey CE. Renal tubular effects of endothelin-B receptor signaling: its role in cardiovascular homeostasis and extracellular volume regulation. *Curr Opin Nephrol Hypertens* 9: 435–439, 2000.
- Ortiz PA and Garvin JL. Autocrine effects of nitric oxide on HCO_3^- transport by rat thick ascending limb. *Kidney Int* 58: 2069–2074, 2000.
- Ortiz PA and Garvin JL. Role of nitric oxide in the regulation of nephron transport. *Am J Physiol Renal Physiol* 282: F777–F784, 2002.
- Pedoto A, Caruso JE, Nandi J, Oler A, Hoffmann SP, Tassiopoulos AK, McGraw DJ, Camporesi EM, and Hakini TS. Acidosis stimulates nitric oxide production and lung damage in rats. *Am J Respir Crit Care Med* 159: 397–402, 1999.
- Plato CF, Pollock DM, and Garvin JL. Endothelin inhibits thick ascending limb chloride flux via ET_B receptor-mediated NO release. *Am J Physiol Renal Physiol* 279: F326–F333, 2000.
- Plato CF, Stoos BA, Wang D, and Garvin JL. Endogenous nitric oxide inhibits chloride transport in the thick ascending limb. *Am J Physiol Renal Physiol* 276: F159–F163, 1999.
- Satlin LM and Schwartz GJ. Cellular remodeling of HCO_3^- -secreting cells in rabbit renal collecting duct in response to an acidic environment. *J Cell Biol* 109: 1279–1288, 1989.
- Schnermann J, Lorenz JN, Briggs JP, and Keiser JA. Induction of water diuresis by endothelin in rats. *Am J Physiol Renal Fluid Electrolyte Physiol* 263: F516–F526, 1992.
- Schuster VL. Function and regulation of collecting duct intercalated cells. *Annu Rev Physiol* 55: 267–288, 1993.
- Schwartz GJ. Plasticity of intercalated cell polarity: effect of metabolic acidosis. *Nephron* 87: 304–313, 2001.
- Schwartz GJ, Barasch J, and Al-Awqati Q. Plasticity of functional epithelial polarity. *Nature* 318: 368–371, 1985.
- Schwartz GJ, Tsuruoka S, Vijayakumar S, Petrovic S, Mian A, and Al-Awqati Q. Acid incubation reverses the polarity of intercalated cell transporters, an effect mediated by hensen. *J Clin Invest* 109: 89–99, 2002.
- Stoos BA, Carretero OA, and Garvin JL. Endothelial-derived nitric oxide inhibits sodium transport by affecting apical membrane channels in cultured collecting duct cells. *J Am Soc Nephrol* 4: 1855–1860, 1994.
- Takemoto F, Uchida S, Ogata E, and Kurokawa K. Endothelin-1 and endothelin-3 binding to rat nephrons. *Am J Physiol Renal Fluid Electrolyte Physiol* 264: F827–F832, 1993.
- Terada Y, Tomita K, Nonoguchi H, and Marumo F. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. *J Clin Invest* 90: 659–665, 1992.
- Tojo A, Guzman NJ, Garg LC, Tisher CC, and Madsen KM. Nitric oxide inhibits bafilomycin-sensitive H^+ -ATPase activity in rat cortical collecting duct. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F509–F515, 1994.
- Tomita K, Nonoguchi H, Terada Y, and Marumo F. Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. *Am J Physiol Renal Fluid Electrolyte Physiol* 264: F690–F696, 1993.
- Tsuruoka S and Schwartz GJ. Adaptation of rabbit cortical collecting duct HCO_3^- transport to metabolic acidosis in vitro. *J Clin Invest* 97: 1076–1084, 1996.
- Tsuruoka S and Schwartz GJ. Metabolic acidosis stimulates H^+ secretion in the rabbit outer medullary collecting duct (inner stripe) of the kidney. *J Clin Invest* 99: 1420–1431, 1997.
- Tsuruoka S, Schwartz GJ, Wakaumi M, Nishiki K, Yamamoto H, Purkerson JM, and Fujimura A. Nitric oxide production modulates cyclosporin A-induced distal renal tubular acidosis in the rat. *J Pharmacol Exp Ther* 305: 840–845, 2003.
- Tsuruoka S, Swenson ER, Petrovic S, Fujimura A, and Schwartz GJ. Role of basolateral carbonic anhydrase in proximal tubular fluid and bicarbonate absorption. *Am J Physiol Renal Physiol* 280: F146–F154, 2001.
- Ujiié K, Yuen J, Hogarth L, Danziger R, and Star RA. Localization and regulation of endothelial NO synthase mRNA expression in rat kidney. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F296–F302, 1994.
- Van Adelsberg J and Al-Awqati Q. Regulation of cell pH by Ca^{2+} -mediated exocytotic insertion of H^+ -ATPases. *J Cell Biol* 102: 1638–1645, 1986.

39. Wang H, Carretero OA, and Garvin JL. Nitric oxide produced by THAL nitric oxide synthase inhibits TGF. *Hypertension* 39: 662–666, 2002.
40. Wang T. Role of iNOS and eNOS in modulating proximal tubule transport and acid-base balance. *Am J Physiol Renal Physiol* 283: F658–F662, 2002.
41. Wang T, Inglis FM, and Kalb RG. Defective fluid and HCO_3^- absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice. *Am J Physiol Renal Physiol* 279: F518–F524, 2000.
42. Wesson DE. Endogenous endothelins mediate increased distal tubule acidification induced by dietary acid in rats. *J Clin Invest* 99: 2203–2211, 1997.
43. Wesson DE and Dolson GM. Endothelin-1 increases rat distal tubule acidification in vivo. *Am J Physiol Renal Physiol* 273: F586–F594, 1997.
44. Wesson DE, Simoni J, and Green DF. Reduced extracellular pH increases endothelin-1 secretion by human renal microvascular endothelial cells. *J Clin Invest* 101: 578–583, 1998.
45. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415, 1988.
46. Yasoshima K, Satlin LM, and Schwartz GJ. Adaptation of rabbit cortical collecting duct to in vitro acid incubation. *Am J Physiol Renal Fluid Electrolyte Physiol* 263: F749–F756, 1992.



Polyamines Affect Polyphosphate Accumulation in *Escherichia coli*

KEI MOTOMURA¹, NOBORU TAKIGUCHI¹, HISAO OHTAKE² and AKIO KURODA^{1,3*}

¹ Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan

² Department of Biotechnology, Osaka University, Osaka 565-0871, Japan

³ SORST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

* TEL: 082-424-7758 FAX: 082-424-7047

* E-mail: akuroda@hiroshima-u.ac.jp

(Received; 4 April, 2006/Accepted; 1 May, 2006)

In the current studies, we showed that a *speABC* and *cadA* mutant of *Escherichia coli*, which fails to produce polyamines such as putrescine, spermidine, and cadaverine, accumulates reduced levels of polyphosphate (polyP) under conditions of amino acid starvation. This was reversed by supplementation of the culture medium with polyamines. In addition, a *speG* mutant, which accumulates polyamines, increased levels of polyP, suggesting that polyamines affect polyP accumulation. Although polyP granules are often found in cellular inclusions containing calcium, those isolated from *E. coli* did not contain calcium. We found that these polyP granules soon disappear when they are placed in a buffer but that they are stabilized by the presence of polyamines. We concluded that polyamines interact with polyP and affect polyP accumulation.

Key words: Polyphosphate, polyamine, *Escherichia coli*, polyphosphate granule

1. Introduction

Increased input of inorganic phosphate (P_i) into lakes, bays, and other surface waters causes the nuisance growth of phytoplankton, called an algal bloom⁶. Hence, considerable attention has been paid to efficient removal of P_i from wastewater^{6,28}. In traditional sewage works, 20% to 40% of P_i in wastewater is typically removed in the sludge by biological conversion to organic phosphate such as DNA, RNA, and phospholipid. Improvement of the ability to accumulate phosphorus compounds in the sludge should therefore help in the removal of P_i from wastewater.

Inorganic polyphosphate (polyP) is a linear polymer of many tens or hundreds of P_i molecules connected by high-energy bonds^{14,15}. PolyP is found in a wide range of prokaryotes and eukaryotes^{14,15}. PolyP has various biological functions, for example, as a P_i reservoir^{14,15}, an alternative source of high-energy bonds^{14,15}, as a buffer against alkaline conditions and metal accumulation²³, and as a regulator for transcription¹⁴ and proteolysis¹⁸. PolyP has an osmotic advantage over free P_i for accumulation of phosphorus in cells. Therefore, enhanced biological phosphorus removal is based on the use of bacteria capable of accumulating polyP.

PolyP kinase (PPK), a tetramer of 80-kDa subunits, is responsible for the processive synthesis of long-chain polyP (750 P_i residues) in *Escherichia coli*¹⁴. PPK catalyzes the reversible reaction of the γ -terminal P_i of ATP to polyP¹⁴. So far, *phoU*, *spoT*, and *relA* genes have been identified as regulators of polyP accumulation^{16,21}. A *phoU* mutant, which derepresses the phosphate regulon, accumulates

polyP²¹. Constitutive expression of the PstSCAB P_i uptake system in the *phoU* mutants is responsible for the elevated levels of polyP²¹. On the other hand, the level of polyP in *E. coli* is very low during the exponential phase but increases up to 1000-fold in response to amino acid starvation¹⁶. An *E. coli relA spoT* mutant that fails to produce guanosine penta- and tetra-phosphate (pppGpp and ppGpp) is deficient in the accumulation of polyP in response to amino acid starvation¹⁶. It was found that the stringent factors pppGpp and ppGpp strongly inhibit polyphosphatase PPX¹⁶. PPK and PPX activities are constitutively expressed, but when pppGpp and ppGpp build up in the cells during amino acid starvation, polyP may accumulate due to inhibition of PPX.

Polyamines, polycationic compounds present in all living organisms, have been implicated in a wide variety of biological reactions, including nucleic acid and protein synthesis^{3,10}. Polyamines are thought to participate in these cellular processes through binding to DNA and RNA^{10,11}. Polyamine content is regulated by its biosynthesis, degradation, uptake, and excretion^{9,22}. A polyamine-deficient strain of *E. coli* has been obtained as a result of deletions in *speA* (arginine decarboxylase), *speB* (agmatine ureohydrolase), *speC* (ornithine decarboxylase), and *cadA* (cadaverine biosynthesis)^{13,29}. Although this strain lacks putrescine, spermidine, and cadaverine, it is still able to slowly but indefinitely grow on polyamine-deficient media^{13,29}. In the presence of polyamines in the medium, an *speABC* and *cadA* mutant can take up polyamines and grow well. On the other hand, a spermidine acetyltransferase gene (*speG*) mutant that fails

to degrade polyamine accumulates approximately 3-fold higher levels of spermidine when grown in a medium containing spermidine⁹. We speculated that polyamines interact not only with DNA and RNA but also polyP, and we demonstrated that polyamines affect polyP accumulation under conditions of amino acid starvation.

2. Materials and Methods

2.1. Plasmid construction

A 2.6-kb *KpnI* fragment containing *ppk* of pBC10 was inserted into the *KpnI* site of pMW119. The orientation of the *ppk* insertion in the resulting plasmid pMWppk was opposite the *lac* promoter of pMW119¹⁷.

2.2. Bacterial strains and growth conditions

E. coli speABC, *cadA*, and *speG* mutants were constructed according to the one-step gene inactivation method described by Datsenko and Wanner⁹. First, DNA fragments containing target and antibiotic genes were prepared by polymerase chain reaction using the primers listed in Table 1. These DNA fragments were then introduced into *E. coli* and replaced with the chromosomal genes. The *speABC cadA* mutant was grown in a 2xYT rich medium (1.6% peptone, 1.0% yeast extract, and 0.5% NaCl) with shaking at 37°C for 12–18 h. This culture was diluted in a MOPS-minimal medium (22.2 mM glucose, 40 mM potassium morpholinopropane sulfonate [pH 7.2], 50 mM NaCl, 9.52 mM NH₄Cl, 4 mM Tricine, 2 mM K₂HPO₄, 0.52 mM MgCl₂, 0.28 mM K₂SO₄, 0.01 mM FeSO₄, 0.0005 mM CaCl₂, and trace metals)¹⁷ containing 2% casamino acid and 0.4 M sorbitol and then cultivated overnight. To deplete endogenous polyamines, this cultivation was repeated, and the culture was inoculated into the MOPS-minimal medium containing 2% casamino acid and 0.4M sorbitol. The fresh medium was supplemented with 1 mM putrescine and spermidine as necessary. After a 6-h incubation, the *E. coli speABC cadA* mutant was collected by centrifugation. The cell

pellet was washed once with the MOPS-minimal medium containing 0.4 M sorbitol and resuspended in the same medium (amino acid-starved condition). The *E. coli speG* mutant was grown to mid-log phase in the 2xYT medium with shaking at 37°C and shifted to the MOPS-minimal medium. PolyP granules were observed under fluorescent microscopy (BX-50, Olympus) after staining with 4',6-diamidino-2-phenylindole (DAPI). PolyP was recovered with silicate glass from cells lysed with guanidine isothiocyanate, and determined by a two-enzyme assay¹¹.

2.3. Preparation of polyP granules from *E. coli*

The *E. coli phoU* mutant was grown to mid-log phase in the 2xYT medium with shaking at 37°C and shifted to the MOPS-minimal medium for 2 h to cause the accumulation of a large amount of polyP-containing granules²¹. PolyP granules were prepared essentially as described by Jacobson¹². Freeze-dried cells (0.1 g), zirconia beads (diameter 2 mm; 3.5 g), and carbon tetrachloride (400 µl) were mixed in a 2-ml tube. The suspension was homogenized twice for 30 s using a Micro Smash (Tomy, Tokyo, Japan) at a speed of 4000 rpm in a cold room. PolyP granules were precipitated by centrifugation at 13,000×g for 10 min. The cell debris was not precipitated under this condition. The pellet containing polyP granules was washed several times with carbon tetrachloride and dried. PolyP granules were observed by scanning electron microscopy, and their chemical composition was analyzed by electron microscopy coupled with energy-dispersive X-ray spectroscopy (EDAX; Nihon Denshi).

2.4. *In vitro* preparation of polyP granules

PolyP granules were formed by mixing 50 mM polyP (average chain-length=65; Sigma), 57 mM KCl, and 39 mM MgCl₂. PolyP granules were collected by centrifugation at 13,000×g for 5 min and washed five times with a solution containing 57 mM KCl and 39 mM MgCl₂.

Table 1. Primers used for gene disruption.

Gene	DNA sequence
<i>speAB</i>	ACTGTTTTACTTAATAAAATAATTGAGGTTTCGCTATGATTCCGGGGATCCGTCGACC (forward)
	TGCGCATCGCATCTGGTCTTACTCGCCCTTTTCGCCGCTGTAGGCTGGAGCTGCTTCG (reverse)
<i>speC</i>	GGCGGTCGGAGCTGGTGACCAGTTTGACCCATATCTCATGATTCCGGGGATCCGTCGACC (forward)
	TGACC'CGTTTTTTTATTCTTACTTCAACACATAACCGTATGTAGGCTGGAGCTGCTTCG (reverse)
<i>cadA</i>	GTGTTGGGAGGGGCTTTTTTACCTGGAGATATGACTATGATTCCGGGGATCCGTCGACC (forward)
	CTTCCCTTGTACGAGCTAATTATTTTTTGCTTCTTCTTTGTAGGCTGGAGCTGCTTCG (reverse)
<i>speG</i>	TAACTGTTATTGATTTAAGGAATGTAAGGACACGTTATGATTCCGGGGATCCGTCGACC (forward)
	CGATCGATTATTAATGCTATTGTGCGGTCGGCTTCAGTGTAGGCTGGAGCTGCTTCG (reverse)

2.5. Stability of polyP granules

PolyP granules prepared *in vitro* were dispersed in 100 mM sodium hydroxyethylpiperazine ethanesulphonate (HEPES-NaOH, pH 7.2) containing each polyamine (putrescine, spermidine, cadaverine), or ammonium chloride at room temperature. After the indicated time, polyP granules were precipitated at $13,000\times g$ for 3 min. PolyP was determined as P_i by the ascorbic acid method after hydrolysis in 1 N HCl at 100°C for 7 min^{2,7}.

3. Results and Discussion

3.1. Polyamines affect polyP accumulation in *E. coli*

PolyP accumulated in response to amino acid starvation in *E. coli* (Fig. 1A). PolyP is a P_i polymer with some similarity to RNA and DNA, and, therefore, it is reasonable that polyP readily interacts with polycationic compounds including polyamines. To determine whether polyamines affect polyP accumulation under conditions of amino acid starvation, we constructed a *speABC* and *cadA* mutant that fails to produce polyamines^{13,29}. The intracellular levels of polyamines in this mutant can be restored by the addition of polyamines to the medium. The levels of polyP in this mutant were also partially restored by addition of polyamines to the medium under conditions of amino acid starvation (Fig. 1A). In addition, introduction of a low-copy plasmid encoding *ppk* (pMWppk) increased the levels of polyP. Under conditions of amino acid starvation, an *speABC* and *cadA* mutant carrying pMWppk accumulated more polyP in the presence than in the absence of polyamines in the medium (Fig. 1B). On the other hand, accumulation of polyamines by a *speG* mutant was enhanced three-fold by the addition of polyamines to the medium.

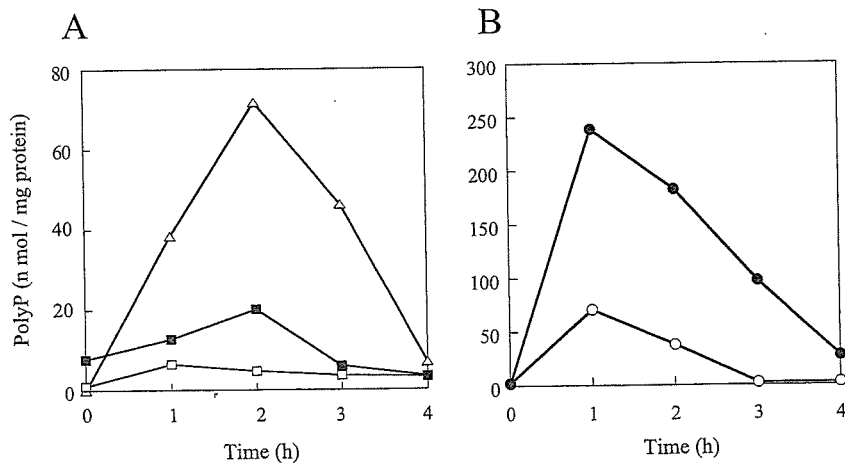


Fig. 1. PolyP accumulation in a *speABC cadA* mutant under conditions of amino acid starvation and in the presence and absence of polyamines.

(A) Intracellular polyP concentrations were measured in the *E. coli* wild type at the indicated times after shifting to amino acid-starved conditions (open triangles). The polyP concentrations were also measured in an *E. coli speABC cadA* mutant at the indicated times after shifting to amino acid-starved conditions with (closed squares) or without (open squares) added polyamines (1 mM putrescine and 1 mM spermidine). (B) The same experiment was performed in the *E. coli speABC cadA* mutant carrying pMWppk in the presence (closed circular) or absence (open circular) of polyamines.

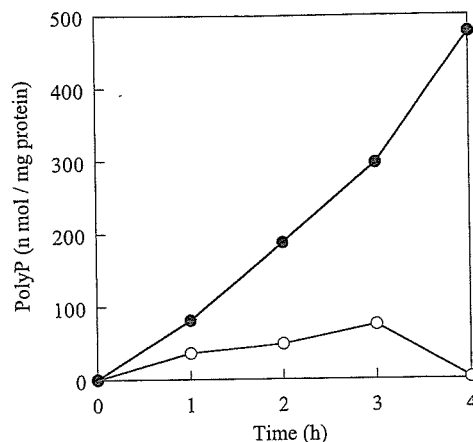


Fig. 2. PolyP accumulation in a *speG* mutant and the wild type under conditions of amino acid starvation in the presence of polyamines.

Intracellular polyP concentrations were measured for *E. coli* MG1655 (open circles) and a *speG* mutant (closed circles) at the indicated times after shifting to MOPS medium containing polyamines (1 mM putrescine and 1 mM spermidine).

This mutant also accumulated more polyP than the wild type (Fig. 2). After 3 h, the wild type restarted to grow under this condition and its polyP accumulation reduced (Fig. 2). However, this mutant could not adapt to amino acid starvation and thus polyP accumulation might prolong even after 3 h. These results suggested that polyamines affect polyP accumulation.

3.2. Analysis of polyP granules prepared from *E. coli*

PolyP is often found in cellular inclusions known as polyP granules or volutin granules^{19,25,26,27}. Here, we isolated polyP granules from an *E. coli phoU* mutant that accumulates 1000-fold higher levels of polyP than the wild type.

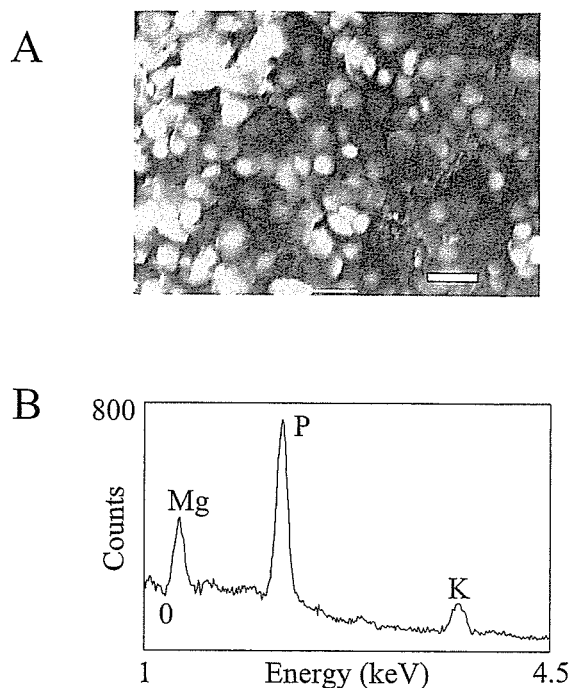


Fig. 3. Scanning electron microscopy (A) and EDAX spectra (B) of polyP granules prepared from *E. coli* cells.

(A) PolyP granules extracted from *E. coli* with carbon tetrachloride were evaporated on the stage, coated with carbon, and then observed by scanning electron microscopy. Bar=2 μm. (B) The elemental composition of polyP granules was analyzed by EDAX.

We examined the polyP granules by microscopy and by scanning electron microscopy with EDAX (Fig. 3). EDAX analysis revealed that polyP granules contained phosphorus, magnesium, and potassium in a ratio of 1.0:0.31:0.19. Although polyP granules are often found in cellular inclusions containing calcium¹⁴), those isolated from *E. coli* did not contain calcium.

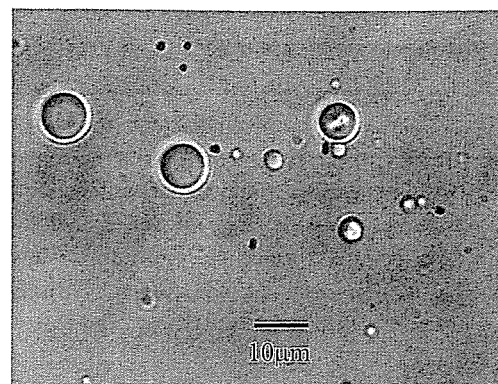


Fig. 4. Microscopic analysis of synthesized polyP granules. The synthesized polyP granules were observed under phase-contrast microscopy.

We prepared polyP granules *in vitro* by mixing polyP, KCl, and MgCl₂ as to approximately the same elemental ratio as the polyP granules prepared from *E. coli* cells. The synthetic polyP granules were spherical with diameters from several hundreds of nanometers to tens of micrometers (Fig. 4). However, these synthetic polyP granules dissolved within 50 min when they were placed in HEPES buffer (data shown in Fig. 5B).

3.3. Polyamines protect polyP granules from dissolution

We suspected that polyamines protect polyP granules from dissolution in *E. coli*. We found that the polyamines such as putrescine, spermidine, and cadaverine protect the polyP granules from dissolution but that ammonium chloride does not (Fig. 5A). Igarashi and Kashiwagi predicted that 12.5 mM putrescine and 0.26 mM spermidine exist in a free form in *E. coli*²⁰). The polyP granules were completely protected against dissolution by a combination of 12.5 mM putrescine and 0.26 mM spermidine (Fig. 5B).

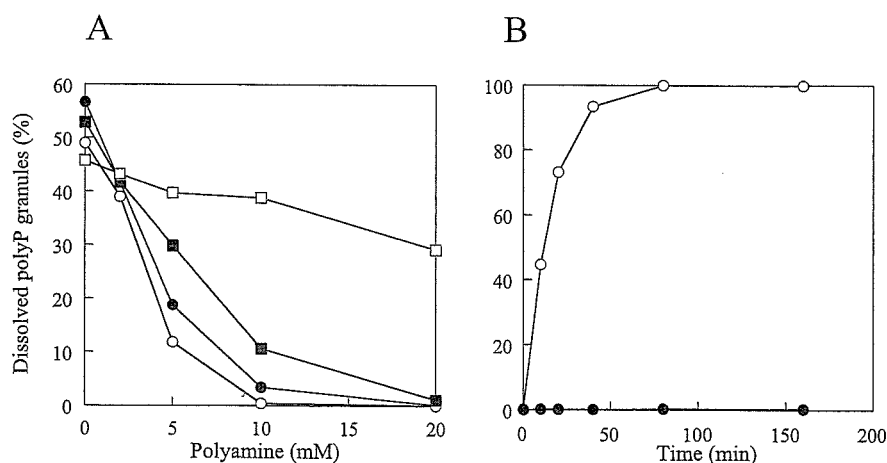


Fig. 5. Polyamines stabilize polyP granules.

(A) PolyP granules prepared *in vitro* (0.5 mg) were precipitated by centrifugation and placed in HEPES buffer containing various concentrations of putrescine (closed circular), spermidine (open circular), cadaverine (closed square), or ammonium chloride (open square). Dissolved polyP could no longer be precipitated by centrifugation. The percentage of dissolved polyP after 10-min incubation vs. total polyP granules was plotted as a function of the polyamine concentration. (B) PolyP granules prepared *in vitro* (0.5 mg) were incubated in HEPES buffer in the presence (closed) and absence (open) of 12.5 mM putrescine and 0.26 mM spermidine. The percentage of dissolved vs. total polyP granules was plotted as a function of the incubation time.

We prepared polyP granules *in vitro* by mixing polyP, KCl, and MgCl₂ in the presence of 10 mM spermidine. The synthetic polyP granules were very stable even when they were placed in HEPES buffer in the absence of polyamine (data not shown). These results suggested that the polyamines also interact with polyP when polyP granules are formed.

PolyP was first identified in microorganisms as metachromatic granules that are stained pink with basic blue dyes, and they were originally referred to as "volutin granule"¹⁴. PolyP granules from unicellular eukaryotes (e.g., *Chlamydomonas reinhardtii*²⁵) and *Dictyostelium discoideum*¹⁹), a number of human pathogens (e.g., malaria parasites and trypanosomatids²⁶), and human platelets²⁷) were recently found to be surrounded by a membrane with a number of pumps and exchangers and were named acidocalcisomes. Although polyP granules are often found in cellular inclusions containing calcium¹⁴), those isolated from *E. coli* did not contain calcium. We found that these polyP granules soon disappeared when added to buffer, although they are stabilized by polyamines (putrescine, spermidine, and spermine), which are known to be present at millimolar concentrations in both prokaryotic and eukaryotic cells^{3,10}. We concluded that polyamines may interact with polyP *in vivo* and therefore affect polyP accumulation.

Our findings also suggested that, conversely, polyP accumulation may affect the intracellular levels of free polyamine. A decrease or increase in polyamine content greatly diminishes cell growth in *E. coli*^{8,24}), and their intracellular levels are closely regulated by control of synthesis, degradation, uptake, and excretion^{9,22}). In *E. coli*, approximately 50% of putrescine and 90% of spermidine exist as polyamine-RNA complexes, and they act at the level of translation to enhance the synthesis of OppA, Cya, RpoS, FeCl, and Fis¹¹). Further investigations are needed to clarify the role of polyP in polyamine metabolism.

Acknowledgments

This work was supported in part by the Research and Development Program for New Bio-industry Initiatives of Bio-oriented Technology Research Advancement Institution Japan.

References

- 1) Ault-Riche, D., C.D. Fraley, C.M. Tzeng, and A. Kornberg. 1998. Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. *J. Bacteriol.* 180: 1841–1847.
- 2) Clesceri, L.S., A.E. Greenberg, and R.R. Trussell. 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington DC.
- 3) Cohen, S.S. 1998. A guide to the polyamines. Oxford University Press, Oxford, UK.
- 4) Datsenko, K.A., and B.L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97: 6640–6645.
- 5) Fukuchi, J., K. Kashiwagi, M. Yamagishi, A. Ishihama, and K. Igarashi. 1995. Decrease in cell viability due to the accumulation of spermidine in spermidine acetyltransferase-deficient mutant of *Escherichia coli*. *J. Biol. Chem.* 270: 18831–18835.
- 6) Hammond, A.L. 1971. Phosphate replacements: problems with the washday miracle. *Science.* 172: 361–363.
- 7) Harold, F.M. 1960. Accumulation of inorganic polyphosphate in mutants of *Neurospora crassa*. *Biochim. Biophys. Acta.* 45: 172–188.
- 8) Igarashi, K., K. Kashiwagi, K. Kishida, T. Kakegawa, and S. Hirose. 1981. Decrease in the S1 protein of 30-S ribosomal subunits in polyamine-requiring mutants of *Escherichia coli* grown in the absence of polyamines. *Eur. J. Biochem.* 114: 127–131.
- 9) Igarashi, K., and K. Kashiwagi. 1999. Polyamine transport in bacteria and yeast. *Biochem. J.* 344. Pt. 3: 633–642.
- 10) Igarashi, K., and K. Kashiwagi. 2000. Polyamines: mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun.* 271: 559–564.
- 11) Igarashi, K., and K. Kashiwagi. 2006. Polyamine Modulon in *Escherichia coli*: Genes Involved in the Stimulation of Cell Growth by Polyamines. *J. Biochem. (Tokyo)* 139: 11–16.
- 12) Jacobson, L., M. Halmann, and J. Yariv. 1982. The molecular composition of the volutin granule of yeast. *Biochem. J.* 201: 473–479.
- 13) Jung, I.L., T.J. Oh, and I.G. Kim. 2003. Abnormal growth of polyamine-deficient *Escherichia coli* mutant is partially caused by oxidative stress-induced damage. *Arch. Biochem. Biophys.* 418: 125–132.
- 14) Kornberg, A. 1995. Inorganic polyphosphate: toward making a forgotten polymer unforgettable. *J. Bacteriol.* 177: 491–496.
- 15) Kulaev, I.S., V. Vagapov, and T. Kulakovskaya. 2004. The Biochemistry of Inorganic Polyphosphates. Wiley and Sons, NJ.
- 16) Kuroda, A., H. Murphy, M. Cashel, and A. Kornberg. 1997. Guanosine tetra- and pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia coli*. *J. Biol. Chem.* 272: 21240–21243.
- 17) Kuroda, A., S. Tanaka, T. Ikeda, J. Kato, N. Takiguchi, and H. Ohtake. 1999. Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 96: 14264–14269.
- 18) Kuroda, A., K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, and A. Kornberg. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science.* 293: 705–708.
- 19) Marchesini, N., F.A. Ruiz, M. Vieira, and R. Docampo. 2002. Acidocalcisomes are functionally linked to the contractile vacuole of *Dictyostelium discoideum*. *J. Biol. Chem.* 277: 8146–8153.
- 20) Miyamoto, S., K. Kashiwagi, K. Ito, S. Watanabe, and K. Igarashi. 1993. Estimation of polyamine distribution and polyamine stimulation of protein synthesis in *Escherichia coli*. *Arch. Biochem. Biophys.* 300: 63–68.
- 21) Morohoshi, T., T. Maruo, Y. Shirai, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, and A. Kuroda. 2002. Accumulation of inorganic polyphosphate in *phoU* mutants of *Escherichia coli* and *Synechocystis* sp. strain PCC6803. *Appl. Environ. Microbiol.* 68: 4107–4110.
- 22) Pegg, A.E. 1988. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer. Res.* 48: 759–774.
- 23) Pick, U., M. Bental, E. Chitlaru, and M. Weiss. 1990. Polyphosphate-hydrolysis—a protective mechanism against alkaline stress? *FEBS. Lett.* 274: 15–18.
- 24) Raj, V.S., H. Tomitori, M. Yoshida, A. Apirakaramwong, K. Kashiwagi, K. Takio, A. Ishihama, and K. Igarashi. 2001. Properties of a revertant of *Escherichia coli* viable in the

- presence of spermidine accumulation: increase in L-glycerol 3-phosphate. *J. Bacteriol.* 183: 4493–4498.
- 25) Ruiz, F.A., N. Marchesini, M. Seufferheld, Govindjee, and R. Docampo. 2001. The polyphosphate bodies of *Chlamydomonas reinhardtii* possess a proton-pumping pyrophosphatase and are similar to acidocalcisomes. *J. Biol. Chem.* 276: 46196–46203.
- 26) Ruiz, F.A., C.O. Rodrigues, and R. Docampo. 2001. Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation, and environmental stress in *Trypanosoma cruzi*. *J. Biol. Chem.* 276: 26114–26121.
- 27) Ruiz, F.A., C.R. Lea, E. Oldfield, and R. Docampo. 2004. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J. Biol. Chem.* 279: 44250–44257.
- 28) Sedlak, R.I. 1991. Phosphorus and nitrogen removal from municipal wastewater, 2nd ed. Lewis publishers, Florida.
- 29) Tabor, H., E.W. Hafner, and C.W. Tabor. 1980. Construction of an *Escherichia coli* strain unable to synthesize putrescine, spermidine, or cadaverine: characterization of two genes controlling lysine decarboxylase. *J. Bacteriol.* 144: 952–956.



INORGANIC POLYPHOSPHATE STIMULATES LON-MEDIATED PROTEOLYSIS OF NUCLEOID PROTEINS IN *ESCHERICHIA COLI*

K NOMURA¹, J KATO¹, N TAKIGUCHI¹, H OHTAKE², and A KURODA^{1,3,✉}

¹Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan
Fax: (+81)82-424-7047; E-mail: akuroda@hiroshima-u.ac.jp

²Department of Biotechnology, Osaka University, Osaka 565-0871, Japan

³PRESTO, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

Received ??th, 2005; Accepted ??th, 2005; Published May 30th, 2006

Abstract – Inorganic polyphosphate (polyP) accumulates in response to amino acid starvation in *Escherichia coli*. Previously, we found that the complex formation of Lon with polyP stimulates proteolysis of free ribosomal proteins. In the current studies, we examined the effects of polyP on the degradation of major nucleoid proteins. Fusions of green fluorescent protein with HimA, Fis, HupA, and HupB were clearly associated with polyP *in vivo*. Lon degraded His-tagged HimA protein only in the presence of polyP *in vitro* as well as *in vivo*. Whereas, when HimA and HimD formed a heterodimer, Lon could not degrade it even in the presence of polyP. In addition, Lon degraded His-tagged Fis protein in the presence of polyP. However, *in vivo*, Lon did not efficiently degrade the Fis protein even when cells accumulated polyP in response to amino acid starvation. It appears that this is due to tighter binding of Fis to DNA than to polyP and resistance of the DNA-Fis to Lon-mediated proteolysis. Indeed, we found that at least a five-fold excess of polyP was necessary to displace DNA from the DNA-Fis complex. Furthermore, Lon degraded His-tagged HupA protein efficiently in the presence of polyP. We also showed that degradation of the translational initiation factor InfC depends on polyP.

Key words: Polyphosphate, Lon, Nucleoid protein.

INTRODUCTION

In prokaryotes such as *Escherichia coli*, amino acid starvation causes coupled cessation of protein and stable RNA synthesis, a process called the “stringent response” (5). In addition, the rate of protein degradation increases under conditions of amino acid starvation (26,36). If the environment is suddenly depleted of amino acids, new enzymes can be synthesized only from amino acids liberated by protein turnover (38). These metabolic responses are very important for allowing cells to escape from amino acid starvation and to adapt to new environmental conditions.

Previously, we found that polyphosphate (polyP), which is a linear polymer of many hundreds of orthophosphate residues, accumulates during the stringent response in *E. coli* (23). An *E. coli* mutant deficient in polyP kinase (PPK), the principal enzyme for the synthesis of polyP, fails to increase protein turnover and shows an extended lag in growth when shifted from a nutrient-rich to a nutrient-poor medium (nutritional downshift) (25). These studies suggested that protein degradation is triggered by the accumulation of polyP in *E. coli*. Starvation-induced proteolysis is an energy-dependent

process requiring the hydrolysis of ATP (27). The ATP-dependent protease Lon, conserved from bacteria to humans, is responsible for the rapid turnover of both abnormal and naturally unstable proteins (16,25). We found that mutations in both the Lon and Clp proteases produce the same phenotype as *ppk* mutations and that addition of amino acids overcomes the block in these cases (24). PolyP forms a complex with Lon, which enables Lon to degrade free ribosomal proteins (24). Certain very abundant ribosomal proteins could be the principal sacrificial substrates targeted for degradation at the onset of the downshift. However, the increase in total protein turnover during starvation could not be ascribed only due to the degradation of free ribosomal proteins.

Previously, we found that the interaction of ribosomal proteins with polyP seems necessary for the stimulation of Lon-mediated proteolysis (24,30). PolyP in the polyP-Lon complex may function as an adaptor molecule to capture substrates that are able to bind to polyP. PolyP is a phosphate polymer with some similarity to RNA and DNA, and, therefore, it seems reasonable that polyP readily interacts with basic proteins such as histones (21). Currently, four structural proteins, IHF (integration host factor), Fis (factor for inversion stimulation), H-NS (histone-like nucleoid structuring protein), and HU (heat-stable nucleoid protein composed of HupA and HupB) as well as one stationary-phase-specific DNA-binding protein, Dps (DNA binding protein

Abbreviations: DAPI: 4',6-diamidino-2-phenylindole; polyP: inorganic polyphosphate; IPTG: isopropyl- β -D-thiogalactopyranoside; GFP: green fluorescence protein; IHF: HimA and HimD heterodimer; HU: HupA and HupB heterodimer; MOPS: 3-(N-Morpholino) propanesulfonic acid; PPK: polyphosphate kinase.

from starved cells), are thought to be the major components of the *E. coli* nucleoid (8,33). Here, we examined whether these nucleoid proteins are associated with polyP *in vivo* and whether they are degraded by the polyP-Lon complex. We also tested seven additional nucleoid-associated proteins (2), including CbpA (curved DNA-binding protein A), CbpB (curved DNA-binding protein B), DnaA (DNA replication protein A or initiator protein), Hfq (host factor for phage Q), IciA (inhibitor of chromosome initiation A), Lrp (leucine-responsive regulatory protein), and StpA (suppressor of Td phenotype) for association with polyP and degradation by the polyP-Lon complex.

MATERIALS AND METHODS

Monitoring of Subcellular Localization of Nucleoid Protein-Green Fluorescent Protein (GFP) Fusions.

A complete set of *E. coli* K-12 open reading frame archive (ASKA) library with GFP and His tags was constructed at the Nara Institute of Science and Technology, Japan (20). From this library, plasmids encoding HimA, HimD, Fis, Hns, HupA, HupB, Dps, CbpA, CbpB, DnaA, Hfq, IciA, Lrp, and StpA proteins fused with GFP were selected and then transferred into *E. coli* MT4 (*phoU*) (28). The transformants were grown on a 3-(N-morpholino) propanesulfonic acid (MOPS) minimal medium (25) containing 0.1mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 20mg/l chloramphenicol for 4hrs at 37°C. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 5mg/l and kept on ice for 30min. Cells were fixed with 1% agarose and then observed under fluorescent microscopy. Fluorescence micrographs were recorded on an Olympus BX60 microscope equipped with a 100X UPlanApo objective. Two filter sets were used: a MNIBA filter for GFP (470-490nm) and a MWU filter for DAPI (330-385nm). Images were captured using a cooled charge-coupled device camera DP70 (Olympus Co., Tokyo, Japan). Images were processed using Adobe Photoshop 6.0.

Protein Purification

DNA fragments of *himA*, *himD*, *hupA*, and *hupB* which were amplified by PCR using *himA1* and *himA2*, *himD1* and *himD2*, *hupA1* and *hupA2*, and *hupB1* and *hupB2* primers, respectively (Table 1), were inserted into the *NcoI* and *XhoI* sites of pET21-b (Novagen, San Diego, CA).

Table 1 DNA sequence of primers

Primer	DNA sequence
<i>himA1</i>	TAACCATGGCGCTTACAAAAGCTGA
<i>himA2</i>	CCGCTCGAGCTCGTCTTTGGGCGAAGCGT
<i>himA3</i>	TGTTCTAGAACTCGTCTTTGGGCGAAGCGT
<i>himA4</i>	CCGAGATCTGCTAACAGGAGGAATTAAC
<i>himA5</i>	ACGGAAATCTCACAGATCCTCTCTGAGATGA
<i>himD1</i>	ACTGGTCTCACATGACCAAGTCAGAATTGA
<i>himD2</i>	CCGCTCGAGACCGTAAATATTGGCGCGATC
<i>himD3</i>	TGTTCTAGAAAACCGTAAATATTGGCGCGATC
<i>himD4</i>	CCGAGATCTTCAGTGGTGGTGGTGGTGGT
<i>hupA1</i>	TAACCATGGGGAACAAGACTCAACTGATTGA
<i>hupA2</i>	CCGCTCGAGCTTAAGTGGCTCTTTCAAGTG
<i>hupA3</i>	TGTTCTAGAACTTAAGTGGCTCTTTCAAGTG
<i>hupB1</i>	TAACCATGGTGAATAAATCTCAATTGATC
<i>hupB2</i>	CCGCTCGAGGTTTACCGGCTCTTTCAAGTG

These plasmids were designated pET-HimA, pET-HimD, pET-HupA, and pET-HupB, respectively. HimA, HimD, HupA, and HupB proteins with C-terminal His tags were expressed in *E. coli* recombinants that harbored pET-HimA, pET-HimD, pET-HupA, and pET-HupB, respectively. To express the HimA and HimD heterodimer (IHF), both the *himA* fragment amplified

with *himA4* and *himA5* primers and the *himD* fragment amplified with *himD1* and *himD4* primers were inserted into *NcoI* and *EcoRI* sites of pBAD/Myc-HisB (Invitrogen, Carlsbad, CA). This plasmid was designated pBAD-IHF. His tagged proteins were purified using chromatography on a HiTrap Chelating column (Amersham Biosciences, Piscataway, NJ). The fractions containing nucleoid proteins were dialyzed against a 50mM HEPES-NaOH (pH 7.4) buffer containing 1mM EDTA (pH 7.4) and 20% glycerol solution, and then applied to HS/M cation exchange column (POROS, Perspective Biosystems, Cambridge, MA). The nucleoid proteins were eluted from the column with a linear gradient of 0 to 1M NaCl in the buffer.

Protein Degradation

Purified proteins (5 μ g) and Lon (1 μ g) were incubated in a 10 μ l buffer containing 25mM Tris-HCl (pH 7.8), 5mM MgCl₂, and 4mM ATP in the presence and absence of polyP700 (1 μ g, 14pmol as a polymer) at 37°C and then separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. PolyP700 was synthesized by PPK with ATP as a substrate as described previously (23). After electrophoresis, proteins were stained with SYPRO Orange (Molecular Probes, Eugene, OR) and visualized with a Typhoon image analyzer (Amersham Biosciences).

Monitoring *in vivo* Degradation

DNA fragments of *himA* and *himD*, which were amplified by PCR with *himA1* and *himA3* primers and *himD1* and *himD3* primers, respectively (table 1), were inserted into the *NcoI* and *XbaI* sites of pBAD/Myc-HisB (Invitrogen, Carlsbad, CA). These plasmids were designated pBAD-HimA and pBAD-HimD, respectively. HimA and HimD with C-terminal myc epitope fusion were expressed in the wild-type, *ppk*, *lon*, and *clp* mutants of *E. coli* harbouring pBAD-HimA and pBAD-HimD on 2xYT medium (34) for 2hrs in the presence of 0.2% L-arabinose. To express HimA and HimD simultaneously, pBAD-IHF was introduced into the wild-type and *ppk* mutant cells. To express HupA, a *hupA* DNA amplified by PCR with *himA1* and *himA3* primers was inserted into the *NcoI* and *XbaI* sites of pBAD/Myc-HisB. This plasmid was designated as pBAD-HupA. Plasmids pHis-Fis and pHis-InfC were constructed from pHis-fis-GFP and pHis-InfC-GFP (ASKA library) by removing the *NotI* fragment that encodes GFP. Fis and InfC proteins with an N-terminal His tag were expressed on 2xYT medium for 2hrs in the presence of 20 μ M IPTG. Cells were collected by centrifugation and resuspended in the MOPS minimal medium. At the indicated times, total protein (100 μ l of the culture) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Western analysis was performed with an antibody to His tag or myc epitope (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and ECL Western blotting detection reagents (Amersham Bioscience). Immunoreactive proteins were detected with X-ray film. Gel Shift Assay. A synthetic double-stranded *rmB*-P1-DNA (AGGCTGATTTGGTTGAATGTTGCGCGGTCAGAAAA) was used for the gel shift assay. Fis protein, *rmB*-P1-DNA, and polyP were mixed in a 10 μ l solution of 25mM Tris-HCl (pH 7.8) and 5mM MgCl₂ for 5min at room temperature and then separated by electrophoresis on a polyacrylamide gel. Electrophoresis was performed with a buffer of 25mM Tris and 192mM glycine, and then stained with ethidium bromide to detect DNA.

RESULT

In vivo PolyP-Binding Assay for Nucleoid Proteins.

The level of polyP accumulated in *E. coli* MG1655 (wild-type) growing on a rich medium is approximately 1nmol/mg protein. When shifted to a

nutrient-poor medium (e.g., MOPS medium), the wild-type accumulates approximately 50-100nmol polyP/mg protein. Following 4hrs of this nutritional downshift, the level decreases 2-3nmol polyP/mg protein (25). While, *E. coli* MT4 (*phoU*) mutant accumulates approximately 200-500nmol polyP/mg protein constitutively (28) and forms polyP particles that can be observed easily by fluorescent microscopy (green-yellow fluorescence in fig. 1).

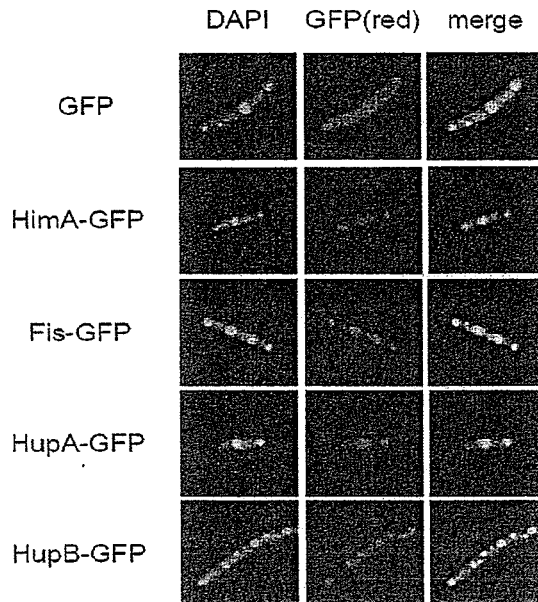


Fig. 1. *In vivo* polyP-binding assay using a polyP-accumulating strain and a GFP-fusion protein. *E. coli* MT4 (*phoU*) carrying a GFP fusion gene was grown on a MOPS minimal medium. DAPI fluorescence was used to detect nucleoids (blue) and polyP (green-yellow) under UV light (330-385nm). GFP shows green fluorescence under blue light (470-490nm) but was changed to red using Adobe Photoshop.

These cells were also costained with DAPI to detect nucleoids (blue fluorescence). GFP, in contrast, was found throughout the cell because it does not bind to polyP. Therefore, GFP fusion proteins can be used to assess polyP binding *in vivo*. Specifically, if a test protein is associated with polyP *in vivo*, its GFP fusion would be localized in the polyP particles. Therefore, we grew *E. coli* MT4 carrying GFP fusion genes on the MOPS minimal medium and observed them under fluorescent microscopy. We found that HimA, Fis, HupA, and HupB fusion proteins linked at their C-termini to GFP were localized in the polyP granules (fig. 1). In contrast, HimD, Hns, Dps, CbpA, CbpB, DnaA, Hfq, IciA, Lrp, and StpA were not localized in the polyP particle.

Degradation of HimA Protein by Lon

Because our initial results suggested that HimA protein, but not HimD protein, is a polyP-binding protein *in vivo*, we examined their ability to be

cleaved by Lon-dependent proteolysis in the absence and presence of polyP. Lon degraded purified HimA with C-terminal His tag (HimA-His) only in the presence of polyP (fig. 2). Lon degraded purified HimD with C-terminal His tag (HimD-His), although the cleavage was not dependent on polyP (fig. 2).

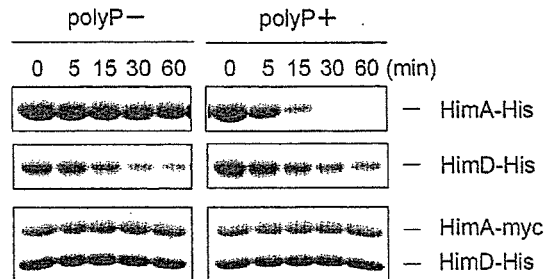


Fig. 2 *In vitro* degradation of HimA protein by Lon in the absence and presence of polyP. HimA-His, HimD-His, or IHF (HimA-myc and HimD-His dimer) (5 μ g) were mixed with Lon (1 μ g) in a 10 μ l solution containing 25mM Tris-HCl (pH 7.8), 5mM MgCl₂, and 4mM ATP. PolyP (1 μ g) was used to stimulate Lon-mediated protein degradation.

In vivo, HimA exists not only as a homodimer but also as a heterodimer with HimD, which is called IHF. We simultaneously expressed HimA with a C-terminal myc epitope (HimA-myc) and HimD-His, and then purified the IHF heterodimer using a HiTrap Chelating column. We used the HimA-myc to omit contamination of the HimA dimer under these conditions. Interestingly, Lon did not degrade IHF heterodimer (fig. 2). Thus, HimA was degraded in the presence of polyP but not when it formed a heterodimer with HimD. To monitor *in vivo* degradation, we expressed HimA protein with a C-terminal myc epitope (HimA-myc-His) in the wild-type, *ppk*, *lon*, and *clp* mutant cells. These cells were then subjected to amino acid starvation. Degradation of HimA-myc-His protein was monitored by Western blotting. As expected, degradation of HimA-myc-His protein was dependent on both PPK and Lon *in vivo* (fig. 3).

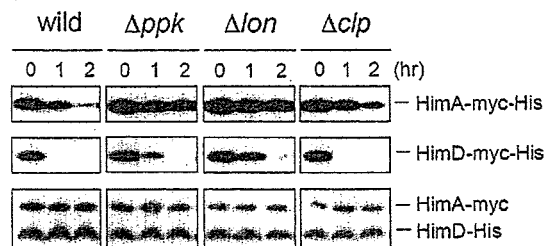


Fig. 3 *In vivo* degradation of HimA and HimD proteins in response to nutritional downshift. HimA-myc-His, HimD-myc-His, and IHF (HimA-myc, HimD-His) proteins were expressed in the wild-type, *ppk*, *lon*, and *clp* mutant strains on a rich medium and then shifted to a MOPS minimal medium (nutritional downshift). Western blot analysis was performed using an antibody against His-tag or myc epitope.

HimD protein with C-terminal myc epitope (HimD-myc-His) was degraded much more quickly *in vivo* than HimA-myc-His. When the IHF heterodimer was formed, it was very stable even in the wild-type (fig. 3).

Degradation of Fis Protein by Lon

Lon also degraded purified Fis proteins with N-terminal His tags (His-Fis) only in the presence of polyP (Fig. 4).

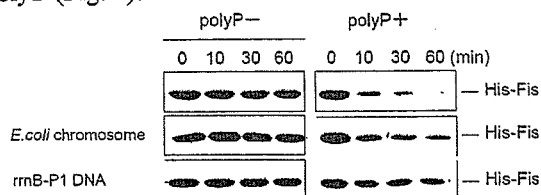


Fig. 4 *In vitro* degradation of Fis protein by Lon in the absence and presence of polyP. His-Fis protein (4 μ g; 235pmol) was mixed with Lon (1 μ g; 11pmol) in a 10 μ l solution containing 25mM Tris-HCl (pH 7.8), 5mM MgCl₂, and 4mM ATP. PolyP (1 μ g; 14pmol) was used to stimulate Lon-mediated protein degradation. *E. coli* chromosomal DNA (1.2 μ g) and *rrnB*-P1 DNA (1.2 μ g; 52pmol) was used to inhibit proteolysis.

To monitor *in vivo* degradation of Fis, we expressed the His-tagged Fis protein in wild-type and *ppk* mutant cells. Unexpectedly, it was stable even in the wild-type cells (fig. 5).

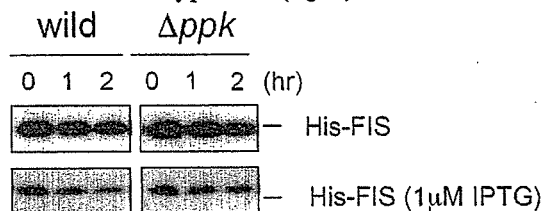


Fig. 5 *In vivo* degradation of Fis protein. His-Fis protein was expressed in the wild-type and *ppk* mutant strains on a rich medium and then subjected to the downshift. Western blot analysis was performed using an antibody against His tag.

In addition, when it was induced by a low concentration of IPTG (1 μ M), *ppk* had very little effect on its degradation *in vivo* (Fig. 5). *E. coli* chromosomal DNA partially rescued polyP-Lon-mediated proteolysis of His-Fis (Fig. 4). It is known that Fis protein specifically binds to *rrnB*-P1 DNA (19). The *rrnB*-P1 DNA more strongly rescued polyP-Lon-mediated proteolysis (Fig. 4). We examined the formation of a DNA-Fis complex using a gel shift assay. In the presence of a five-fold excess of His-Fis to DNA, almost all of the *rrnB*-P1 DNA was shifted (Fig. 6A). Because polyP binds to Fis, it is able to compete with DNA for the binding to Fis. However, at least a five-fold molar excess of polyP to DNA is necessary to inhibit the formation of the *rrnB*-P1 DNA-Fis complex (Fig. 6B).

Therefore, when 14pmol of polyP was mixed with 52pmol *rrnB*-P1 DNA (the condition in fig. 4), the majority of Fis was complexed with DNA. These results indicated that the polyP-Fis is degraded by Lon, but the DNA-Fis complex is resistant to Lon-mediated proteolysis.

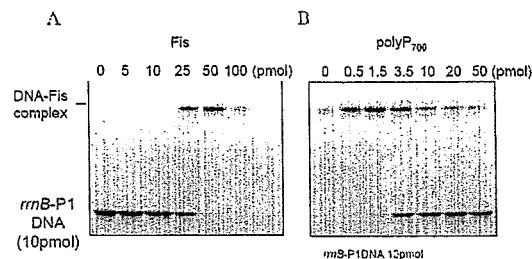


Fig. 6 DNA-Fis complex in the presence of polyP. *A*, A synthesized *rrnB*-P1 DNA (10pmol) and various amount of His-Fis protein were mixed and then subjected to polyacrylamide gel electrophoresis. *B*, The synthesized *rrnB*-P1 DNA (10pmol), Fis protein (100pmol), and various amount of polyP were mixed and then subjected to polyacrylamide gel electrophoresis. DNA was stained with ethidium bromide.

Degradation of HupA and HupB Proteins by Lon

Lon degraded purified HupA with a C-terminal His tag (HupA-His) and polyP stimulated its cleavage (fig. 7). Lon degraded purified HupB with a C-terminal His tag (HupB-His), although the cleavage was not dependent on polyP (fig. 7).

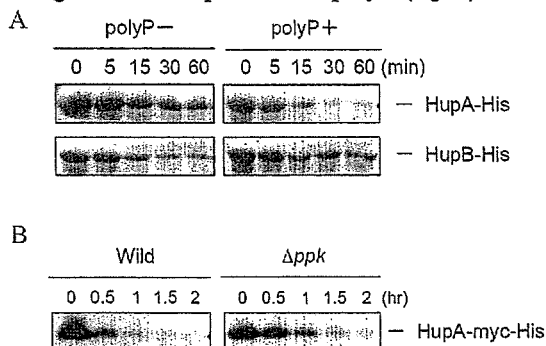


Fig. 7 Degradation of HupA and HupB proteins. *A*, HupA-His and HupD-His (5 μ g) were mixed with Lon (1 μ g) in a 10 μ l solution containing 25mM Tris-HCl (pH 7.8), 5mM MgCl₂, and 4mM ATP. PolyP (1 μ g) was used to stimulate Lon-mediated protein degradation. *B*, HupA-myc-His protein was expressed in the wild-type and *ppk* mutant strains on a rich medium and then subjected to the downshift. Western blot analysis was performed using an antibody against myc epitope.

In vivo, degradation of HupA protein with a C-terminal myc epitope (HupA-myc-His) in the wild-type was faster than in the *ppk* mutant cells during amino acid starvation (fig. 7).

PolyP-dependent Degradation of InfC

In vitro experiments in Kornberg's (A. Kornberg and R. Ohtomo, personal communication) and our

laboratories have identified not only histone-like proteins as polyP-binding proteins, but also InfC and InfB (translational initiation factors); Lon and ClpB (chaperon and protease); RpoD, SrmB, Rnr, RluC and Gyr (RNA- and DNA-related enzymes); YabC and YbiB (unknown function); LpdA (pyruvate dehydrogenase complex); and SucA (2-oxoglutarate dehydrogenase). We therefore analyzed whether these proteins are associated with polyP and whether their degradation *in vivo* is dependent on polyP. Our *in vivo* assay showed that C-terminal GFP fusions of LpdA, RluC, and InfC associated with polyP. In addition, InfC protein with an N-terminal His tag was degraded in the wild-type after the nutritional downshift, whereas it was very stable in the *ppk* mutant (fig. 8). These results indicated that polyP-Lon degrades InfC.

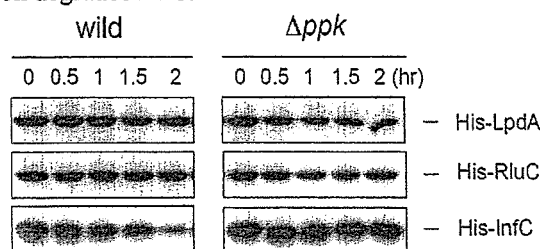


Fig. 8 *In vivo* degradation of InfC protein. His-LpdA, His-RluC, and His-InfC were expressed on a rich medium and then subjected to the downshift. The Western analysis was performed using an antibody against His tag.

DISCUSSION

Cells balance energy-efficient growth with the ability to adapt rapidly to sudden changes in their environment. In an environment rich in amino acids, cells do not expend energy for making amino acid biosynthetic enzymes. If the environment becomes depleted of amino acids, cells use their own proteins as sources of amino acids to generate amino acid biosynthetic enzymes and to adapt to amino acid starvation. In the recent studies (23,24,25,30), we provide several lines of evidence that polyP and Lon protease are involved in this starvation-induced proteolysis in *E. coli*: i) the *ppk* and *lon clp* mutants fail to activate starvation-induced proteolysis; ii) polyP, which accumulates in response to amino acid starvation, forms a complex with Lon; and iii) formation of the complex enables Lon to degrade free ribosomal proteins. Certain very abundant ribosomal proteins could be the sacrificial substrates targeted for degradation at the onset of the downshift. However, we could not ascribe the increase in total protein turnover during starvation exclusively to degradation of free ribosomal proteins. Indeed, in the current studies, we found that polyP-Lon complex degrades major nucleoid

proteins, such as HimA, Fis, and HupA, and the translational initiation factor, InfC. The degradation of these proteins may contribute to the amino acid supply when the cells are starved for amino acids. HimA is a subunit of IHF, which was originally discovered as an essential protein for the integration of phage lambda DNA into the chromosome (29). Another subunit of IHF is HimD, which show 30% amino acid sequence identity with HimA. IHF is now known to be involved in a variety of other processes, such as activation of transcription in phages (15,17) and *E. coli* (10), transposition (31,35), DNA replication (13,19), partitioning (12), and lambda packaging (22). Although IHF is mainly a heterodimeric protein, HimA and HimD can also exist as homodimers, which specifically recognize the same *ihf* sequence (39). However, neither HimA nor HimD homodimers can substitute for the function of the heterodimer *in vivo* (11). Therefore, it is unclear whether the Lon-mediated proteolysis of the HimA homodimer directly affects the function of IHF. IHF also shares significant homology with the subunits of another heterodimeric histone-like protein, HU, which is composed of HupA and HupB (8). HU is also involved in many cellular processes, such as DNA replication, recombination, and transcription as likely as IHF (8). In contrast to IHF, the HupA and HupB homodimers are functionally active and the composition of HU varies throughout the growth phase. Although the HupA homodimer prevails during exponential growth phase, the HupA and HupB heterodimer becomes predominant in stationary phase (7). Furthermore, the function of the heterodimer is required for long-term survival (7). Recently, it was also reported that the composition of HU changes during cold shock (14). Therefore, Lon-mediated proteolysis of the HupA homodimer might be important in the regulation of HU. Fis is the most abundant nucleoid protein in exponentially growing *E. coli* cells (60000 molecules/cell or 1 Fis dimer for every 200 to 300bp DNA segment) (1). Fis binds to AT-rich DNA sequences, which are located upstream of genes, induces DNA-bending, and enhances the expression of genes such as rRNA and tRNA in rapidly growing cells (9,37). It is known that the intracellular levels of Fis in growing cells decreases in the stationary phase. Because the polyP-Lon complex degraded the His-Fis protein *in vitro*, we expected that the His-Fis fusion protein is rapidly degraded after the nutritional downshift. However, His-Fis was not degraded rapidly even in the wild-type after the downshift. Furthermore, we found that a DNA-Fis complex is resistant to Lon-mediated proteolysis. Although polyP competed with DNA for the binding to Fis, the intracellular level of polyP might not be high enough to remove

Fis from DNA-Fis complexes. Recently, we found that ClpAP is involved in the rapid degradation of Fis under starvation conditions (K. Nomura and A. Kuroda, unpublished results), and we are now investigating the mechanism. InfC is the translational initiation factor, IF3, that is believed to be essential for selection of the initiation codon by monitoring the pairing between the initiator fMet-tRNA and the initiation codon (18). If this pairing is good, as in an AUG start codon, IF3 stabilizes the translation initiation complex and translation initiation proceeds. If the codon is suboptimal, IF3 destabilizes this complex and translation initiation is disfavored (3). IF3 is autoregulated through use of its unique AUU initiation codon (4). IF3 may also be targeted for Lon-mediated proteolysis as the free ribosomal proteins were degraded during amino acid starvation.

Acknowledgments – We thank Drs. H. Mori and H. Niki for providing ASKA library, and Drs A. Kornberg and R. Ohtomo for providing unpublished results on polyP-binding proteins. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1. Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S. and Ishihama, A., Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J. Bacteriol.* 1999, **181**:6361-6370.
2. Azam, T. A., Hiraga, S. and Ishihama, A., Twotypes of localization of the DNA-binding proteins within the *Escherichia coli* nucleoid. *Genes Cells.* 2000, **5**:613-626.
3. Berkhout, B., van der Laken, C. J. and van Knippenberg, P. H., Formylmethionyl-tRNA binding to 30 S ribosomes programmed with homopolynucleotides and the effect of translational initiation factor 3. *Biochim. Biophys. Acta.* 1986, **866**:144-153.
4. Butler, J. S., Springer, M. and Grunberg-Manago, M., AUU-to-AUG mutation in the initiator codon of the translation initiation factor IF3 abolishes translational autocontrol of its own gene (*infC*) *in vivo*. *Proc. Natl. Acad. Sci. U S A* 1987, **84**:4022-4025.
5. Cashel, M. and Kalbacher, B., The control of ribonucleic acid synthesis in *Escherichia coli*. V. Characterization of a nucleotide associated with the stringent response. *J. Biol. Chem.* 1970, **245**:2309-2318.
6. Chung, C. H. and Goldberg, A. L., DNA stimulates ATP-dependent proteolysis and protein-dependent ATPase activity of protease La from *Escherichia coli*. *Proc. Natl. Acad. Sci. U S A* 1982, **79**:795-799.
7. Claret, L. and Rouviere-Yaniv, J., Variation in HU composition during growth of *Escherichia coli*: the heterodimer is required for long term survival. *J. Mol. Biol.* 1997, **273**:93-104.
8. Drlica, K. and Rouviere-Yaniv, J., Histone-like proteins of bacteria. *Microbiol. Rev.* 1987, **51**:301-319.
9. Finkel, S. E. and Johnson, R. C., The Fis protein: it's not just for DNA inversion anymore. *Mol. Microbiol.* 1992, **6**:3257-3265.
10. Freundlich, M., Ramani, N., Mathew, E., Sirko, A. and Tsui, P., The role of integration host factor in gene expression in *Escherichia coli*. *Mol. Microbiol.* 1992, **6**:2557-2563.
11. Friedman, D. I., Integration host factor: a protein for all reasons. *Cell* 1988, **55**:545-554.
12. Funnell, B. E., Participation of *Escherichia coli* integration host factor in the P1 plasmid partition system. *Proc. Natl. Acad. Sci. U S A* 1988, **85**:6657-6661.
13. Gamas, P., Burger, A. C., Churchward, G., Caro, L., Galas, D. and Chandler, M., Replication of pSC101: effects of mutations in the *E. coli* DNA binding protein IHF. *Mol. Gen. Genet.* 1986, **204**:85-89.
14. Giangrossi, M., Giuliodori, A. M., Gualerzi, C. O. and Pon, C. L., Selective expression of the beta-subunit of nucleoid-associated protein HU during cold shock in *Escherichia coli*. *Mol. Microbiol.* 2002, **44**:205-216.
15. Giladi, H., Gottesman, M. and Oppenheim, A. B., Integration host factor stimulates the phage lambda pL promoter. *J. Mol. Biol.* 1990, **213**:109-121.
16. Goldberg, A. L., The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur. J. Biochem.* 1992, **203**:9-23.
17. Goosen, N. and van de Putte, P., Regulation of Mu transposition. I. Localization of the presumed recognition sites for HimD and Ner functions controlling bacteriophage Mu transcription. *Gene* 1984, **30**:41-46.
18. Gualerzi, C., Risuleo, G. and Pon, C. L., Initial rate kinetic analysis of the mechanism of initiation complex formation and the role of initiation factor IF-3. *Biochemistry* 1977, **16**:1684-1689.
19. Hwang, D. S. and Kornberg, A., Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. *J. Biol. Chem.* 1992, **267**:23083-23086.
20. Kitagawa, M., Ara, T., Arifuzzaman, M., Iokanaka, M., Inamoto, E., Toyonaga, H. and Mori, H., Complete set of ORF clones of *Escherichia coli* ASKA library (A Complete Set of *E. coli* K-12 ORF Archive): Unique Resources for Biological Research. *DNA Research* 2005, in press.
21. Kornberg, A., Inorganic polyphosphate: toward making a forgotten polymer unforgettable. *J. Bacteriol.* 1995, **177**:491-496.
22. Kosturko, L. D., Daub, E. and Murialdo, H., The interaction of *E. coli* integration host factor and lambda cos DNA: multiple complex formation and protein-induced bending. *Nucleic Acids Res.* 1989, **17**:317-334.
23. Kuroda, A., Murphy, H., Cashel, M. and Kornberg, A., Guanosine tetra- and pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia coli*. *J. Biol. Chem.* 1997, **272**:21240-21243.
24. Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H. and Kornberg,

- A., Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* 2001, **293**:705-708.
25. Kuroda, A., Tanaka, S., Ikeda, T., Kato, J., Takiguchi, N. and Ohtake, H., Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 1999, **96**:14264-14269.
26. Mandelstam, J., Turnover of protein in growing and non-growing populations of *Escherichia coli*. *Biochem. J.* 1958, **69**: 110-119.
27. Maurizi, M. R., Proteases and protein degradation in *Escherichia coli*. *Experientia* 1992, **48**:178-201.
28. Morohoshi, T., Maruo, T., Shirai, Y., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H. and Kuroda, A., Accumulation of inorganic polyphosphate in *phoU* mutants of *Escherichia coli* and *Synechocystis sp.* strain PCC6803. *Appl. Environ. Microbiol.* 2002, **68**:4107-4110.
29. Nash, H. A. and Robertson, C. A., Purification and properties of the *Escherichia coli* protein factor required for lambda integrative recombination. *J. Biol. Chem.* 1981, **256**:9246-9253.
30. Nomura, K., Kato, J., Takiguchi, N., Ohtake, H. and Kuroda, A., Effects of inorganic polyphosphate on the proteolytic and DNA-binding activities of Lon in *Escherichia coli*. *J. Biol. Chem.* 2004, **279**:34406-34410.
31. Prentki, P., Chandler, M. and Galas, D. J., *Escherichia coli* integration host factor bends the DNA at the ends of IS1 and in an insertion hotspot with multiple IHF binding sites. *EMBO J.* 1987, **6**:2479-2487.
32. Ross, W., Thompson, J. F., Newlands, J. T. and Gourse, R. L., *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J.* 1990, **9**:3733-3742.
33. Rouviere-Yaniv, J., Yaniv, M. and Germond, J. E., *E. coli* DNA binding protein HU forms nucleosomelike structure with circular double-stranded DNA. *Cell* 1979, **17**:265-274.
34. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular cloning: a laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1989.
35. Surette, M. G. and Chaconas, G., A protein factor which reduces the negative supercoiling requirement in the Mu DNA strand transfer reaction is *Escherichia coli* integration host factor. *J. Biol. Chem.* 1989, **264**:3028-3034.
36. Sussman, A. J. and Gilvarg, C., Protein turnover in amino acid-starved strains of *Escherichia coli* K-12 differing in their ribonucleic acid control. *J. Biol. Chem.* 1969, **244**:6304-6306.
37. Verbeek, H., Nilsson, L., Baliko, G. and Bosch, L., Potential binding sites of the trans-activator FIS are present upstream of all rRNA operons and of many but not all tRNA operons. *Biochim. Biophys. Acta.* 1990, **1050**:302-306.
38. Yen, C., Green, L. and Miller, C. G., Degradation of intracellular protein in *Salmonella typhimurium* peptidase mutants. *J. Mol. Biol.* 1980, **143**:21-33.
39. Zulianello, L., de la Gorgue de Rosny, E., van Ulsen, P., van de Putte, P. and Goosen, N., The HimA and HimD subunits of integration host factor can specifically bind to DNA as homodimers. *EMBO J.* 1994, **13**:1534-1540.