RppH-dependent pyrophosphohydrolysis of mRNAs is regulated by direct interaction with DapF in Escherichia coli

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ABSTRACT

Similar to decapping of eukaryotic mRNAs, the RppH-catalyzed conversion of 5′-terminal triphosphate to monophosphate has recently been identified as the rate-limiting step for the degradation of a subset of mRNAs in Escherichia coli. However, the regulation of RppH pyrophosphohydrolase activity is not well understood. Because the overexpression of RppH alone does not affect the decay rate of most target mRNAs, the existence of a mechanism regulating its activity has been suggested. In this study, we identified DapF, a diaminopimelate (DAP) epimerase catalyzing the stereoinversion of L,L-DAP to meso-DAP, as a regulator of RppH. DapF showed a high affinity interaction with RppH and increased its RNA pyrophosphohydrolase activity. The simultaneous overexpression of both DapF and RppH increased the decay rates of RppH target RNAs by about a factor of two. Together, our data suggest that the cellular level of DapF is a critical factor regulating the RppH-catalyzed pyrophosphate removal and the subsequent degradation of target mRNAs.

INTRODUCTION

Nudix (nucleoside diphosphate X) hydrolases are widespread among eukaryotes, bacteria, archaea and viruses and hydrolyze a wide range of organic pyrophosphates. Substrates of nudix hydrolases include nucleoside di- and tri-phosphates, nucleotide sugars and alcohols, dinucleoside polyphosphates (NPₙN), dinucleotide coenzymes and capped RNAs, with varying degrees of substrate specificity (1). Nudix hydrolases have the conserved 23-amino acid Nudix motif (Nudix box) GX₅EX²REUXEEXGU, where X represents any amino acid and U is usually a bulky hydrophobic amino acid, such as Leu, Val or Ile (2). In Escherichia coli, 13 Nudix hydrolase genes have been found, and the physiological functions of some of these proteins were studied genetically and enzymatically (1–6). Among these genes, the nudH gene forms an operon with the downstream gene ptsP encoding the first component (enzyme I⁰) of the nitrogen-regulatory phosphoenolpyruvate-dependent phosphotransferase system (PTS⁰) (7–9). nudH was first identified as a gene associated with the invasiveness of E. coli K1 causing neonatal meningitis (10). Genetic experiments with several pathogenic bacteria have shown important roles for NudH and its orthologs in invasiveness and virulence (11–14). Studies on purified NudH have shown that it can catalyze the hydrolysis of diadenosine tetra-, penta- and hexa-phosphates with a preference for diadenosine penta-phosphate (Ap₄A) (15). However, the physiological importance of this activity is not clear.

Recently, NudH was renamed RppH because it has RNA pyrophosphohydrolase activity (16). RppH cleaves pyrophosphate from the 5′-terminal triphosphate of mRNA and this RppH-catalyzed conversion of the 5′-terminal triphosphate to monophosphate triggers endonucleolytic cleavage by RNase E in E. coli and 5′-exonucleolytic degradacion by RNase J in Bacillus subtilis (16,17). The RNA pyrophosphohydrolase activity is independent of the identity of the 5′-terminal nucleotide in vitro, and microarray analysis revealed that E. coli RppH induces the degradation of hundreds of transcripts.

The ability of RppH to remove a protective structure at the 5′ terminus is functionally similar to the removal of the cap structure from the 5′ ends of eukaryotic mRNAs. In both cases, the 5′-terminus of the 5′-proximal triphosphate is cleaved to produce a monophosphorylated intermediate vulnerable to attack by a 5′-monophosphate-dependent ribonuclease (16,18). The most well-studied and conserved eukaryotic decapping enzyme is Dcp2 (19). Although Dcp2 shares little sequence homology with RppH, Dcp2 is also a member of the Nudix hydrolase family (20). Whereas many

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

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co-factors and decapping enzymes regulating the catalytic activity of Dcp2 have been identified (19), the regulation of RppH activity has not been studied. In this study, we show that DapF, the dianinopimelate (DAP) epimerase catalyzing the biosynthesis of lysine and peptidoglycan (21), forms a tight complex with RppH to stimulate its RNA pyrophosphohydrolase activity both in vitro and in vivo. Our data suggest that the cellular level of DapF is an important factor regulating the RppH-catalyzed pyrophosphohydrolase removal, which is the rate-limiting step for the degradation of several hundred mRNAs in E. coli.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. All plasmids were constructed using standard polymerase chain reaction (PCR)-based cloning procedures and verified by sequencing. Bacterial cells were grown as described previously (22). The rppH deletion mutant was constructed using E. coli DY330 as described previously (23). The rppH gene (from the start codon to the stop codon) was replaced by the neo gene. The neo gene was amplified by PCR from the CR501 strain (22) using the following primers: forward primer, 5'-CATATGCAGTTCTCGAAAA-3' and reverse primer, 5'-GGATCCTGGTGATTGGAGTAAATGTTAAGACCAGTAGGATTAAGC-3' (mutated bases underlined). The expression vector pHRppH(E56&57A) for the overproduction of His6-RppH(E56&57A) was generated similarly using an additional mutagenic primer pair to amplify the region encoding Glu56 and Glu57: forward primer, 5'-GCATAATCCTACTGCAGGAACAAATCTACG-3'; reverse primer, 5'-CGTAGATTGTTTGCTGAGTAGAATTAGGC-3' (mutated bases underlined).

Purification of overexpressed proteins

Purification of His-tagged proteins (His-RppH, His-DapF, His-RppH(E56&57A), His-DapF(C73&217A) and His-EIIA<sup>Neo</sup>) was performed as previously described with some modifications (7). E. coli GI698 strains harboring pREI-based expression vectors were grown and protein expression was induced as described previously (25). The pellet of cells overexpressing each His-tagged protein was resuspended in binding buffer (50-mM Tris-HCl, pH 8.0, containing 300-mM NaCl) and then passed two times through a French pressure cell at 10 000 p.s.i. The lysate was cleared of cell debris by centrifugation at 100 000 x g for 90 min. The soluble fraction was loaded onto a BD TALON<sup>TM</sup> metal affinity resin (BD Biosciences Clontech) and bound proteins were eluted with binding buffer containing 200-mM imidazole. The fractions containing His-tagged protein were pooled and concentrated in an Amicon Ultracel-3K centrifugal filter (Millipore). To remove imidazole and to purify the protein to homogeneity (>98% pure), the concentrated pool was chromatographed on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare Life Sciences) equilibrated with 50-mM Tris-HCl (pH 8.0) containing 100-mM NaCl. The fractions containing the protein were pooled and concentrated as described above. The purified protein was stored at −80°C until use.

Ligand-fishing experiments using metal affinity chromatography

E. coli MG1655 cells grown overnight in 500 ml of Luria-Bertani (LB) medium were harvested, washed with binding buffer in the presence of 100 μg/ml phenylmethanesulfonyl fluoride (PMSF) and resuspended in 30 ml of the same buffer. The cell pellet was disrupted by passing it twice through a French pressure cell at 10 000 p.s. folowed by centrifugation at 100 000 x g for 60 min at 4°C. The supernatant was divided into aliquots and mixed with either binding buffer as control or 500 μg of His-RppH as bait. Each mixture was incubated with 500 μl of BD TALON<sup>TM</sup> metal affinity resin in a column for 30 min. The column was washed with 3 ml of binding buffer containing 5 mM imidazole three times, and the bound proteins were eluted with binding buffer containing 200 mM imidazole. Aliquots of the eluted protein sample (10 μl each) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R. The protein band specifically bound to His-RppH was excised from the gel, and in-gel digestion and peptide mapping of tryptic digests were performed as previously described (26).
Surface plasmon resonance spectroscopy

Real-time interaction of RppH with DapF was monitored by Surface Plasmon Resonance (SPR) detection using a BIACore 3000 (BIACore AB) instrument as previously described with some modifications (27–29). RppH was immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip. RppH (100 μL, 5 μg/ml) in coupling buffer (10 mM sodium acetate, pH 5.0) was flowed over the sensor chip at 5 μl/min to couple the proteins to the matrix by an N-hydroxysuccinimide/N-ethyl-

Gel filtration chromatography of the RppH–DapF complex

Gel filtration chromatography was performed in an ÄKTA-FPLC system (GE Healthcare Life Sciences). Samples containing 50 μg of RppH, 400 μg of DapF or both proteins in 250 μl of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl were incubated for 10 min on ice and injected through a Superose 12 10/300 GL column (GE Healthcare Life Sciences) equilibrated with the same buffer. Gel filtration was performed at room temperature at a flow rate of 0.5 ml/min and the elution profiles were monitored by measuring the absorbance at 280 nm. Fractions of 0.5 ml were collected, and each fraction (20 μl) was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R.

in vitro assay of RppH activity on synthetic RNAs

Triphosphorylated GCA and ACG mRNAs were synthesized from two strong promoters using the E. coli σ70-RNA polymerase holoenzyme. DNA sequences near the transcription start sites of the tyrT and rrsA promoters were slightly modified to make transcription begin with GCAT and ACAT, respectively, as shown in Supplementary Figure S1 and the regions spanning -60 to +25 relative to the transcription start sites were amplified by PCR. The two small mRNAs were synthesized in a 50-μl reaction containing 40 mM Tris-HCl, pH 8.0, 200 mM potassium glutamate, 5 mM dithiothreitol (DTT), 10 mM MgSO4, 50 ng of linear DNA template and 10 units of the E. coli σ70-RNAP holoenzyme. After 1 mM of adenosine triphosphate (ATP), cytosine triphosphate (CTP) and guanosine triphosphate (GTP) were added, the reaction mixtures were incubated at 37°C for 2 h, and the synthesized transcripts were separated by HPLC. Because uridine 5′-triphosphate (UTP) was not added to the reaction, transcription was terminated at the third base. Hydrolysis of small RNAs by RppH was analyzed as described for Ap5A except that triphosphorylated mRNAs and their monophosphorylated forms were separated using a linear gradient of 0–20% 20 mM ammonium acetate in methanol for 20 min.

Measurement of the phosphorylation state of mRNAs using exonuclease

The phosphorylation state of mRNAs after the RppH pyrophosphohydrolase reaction was tested using the Terminator 5′-phosphate-dependent exonuclease (Epicentre Biotechnologies), which digests RNAs that have 5′-monophosphate but not triphosphate ends. To test the pyrophosphohydrolase activity of RppH on the rpsT P1 transcript, the total RNA (10 μg) isolated from the E. coli MG1655 strain was incubated at 37°C for 1 h in reaction buffer containing 50 mM Tris-HCl (pH 8.8) and 5 mM MgCl2 with either purified RppH (0.4 or 0.8 μg), DapF (2.5 μg) or both. After phenol extraction, the RNAs were digested with the Terminator 5′-phosphate-dependent exonuclease at 30°C for 3 h followed by northern blotting with an rpsT-specific digoxigenin (DIG)-labeled probe. The decrease in the rpsT P1 transcript level corresponds to the level of dephosphorylation by RppH enzyme activity.

Northern blotting

E. coli cells were grown to an OD600 of 0.8 in LB medium, and the total RNA was extracted by the hot-phenol method (30). The purity and amount of RNA were verified by electrophoresis on a 1.6% agarose gel followed by staining with ethidium bromide. Northern blot analysis was performed as previously described (31). The total cellular RNA was separated on a 6% urea polyacrylamide gel and transferred to a positively charged nylon membrane (Roche Diagnostics) by electroblotting using the Mini Trans-Blot Cell (Bio-Rad). The blots were hybridized with a DIG-labeled oligonucleotide probe complementary to a specific mRNA at room temperature overnight using the DIG Northern Starter kit (Roche Diagnostics) according to manufacturer’s instructions. DIG-labeled probes were prepared by PCR amplifica-
tion using E. coli MG1655 genomic DNA as template, DIG-11-dUTP (Roche) and dNTPs as substrates, gene-specific forward and reverse primers and Ex Taq DNA polymerase (Takara). For the detection of the DIG-labeled probe, the membrane was treated with the anti-DIG-AP antibody and NBT/BCIP (Roche), and the image was scanned using the LAS-4000 IR multi-color imager (Fuji Film).

**RNA half-life measurements**

E. coli cells were grown to mid-logarithmic phase at 37°C, and the total cellular RNA was extracted at different time intervals after inhibiting transcription by the addition of rifampin (0.2 mg/ml). For quantitative real-time (qRT)-PCR analysis, cDNA was synthesized from 1 μg of DNAse I-treated total RNA using cDNA EcoDry Premix (Clontech), according to the manufacturer’s instructions. qPCR was performed in triplicate in a reaction volume of 20 μl, which included 10 μl of SYBR Premix Ex TaqII (Takara). Specific products were amplified and detected using the CFX96 Real-Time System (Bio-Rad). The 16S rRNA gene was used as a reference for normalization of the transcript level. The relative expression level was calculated as the difference between the threshold cycle (Ct) of the target gene and the Ct of the reference gene for each template.

**Bacterial two-hybrid assays**

Protein–protein interaction in live E. coli cells was assayed using the bacterial two-hybrid (BACTH) system based on the reconstitution of adenyl cyclase activity as described previously (32). Briefly, the cya-deficient E. coli strain BTH101 was co-transformed with pUT18c-derived plasmids and pKT25 derivatives. pUT18c and pKT25 encode the T18 and T25 fragments of *Bordetella pertussis* adenyl cyclase, respectively. The transformants were spotted on LB plates containing 100 μg/ml streptomycin, 100 μg/ml ampicillin and 50 μg/ml kanamycin with 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the color indicator for β-galactosidase activity and then incubated at 30°C overnight.

**RESULTS**

**Specific interaction between RppH and the DAP epimerase DapF**

Although the disruption of the gene encoding RppH affected the stability of hundreds of mRNAs, the overexpression of RppH did not influence the longevity of most target mRNAs (33). This observation suggests the existence of a prior event or another cellular factor regulating the activity of RppH. Genes encoded within the same operon often function in the same physiological context. The *rppH* gene is in the same operon as *ptsP*, which encodes enzyme I*N* of the PTS^N*, which regulates diverse physiological processes, such as amino acid metabolism and potassium homeostasis, by sensing nitrogen availability (8,9). Therefore, we envisioned that the regulation of RppH might be related to nitrogen metabolism.

To elucidate the mechanism regulating the activity of RppH, we used a ligand-fishing strategy (34,35). A crude extract from *E. coli* MG1655 was mixed with RppH or a 6His-tagged form of RppH (His-RppH) and subjected to pull-down assays using a metal affinity resin. We observed a protein band (apparent molecular mass of ~30 kDa) specifically eluting in a fraction containing His-tagged RppH (Figure 1A). Peptide mapping of the protein band indicated that it corresponds to the diaminopimelate (DAP) epimerase DapF, which catalyzes the stereoinversion of L,L-DAP to meso-DAP, the penultimate step in the lysine biosynthetic pathway (21,36). In most Gram-negative organisms and some Gram-positive organisms, meso-DAP is also used as a component of the pentapeptide in the biosynthesis of peptidoglycan (37).

To confirm the interaction between RppH and DapF, DapF with an N-terminal 6His-tag (His-DapF) was constructed. A crude extract of wild-type MG1655 cells was mixed with purified DapF or His-DapF and subjected to pull-down assays using TALON metal affinity resin. As shown in Supplementary Figure S2A, the protein band corresponding to RppH was detected in the eluate of this wild-type cell extract mixed with His-DapF but not in the eluate of the same extract mixed with untagged DapF. In addition, when ligand-fishing experiments were performed with the crude extract of RppH-overexpressing cells after incubating with DapF or His-DapF, a much higher amount of RppH was pulled down by His-DapF, but not by DapF, as expected (Supplementary Figure S2B). These data support the interaction of RppH with DapF.

To test the specificity of the interaction between RppH and DapF, purified DapF was mixed with varying amounts of His-RppH or the control protein His-enzyme IIA^N* and subjected to pull-down assays (Supplementary Figure S3). Whereas the amount of the DapF protein bound to the column was independent of the amount of His-enzyme IIA^N* pre-bound to the affinity resin, DapF binding increased with increasing amounts of His-RppH added to the column, confirming that DapF specifically interacts with RppH. The band intensities of the two proteins (Supplementary Figure S3) suggested that RppH interacts with DapF in a 1:1 ratio.

The tight interaction between DapF and RppH was also confirmed by gel filtration analysis. The elution profile of the complex from a Superose 12 gel filtration column (10 × 300 mm) was compared with those of the individual proteins. RppH was eluted with a symmetrical peak at 14 ml, corresponding to the monomeric form (20 kDa), while purified DapF was eluted at ~12 ml, corresponding to the dimeric form (62 kDa) (Figure 1B). This is consistent with previous studies on *E. coli* and *Haemophilus influenzae* in which the dimeric form of DapF was observed (36,38). When a mixture of the two proteins was loaded onto the column, the elution peak of RppH shifted to ~11.6 ml (Figure 1B and C), indicating that the molecular mass of the RppH–DapF complex is ~100 kDa (Supplementary Figure S4), corresponding to a heterotetramer with a 2:2 stoichiometry.

Kinetic parameters for the binding of DapF to RppH were determined using RppH immobilized to a sensor chip in the BIAcore system. Three different concentrations (20, 50 and 70 μg/ml) of purified DapF were used for the binding analysis (Supplementary Figure S5). Using the BIAe-
DapF stimulates RppH activity in vitro

The tight interaction between DapF and RppH suggested that DapF may regulate the activity of RppH. To test this hypothesis, we examined the effect of DapF on RppH activity. RppH catalyzes the hydrolysis of diadenosine pentaphosphate (Ap5A) in vitro (15). Consistent with the previous study, we observed that purified RppH hydrolyzed Ap5A to ATP, adenosine 5'-diphosphate (ADP) or adenosine 5'-monophosphate (AMP) in vitro (Figure 2). Notably, the addition of DapF to the reaction mixture significantly increased the Ap5A hydrolyase activity of RppH. DapF alone did not show any pyrophosphohydrolase activity, implying that the apparent activation of RppH by DapF does not reflect contamination with RppH during the preparation of DapF.

We then tested the RNA pyrophosphohydrolase activity of RppH using a triphosphorylated short mRNA (5'-pppGpCpA) as a substrate. A recent study showed that *B. subtilis* RppH requires at least two unpaired nucleotides at the 5' end of its RNA substrates (39). RppH prefers three or more nucleotides and the difference between three and four or more nucleotides was negligible. Therefore, we measured the activity of RppH using three nucleotides. The GCA sequence represents the first three nucleotides of the *efp* transcript, which is a known substrate of RppH (16). Upon incubation with purified wild-type RppH, pppGpCpA was converted to a monophosphorylated form, whereas the purified RppH(E56&57A) protein, in which the 56th and 57th glutamates of RppH were mutated to alanine, could not remove pyrophosphate from the substrate (Figure 3A and Supplementary Figure S6). When incubated with the Terminator™ 5'-monophosphate-dependent exonuclease, the reaction product, but not the substrate, was degraded (Supplementary Figure S7). This result confirms that the 5’-monophosphorylated form is the reaction product. Notably, most *E. coli* gene transcripts downregulated by RppH begin with G or A (16). When we tested the pyrophosphohydrolase activity of RppH on pppApCpG, the same results were obtained (Figure 3B). As for the Ap5A hydrolyase activity, DapF stimulated mRNA pyrophosphohydrolase activity of RppH, irrespective of the first nucleotide (Figure 3). The observed activation of RppH activity is unlikely to be due to a carrier effect of DapF, since the same amount of bovine serum albumin (BSA), a known carrier protein, did not activate the pyrophosphohydrolase activity of RppH. Notably, RppH was slightly more active on the triphosphorylated RNA starting with A rather than G (compare Figure 3A with B); this observation is consistent with the results from recent studies on RppH in *B. subtilis* (39,40).

The DapF enzyme catalyzes the conversion of L,L-DAP to meso-DAP, which is an intermediate for the biosynthesis of lysine and peptidoglycan (21,41). To study whether RppH activation by DapF is dependent on DapF enzymatic activity, the two catalytic cysteine residues of DapF, C73
Figure 2. Effect of DapF on the Ap5A-hydrolysis activity of RppH. The Ap5A-hydrolysis activity of RppH (0.5 μg) was assayed in a 200-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 20 mM MgCl2 and 100 μM Ap5A with and without DapF (1.5 μg). After incubation at 37°C for 10 min, the reaction was terminated by adding 20 μl of 20% trifluoroacetic acid. A 10-μl reaction mixture was applied to a Hypersil Gold C18 reverse phase chromatography column (Thermo Scientific) equilibrated with 20 mM ammonium acetate buffer (pH 5.0) in water and then chromatographed using a linear gradient of 0–50% 20 mM ammonium acetate in methanol at a flow rate of 1 ml/min for 20 min using a Varian dual pump HPLC system. Ap5A and the reaction products were monitored by measuring the absorbance at 254 nm (A254). Relative peak areas are shown on the top of each peak with the peak of Ap5A in the control sample assigned a value of 100.

To test the pyrophosphohydrolase activity of RppH on an in vivo substrate and to confirm the stimulatory effect of DapF on RppH activity, total RNA was extracted from wild-type cells grown in LB medium to the exponential phase. The extracted RNA was incubated with various concentrations of purified RppH, DapF or both, and each reaction mixture was treated with the Terminator 5′-phosphate-dependent exonuclease. By northern blot analysis using a specific probe, we measured the level of 5′-triposphorylated rpsT P1 mRNA, which is a known substrate of RppH (16). RppH alone decreased the amount of 5′-triposphorylated rpsT P1 mRNA in a concentration-dependent manner (compare lanes 1, 3 and 5 in Figure 5). Whereas DapF alone did not alter the transcript level, it significantly stimulated the RppH-mediated decay of the rpsT P1 transcript. The stimulation of the RppH-catalyzed pyrophosphate removal from the same transcript by DapF was further confirmed using different concentrations of RppH and DapF by both northern blot experiment and qRT-PCR (Supplementary Figure S8).

DapF forms a tight complex with RppH and potentiates RppH activity in vivo

The in vivo interaction between DapF and RppH was tested using two methods. First, the pDuet-RD vector was constructed from the pET-duet vector to co-express DapF and His-RppH for pull-down assays. When the crude extract prepared from E. coli ER2566 cells harboring pDuet-RD was loaded onto the TALON™ metal affinity column, DapF co-eluted with His-RppH; however, DapF was barely detectable in the eluate when the crude extract prepared from the cells overexpressing DapF alone was loaded (Supplementary Figure S9A). Based on the band intensity on the SDS-PAGE gel, RppH might interact with DapF to form a complex with a 1:1 molar ratio, which is consistent with the gel filtration chromatography data showing that DapF and RppH can form a heterotetramer with a 2:2 stoichiometry (Figure 1C).

The in vivo interaction between DapF and RppH was also confirmed using BACTH assays. For the BACTH assays, DapF and RppH were fused to the C-terminal ends of the T18 and T25 fragments of B. pertussis adenylyl cyclase, respectively. Whereas cells co-expressing the unfused T25- and T18-fragments did not develop a color, cells co-producing T18-DapF and T25-RppH developed a blue color similar to the positive control strain expressing the T25- and T18-fragments fused to the leucine zipper of the transcription factor GCN4 (Supplementary Figure S9B). Therefore, these results demonstrate that the tight interaction between RppH and DapF occurs in vivo.

In bacteria, concentrations of diadenosine oligophosphates increase more than 100-fold over the endogenous level in response to heat or oxidative stress (44). Furthermore, RppH levels decrease during cellular stress (45), suggesting that RppH might be involved in stress responses. To determine the phenotype associated with RppH activity, we constructed an rppH mutant and strains overproducing the wild-type and mutant forms of RppH and tested the response of these strains to various stresses, including thermal, oxidative and osmotic stresses. Cells with different ex-
Figure 3. Effect of DapF on RppH pyrophosphohydrolase activity. The pyrophosphohydrolase activity of RppH (0.5 μg) was measured in vitro using two synthetic RNAs (pppGpCpA (A) and pppApCpG (B)) in the presence and absence of 1.5 μg of DapF or BSA (as control). Triphosphorylated and monophosphorylated RNAs were separated on a Hypersil Gold C18 reverse phase chromatography column (Thermo Scientific) equilibrated with 20 mM ammonium acetate buffer (pH 5.0) in water and then chromatographed using a linear gradient of 0–20% 20 mM ammonium acetate in methanol at a flow rate of 1 ml/min for 20 min using a Varian dual pump HPLC system. The eluted nucleotides were monitored by measuring the A254. Relative amounts of triphosphorylated or monophosphorylated forms of two synthetic RNAs were measured by the relative peak areas (shown on the top of each peak) with a peak area of 100 for the amount of triphosphorylated RNAs in the control sample.

expression levels of RppH did not show any significant difference in growth compared to the wild type when grown in LB medium, irrespective of growth temperature or the addition of 0.2 mM H₂O₂. However, an RppH-overproducing strain had a significant and reproducible difference in sensitivity to osmotic stress compared to the wild-type strain. Notably, the overexpression of RppH caused hypersensitivity to high osmolality (750 mM NaCl), whereas the rppH mutant and the strain overproducing an inactive form of RppH (E56&57A) were as resistant as the wild-type strain (Figure 6). The expression level of mutant RppH was similar to that of the wild-type protein (Supplementary Figure S10). The rppH mutant with the RppH-expressing plasmid was slightly more sensitive than the wild-type and the rppH mutant strain, but more resistant than the wild-type strain harboring the RppH-expressing plasmid to salt stress (Supplementary Figure S11). Therefore, these data suggest that the hypersensitivity of the RppH-overproducing strain to osmotic stress is primarily due to increased enzyme activity.

Considering that DapF stimulates the activity of RppH by direct interaction, we proposed that increased expression of DapF might affect osmotic sensitivity. Overexpression of DapF in a wild-type strain led to hypersensitivity to high osmolality, whereas in the rppH mutant strain, the overexpression of DapF had little effect on osmotic sensitivity. To exclude the possibility that the phenotype of DapF overproduction is due to increased DAP epimerase activity, we performed the same experiment using a strain overproducing DapF(C73&217A), which has no catalytic activity but is still capable of interacting with and stimulating RppH (Figure 4). Wild-type cells overproducing the mutant DapF were as sensitive to high osmolality as the cells overproducing wild-type DapF, but the rppH mutant strain overproducing the mutant DapF did not show osmotic sensitivity (Figure 6). Together, these data suggest that higher RppH activity renders cells more sensitive to osmotic stress, and the effect of DapF overexpression on osmotic sensitivity is exerted through the stimulation of RppH activity.

Stimulation of RppH pyrophosphohydrolase activity by DapF in vivo

Using gene array analysis, the abundance of 382 gene transcripts including the rpsT mRNA was found to be significantly higher in cells containing the mutant RppH compared to the wild-type strain (16). To validate the in vivo effect of DapF on RppH, concentrations of specific substrate mRNAs were measured in the wild-type, rppH mutant and dapF mutant strains by northern blot analysis. Consistent with the previous study (16), the levels of all tested substrate mRNAs were significantly higher in the rppH mutant cells compared to wild-type cells (Figure 7). Notably, the concentrations of the osmY and yeiP transcripts increased in the dapF mutant cells to levels similar to those in the rppH mutant cells. The concentrations of the slyB and rpsT transcripts in the dapF mutant cells were lower than those in the rppH mutant cells but still higher than those in wild-type cells. The concentrations of two rpsT transcripts and the
**Figure 4.** Complex formation with RppH and the activation of RppH are independent of the DAP epimerase activity of DapF. (A) Formation of a complex between RppH and DapF(C73&217A). Samples containing 50 μg of RppH, 100 μg of DapF(C73&217A) or both were injected onto a Superose 12 10/300 GL column, and each fraction (20 μl) was electrophoresed in SDS-PAGE gels and stained with Coomassie Brilliant Blue R. (B) Stimulation of RppH activity by DapF(C73&217A) in a dose-dependent manner. The Ap5A-hydrolysis activity of RppH (0.5 μg) was compared in the presence and absence of DapF(C73&217A) (1.5 μg or 7.5 μg). The reactions were monitored by HPLC as described in the legend to Figure 3. Relative peak areas are shown on the top of each peak.

**Figure 5.** Effect of DapF on RppH-mediated conversion of the rpsT transcript from the triphosphate to monophosphate form. The total RNA isolated from E. coli was incubated with purified RppH, DapF or both and digested with the Terminator 5′-phosphate-dependent exonuclease. The remaining transcript was detected by northern blot analysis using an rpsT-specific probe or a 23S rRNA-specific probe: lane 1, no addition; lane 2, 2.5 μg of DapF; lane 3, 0.4 μg of RppH; lane 4, 0.4 μg of RppH and 2.5 μg of DapF; lane 5, 0.8 μg of RppH; lane 6, 0.8 μg of RppH and 2.5 μg of DapF. Band intensities of the rpsT transcript were analyzed using the Multi Gauge V3.0 software and given below each lane.
Figure 6. Hypersensitivity of strains with increased RppH activity to high osmolarity. Stationary phase cells of the indicated strains grown in LB medium were serially diluted 10-fold from $\sim 10^9$ to $\sim 10^4$ cells/ml, and 1-μl aliquots were spotted onto LB agar plates with (left) and without (right) the addition of 750 mM NaCl. After incubation at 37°C for 16–18 h, the plates were scanned.

Figure 7. Influence of DapF on the levels of RppH substrate mRNAs. The indicated strains were grown in LB medium at 37°C. The total RNA was prepared from each strain at exponential phase and analyzed by northern blotting using standard conditions to analyze the levels of some RppH target mRNAs: (A) osmY, (B) yeiP, (C) rpsT, and (D) slyB. The DIG-labeled hybridization probes are shown to the left of each panel. The blot with the 23S rRNA probe ((A) and (C)) or 5S rRNA probe ((B) and (D)) was used as a loading control.

producing RppH or DapF (Figure 9). In addition, the salt hypersensitivity of the RppH- and DapF-overproducing strains was suppressed by overexpression of the osmY gene. Although further studies are required to fully understand the relationship between RppH-mediated mRNA degradation and the hyperosmotic stress response, our data suggest that the salt sensitivity of RppH- and DapF-overexpressing strains is likely to be due to the decreased level of the osmY transcript. The decrease in the osmY mRNA level could be due, at least in part, to the increased pyrophosphohydrolase activity. In this regard, the significantly decreased expression level of DapF in stationary phase cells compared to exponentially growing cells (Supplementary Figure S14A) might explain the previous result showing that expression of the osmY gene is induced upon entry into stationary phase (47). It should be noted that the RppH level remained relatively constant regardless of growth phase. Therefore, our data suggest that the cellular level of DapF is a critical factor regulating the RppH activity.

DISCUSSION

In parallel with the mechanism of mRNA decay in eukaryotic organisms, the status of the 5′ end is critical to mRNA decay in bacteria (48). In E. coli, the RNA pyrophosphohydrolase RppH initiates mRNA decay by con-
Figure 8. Effect of DapF on the decay rates of RppH target mRNAs. The total RNAs were extracted from the wild-type (closed diamonds), rppH mutant (closed squares), RppH-overexpressing (closed triangles) and DapF(C73&217A)-overexpressing (closed circles) strains, and the strain overexpressing both RppH and DapF(C73&217A) (open squares) at the indicated times after inhibiting transcription by the addition of rifampin. Transcript levels were analyzed by qRT-PCR with primers specific for rpsT P1, yeiP or 16S rRNA. The mRNA levels were normalized to the concentration of 16S rRNA and plotted as a function of time. Average data from two independent experiments are shown.

Figure 9. Salt hypersensitivity of cells with an increased RppH activity is suppressed by overexpression of the osmY gene. Effects of osmY deletion (A) and overexpression (B) on the salt sensitivity of the wild-type strain (WT), and strains harboring RppH and DapF expression vectors were measured. Stationary phase cells of the indicated strains grown in LB medium were serially diluted 10-fold from ~10⁶ to ~10⁴ cells/ml, and 1-µl aliquots were spotted onto LB agar plates with (left) and without (right) the addition of 750 mM NaCl. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM to induce expression of OsmY in (B). After incubation at 37°C for 16–18 h, the plates were scanned.

Together with previous reports, our data suggest that the major physiological role of DapF might be to stimulate RppH activity rather than to produce meso-diaminopimelate (DAP). meso-DAP is an essential component of the cell wall peptidoglycan and is decarboxylated by LysA to generate L-lysine in most Gram-negative bacteria. meso-DAP can be synthesized by two pathways in bacteria: the epimerase pathway in which DapF converts L,L-DAP to meso-DAP and the dehydrogenase pathway in which DAP dehydrogenase directly converts tetrahydrodipicolinate to meso-DAP (37). However, because the dehydrogenase pathway exists only in a small number of Gram-positive bacteria such as Bacillus sphaericus (37), meso-DAP and consequently L-lysine are thought to be generated only through the epimerase pathway in E. coli. However, a dapF deletion mutant did not require meso-DAP or L-lysine for growth and could synthesize meso-DAP, although this mutation accumulated large amounts of L,L-DAP, the substrate of the epimerase reaction (21,41). These data suggest that there might be another enzyme capable of catalyzing the DAP degradation in E. coli. DapF stimulated the mRNA pyrophosphohydrolase activity of RppH by direct protein–protein interaction with unusually high affinity (Kₐ ~ 5.2 × 10⁻⁹ M) and this stimulation was independent of the catalytic activity of DapF (Figures 2–4). Whereas increased expression of RppH alone had only a slight effect on the decay rates of the tested mRNAs, the coexpression of RppH with DapF significantly increased the decay rates of these transcripts. Furthermore, several transcripts were stabilized in the dapF mutant and in the rppH mutant and the overproduction of DapF accelerated the decay of some of these transcripts (Figure 7), suggesting that the cellular level of DapF is a critical factor regulating the RppH-catalyzed pyrophosphate removal and subsequent degradation of target mRNAs.

DapF, which was identified as a regulator of RppH-mediated mRNA degradation in E. coli, stimulated the mRNA pyrophosphohydrolase activity of RppH by direct protein–protein interaction with unusually high affinity (Kₐ ~ 5.2 × 10⁻⁹ M) and this stimulation was independent of the catalytic activity of DapF (Figures 2–4). Whereas increased expression of RppH alone had only a slight effect on the decay rates of the tested mRNAs, the coexpression of RppH with DapF significantly increased the decay rates of these transcripts. Furthermore, several transcripts were stabilized in the dapF mutant and in the rppH mutant and the overproduction of DapF accelerated the decay of some of these transcripts (Figure 7), suggesting that the cellular level of DapF is a critical factor regulating the RppH-catalyzed pyrophosphate removal and subsequent degradation of target mRNAs.

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epimerase reaction at a rate sufficient to sustain growth in *E. coli*.

If the synthesis of *meso*-DAP from L-L-DAP is the major role of DapF, the expression level of DapF should increase in cells grown in a medium deficient in L-lysine compared to cells grown in a medium supplemented with the amino acid. However, the expression level of DapF was higher in *E. coli* cells grown in lysine-containing media (LB and M9 supplemented with casamino acids) compared to cells grown in M9 medium without amino acids (Supplementary Figure S14B). Furthermore, many of the currently identified mRNA targets of RppH are responsible for the synthesis of amino acids such as His, Trp, Tyr, Phe, Thr, Ile and Val (16). Therefore, the expression level of DapF might increase in amino acid-rich medium to stimulate the decay of mRNAs involved in the biosynthesis of amino acids. Further studies are required to elucidate the mechanism regulating DapF expression level and to delineate the connection between RppH-mediated mRNA degradation and amino acid metabolism.

Enolase is a glycolytic enzyme that catalyzes the dehydration of 2-phospho-D-glycerate to form phosphoenolpyruvate and the reverse reaction in gluconeogenesis (49). In *E. coli*, approximately one-tenth of the total enolase is associated with RNase E, the polynucleotide phosphorylase PNPase and the ATP-dependent RNA helicase RhlB to form the RNA degradasome (50). The role of enolase in the degradasome has not been definitively established. However, mutational analyses demonstrated that enolase within the degradasome plays a crucial role in the regulation of *ptsG* mRNA stability in the response to phosphosugar stress (51), which is mediated by the small regulatory RNA SgrS. Another study using DNA microarray analyses suggested that the association of enolase with RNase E in the degradasome affects transcripts that encode enzymes of energy-generating pathways (52). In this study, we provide another instance where a metabolic enzyme is involved in an RNA degradation complex. Like enolase, DapF might specifically target genes involved in certain metabolic pathways such as the biosynthesis of amino acids. Further studies on the regulation of DapF expression and the effect of DapF on the affinity of RppH for their target mRNAs may help to understand why some mRNAs respond differently to RppH and DapF levels.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES


