Mechanism of methionine synthase overexpression in chaperonin-depleted *Escherichia coli*

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The chaperonin GroE (GroEL and the co-chaperonin GroES) is the only chaperone system that is essential for the viability of *Escherichia coli*. GroE is absolutely required for the folding of at least 57 proteins in *E. coli*, referred to as class IV substrates, and assists in the folding of many more. Although GroE is mainly involved in protein folding, when it is depleted, the expression levels of about a hundred further proteins can be seen to increase, most prominently methionine synthase (MetE). Here we investigate the mechanism of *metE* overexpression in GroE-depleted cells. Gene fusion experiments in which the *metE* transcriptional region was fused to an assayable reporter showed that addition of a GroE-independent MetK homologue [MetK synthesizes S-adenosylmethionine (SAM), the *metJ* corepressor] to the system (*E. coli* MetK depends on GroE for folding) almost fully suppressed the increased expression. An analysis of deletion mutants in the *metE* promoter, and overexpression and disruption of the *metR* gene, showed that the absence of MetJ binding and increased levels of the activator MetR resulted in the overexpression of MetE. We conclude that the need of *metE* for MetK, and the need of *metK* for GroE, can explain the overexpression of methionine synthase in GroE-depleted cells.

In parallel with these proteomic studies, the phenotypes of GroE-deficient cells have been investigated. GroE-depleted cells show obvious phenotypes, including cell lysis, cell filamentation, and eventual cell death (Fujiwara & Taguchi, 2007; McLennan & Masters, 1998). Detailed *in vivo* analyses have shown that the dysfunction of class IV proteins caused by GroE depletion is responsible for these phenotypes. In the case of cell lysis and filamentation, DapA and FtsE, respectively, have been identified as the sole class IV substrates responsible (Fujiwara & Taguchi, 2007; McLennan & Masters, 1998).

In addition to these phenotypes, global proteome analyses have shown that GroE depletion or impairment induces about a hundred proteins in cells (Fujiwara et al., 2010; Horwich et al., 1993; Kanemori et al., 1994). The protein most dramatically induced by GroE depletion is methionine synthase (MetE) (Chapman et al., 2006; Fujiwara et al., 2010; Horwich et al., 1993; Masters et al., 2009). In fact, MetE is so strongly induced by GroE depletion that it can be easily detected in Coomassie brilliant blue (CBB)-stained gels (Chapman et al., 2006; Fujiwara et al., 2010; Masters et al., 2009). Since MetE is the only protein that shows such high, detectable levels of overexpression under GroE depletion, studies of the mechanism of MetE overexpression should be useful for understanding the roles of GroE in cells. Chapman and co-workers suggested that two class IV proteins, MetK and MetF, are associated with MetE overexpression in GroE-impaired cells, based on

INTRODUCTION

A subset of proteins in cells absolutely depends on molecular chaperones to fold into the correct tertiary structures (Chapman et al., 2006; Fujiwara et al., 2010; Houry et al., 1999; Kerner et al., 2005). The chaperonin GroE (i.e. GroEL and the co-chaperonin GroES) is a highly conserved molecular chaperone, and is the only chaperone that is essential for the viability of *Escherichia coli* at all temperatures (Fayet et al., 1989; Horwich et al., 1993). Since GroE is the only indispensable chaperone of *E. coli*, studies of the roles of GroE in *E. coli* cells may be able to explain how GroE affects protein homeostasis in cells, which would contribute to our understanding of the roles of chaperones in cells.

In particular, the identification of GroE substrates in cells is critical for clarifying the roles of GroE. Purified GroEL can bind about half of the soluble proteins in *E. coli* lysate (Chapman et al., 2006; Houry et al., 1999; Kerner et al., 2005; Viitanen et al., 1992). In fact, MS-based proteomic studies of GroE-binding proteins have identified hundreds of proteins that interact with GroE in cells (Chapman et al., 2006; Houry et al., 1999; Kerner et al., 2005). Our recent systematic survey revealed that 57 proteins, including six that are essential for viability, are bona fide obligate GroE substrates, which are defined as class IV substrates, in *E. coli* (Fujiwara et al., 2010).

Abbreviations: CBB, Coomassie brilliant blue; RU, relative units; SAM, S-adenosylmethionine; SMM, S-methylmethionine.
their proteomics study (Chapman et al., 2006). We recently found that the heterogeneous expression of MetK from *Ureaplasma urealyticum*, which lacks GroE (Glass et al., 2000), reduced MetE overexpression to levels that could not be detected in CBB-stained gels (Fujiwara et al., 2010), suggesting that the complementation of MetK is sufficient to suppress the overexpression of MetE. However, the details of how GroE depletion causes the overexpression of MetE were still unclear.

In this study, we focused on the mechanism of the overexpression of MetE in GroE-depleted cells. A complementation assay revealed that the *metE* promoter and the dysfunction of MetK, a class IV GroE substrate, are responsible for the overexpression of MetE. We also showed that a co-repressor shortage of the repressor MetJ with overproduction of the activator MetR induces the overexpression of MetE, and that dysfunction of MetF caused by GroE depletion contributes only slightly to the overexpression of MetE.

**METHODS**

**Plasmids.** pMCS, in which the T7 promoter region of the pET vector is replaced with tac, was used as a vector (Fujiwara & Taguchi, 2007). To construct pMCS-ParM-FLAG, a fragment encoding full-length parM was amplified by PCR from pMD137 (Dam & Gerdes, 1994). A FLAG tag (DYKDDDDK) was added at the C terminal of the parM gene by PCR. To construct pMetE240-ParM-FLAG (240 reporter), pMetE240dR-ParM-FLAG (dR reporter), pMetE150dJ-ParM-FLAG (dJ reporter) and pMetR240-ParM-FLAG (MetR reporter), the 240 bp region upstream from the *metE* first codon, the 210 bp region upstream from the *metE* first codon that lacks MetR-binding sites, the 79 bp region from the 240 bp region upstream of the *metE* first codon to downstream fusing the 15 bp region from the *metE* first codon that lacks MetJ-binding sites, or the 240 bp region upstream from the *metR* first codon, respectively (details are given in Fig. 1a), were amplified from *E. coli* K-12 MG1655 chromosomal DNA by PCR. The tac promoter of pMCS-ParM-FLAG was replaced with the amplified fragments after digestion with BglII/NdeI. In all cases, the first codons of the reporter gene were ATG of the NdeI site, and thus the 3 bp before the *metE* first codon (AAA) was replaced with CAT. pMetEp-parM-FLAG was constructed by replacing the parM-FLAG gene with the metK gene of *E. coli* K-12 MG1655.
To construct pACYCtac-UuMetK, the BglII/Xhol-digested fragment of pMCS-UuMetK, which contained the metK gene of *U. urealyticum* regulated by the tac promoter, was cloned into the BglII–Xhol site of pACYC-Duet (Takara). To construct pACYCtac-MmuM, the mmuM gene was amplified from *E. coli* K-12 MG1655 chromosomal DNA by PCR. The amplified MmuM fragment was treated with Ndel/Xhol, and cloned into pACYCtac-UuMetK by replacing the metK gene with the mmuM gene. To construct pACYCtac-MetR, the metR gene was amplified from *E. coli* K-12 MG1655 chromosomal DNA by PCR. The amplified MetR fragment was treated with Ndel/Xhol, and cloned into pACYCtac-UuMetK by replacing the metK gene with the metR gene.

**RESULTS**

**The metE promoter is responsible for the reconstitution of MetE overexpression in GroE-depleted cells**

To investigate the mechanism by which methionine biosynthesis proteins are overexpressed in GroE-depleted cells, we focused on methionine synthase (MetE), which is one of the proteins most highly overexpressed in the presence of GroE depletion (Chapman et al., 2006; Fujiwara et al., 2010; Horwich et al., 1993; Kanemori et al., 1994; Masters et al., 2009). First, we investigated whether this overexpression is driven by the promoter of the *metE* gene. The transcription start site of the *metE* promoter is located 169 bp upstream from the start codon of *metE* (Cai et al., 1989b). Thus, we used the 240 bp region upstream from the MetE first codon, which included the promoter and operator (Fig. 1a) (Cai et al., 1989b; Maxon et al., 1989). A C-terminal FLAG-tagged *parM* gene (parM–FLAG), which is encoded in R1 plasmids but not in the genome of *E. coli*, and the *metK* gene of *E. coli* (EcMetK), a class IV substrate of GroE (Fujiwara & Taguchi, 2007; Fujiwara et al., 2010; Kerner et al., 2005), were fused with the 240 bp promoter (Fig. 1b). After GroE depletion, the overexpression of both proteins was observed by the use of CBB-stained gels (Fig. 1c). While overexpressed ParM–FLAG was soluble, overexpressed EcMetK aggregated. Since overexpressed EcMetK was soluble under GroE-normal conditions (Fujiwara & Taguchi, 2007), the aggregation of EcMetK indicated that the *metE* promoter was activated after GroE depletion. Taken together, these results suggest that the *metE* promoter is responsible for the overexpression of MetE, and that the 240 bp region upstream of the *metE* gene is sufficient for analysing the mechanism of the overexpression of MetE caused by GroE depletion.

**Heterologous expression of Ureaplasma MetK suppresses the overexpression of MetE under GroE-depleted conditions**

As described above, ParM–FLAG folded spontaneously into the soluble form, regardless of the GroE level. This indicated that the difference in GroE expression levels does not affect the levels of ParM–FLAG when the promoter activity is the same. Since it has been suggested that several soluble proteins are inactive in GroE-depleted cells (Chapman et al., 2006; Masters et al., 2009), we avoided enzymic activity-based detection by immunoblotting using horseradish peroxidase-conjugated anti-FLAG monoclonal antibody (Sigma). Bands were detected and quantified with an LAS 4000 imager (Fujifilm). To avoid the saturation of bands, samples were diluted to appropriate concentrations before loading onto gels. To adjust for differences in blotting efficiency and the sensitivity of detection, standard samples were loaded in all experiments. The relative units (RU) per milligram protein in this study were determined by dividing each quantified value by the value for GroE-normal cells containing pMetE240-ParM-FLAG. All experiments were performed with cells cultivated in three independent media.

**Strain.** *E. coli* MGM100 cells [MG1655 groE::araC-P_ABAD groEl Kan’] (McLennan & Masters, 1998) were used to analyse cell physiology under GroE depletion. With this strain, when the sugar in the growth medium was changed from arabinose to glucose, the GroE levels decreased by ~90 % within 2 h (Fujiwara et al., 2010; McLennan & Masters, 1998). To suppress the cell lysis phenotype due to the loss of GAP in GroE-depleted cells, the growth medium was supplemented with 1 mM Dapaminopimelate (Wako) throughout this study.

**Disruption of the metR gene in E. coli MGM100 cells.** The chromosomal metR gene in *E. coli* MGM100 cells was disrupted by pre-ligation methods (Yamada et al., 1995). At first an ~2200 bp fragment, which was from 600 bp upstream of the MetR first codon to 600 bp downstream of the MetR terminal codon, was amplified from *E. coli* K-12 MG1655 chromosomal DNA by PCR. The amplified fragment was treated with BamHI/Xhol, and ligated into the BglII–Xhol site of vector pET15b (Novagen). In these steps, the EcoRI site was added at 600 bp upstream from the metR gene by PCR. Second, the 546 nt position of the metR gene was cut by BglII, and a chloramphenicol-resistance gene, which was amplified from vector pACYC by PCR, was ligated into the BglII-digested site. EcoRI cut the obtained plasmid at two sites, the added EcoRI site and the specific EcoRI site of vector pET15. Thus, the EcoRI-digested fragment was self-ligated, and then introduced into MGM100 cells. Colonies on chloramphenicol plates were picked up, and disruption of the metR gene was confirmed by PCR. The obtained disruptants of metR were used for the present study.

**Preparation of cell extracts for reporter assays.** MGM100 cells harbouring pMetE240-ParM-FLAG, pMetEp240-EcMetK, pMetE240dI-ParM-FLAG, pMetE190dR-ParM-FLAG and pMetR240-ParM-FLAG, and the metR disruptant of MGM100 harbouring pMetE240-ParM-FLAG, were grown in Luria–Bertani (LB) medium containing 200 μg ampicillin ml⁻¹ and 0.2 % arabinose at 37 °C to OD₆₆₀ 0.5. For MGM100 cells harbouring both pACYCtac-UuMetK, pACYCtac-MmuM or pACYCtac-MetR and a reporter plasmid, 0.2 % chloramphenicol was added to the medium. The cells were then washed twice with LB medium. The washed cells were diluted in LB with 1 mM DAP, containing either 0.2 % arabinose or 0.2 % glucose. To harvest cells before the OD₆₆₀ reached 0.8, the dilution rates were 1:5000 for the arabinose medium and 1:250 for the glucose medium. S-Methylmethionine (SMM) (1 mM) was added to the medium to estimate the effect of homocysteine. After 5 h of cultivation, the cells were disrupted by Sonifier (Branson). The obtained cell lysates were used as total lysates. Supernatants and precipitates were obtained by centrifugation at 20000 g. Differences in optical density among samples were much smaller than the differences in expression levels of reporter proteins, except in the case of MetR overexpression with the d) reporter.

**Quantitative Western blotting.** MGM100 cells harbouring one or two of the above plasmids were grown according to the procedure described above. Cells were harvested at 5 h after GroE depletion, suspended in lysis buffer [20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA] to adjust to equivalent OD₆₆₀ units, and sonicated (Branson Sonifier). Total extracts were loaded onto 12 % SDS gels and
Overexpression of a class IV protein before GroE depletion has been used to suppress the deficiency of the protein (Fujiwara & Taguchi, 2007; McLennan & Masters, 1998). We tried such previous overexpression of MetK, but the MetK overexpression completely failed to suppress the MetE overexpression. Thus, we used UuMetK, leaky expression of which can suppress observable MetE overexpression by CBB-stained gels, for further experiments. Thus, MetK of U. urealyticum (UuMetK), which does not require GroE to form an active structure (Fujiwara et al., 2010), was used to evaluate the contribution of MetK dysfunction in GroE depletion. Our recent study showed that soluble EcMetK disappears in GroE-depleted cells, and a low expression level of UuMetK suppresses the overexpression of MetE to some extent (Fujiwara et al., 2010). However, the suppression levels were not quantified. Cells harbouring both the reporter plasmid and the UuMetK-inducible plasmid were cultivated in medium supplemented with IPTG. The reporter assays showed that the activity of the metE promoter was decreased by 99% in the presence of 50 mM IPTG (Table 1). Induction with 1 mM IPTG fully suppressed the overexpression induced by GroE depletion [0.2 RU (mg protein)−1]. The results suggested that no factor other than MetK affects the overexpression of MetE.

**Table 1. Activity of the metE promoter and metE deletion mutants under different GroE conditions**

The differences in the relative units for each sample were all significant (P<0.05), except between MetEp240 in GroE-normal cells and MetEp240dJ in GroE-normal cells, and between MetEp240 in GroE-depleted cells expressing the UuMetK gene in the presence of 50 mM IPTG and MetEp240dJ in GroE-depleted cells. Mean ± SD values from three independent experiments are shown.

<table>
<thead>
<tr>
<th>Region</th>
<th>GroE-normal</th>
<th>Depleted</th>
<th>Depleted + UuMetK*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MetEp240</td>
<td>1.0 ± 0.2</td>
<td>4459 ± 999</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>MetEp240-dJ</td>
<td>92 ± 12</td>
<td>5288 ± 828</td>
<td>213 ± 10</td>
</tr>
<tr>
<td>MetEp240-dR</td>
<td>1.4 ± 0.4</td>
<td>31 ± 5</td>
<td>2.8 ± 7</td>
</tr>
</tbody>
</table>

*GroE-depleted cells harbouring the UuMetK gene, where UuMetK was induced by 50 mM IPTG.
Other than MetJ, MetR was considered to induce the overexpression of MetE. Deletion of MetR-binding regions did not affect the expression levels under GroE-normal conditions, but decreased the expression levels by 99% (31 vs 4459) compared with the overexpression levels in GroE-depleted cells (Table 1). Such a large decrease indicated that MetR actually contributes to the overexpression of MetE. An alternative possibility is that the metE promoter was affected because the deletion of the MetR-binding site was close enough to leave it impaired. However, that would not account for the MetJ binding-independent differences in expression using the dJ reporter. UuMetK expression suppressed transcription from the metE promoter lacking MetR-binding regions at similar levels under GroE-normal conditions (Table 1), which supports the notion that both MetJ and MetR regulate the metE promoter. Thus, MetK activity was either directly or indirectly associated with both the MetJ- and the MetR-mediated regulation of the metE promoter.

**Activation of the metR promoter in GroE-depleted cells**

Even in the metE promoter in which the MetJ-binding sites had been deleted, UuMetK expression suppressed the overexpression of MetE in GroE-depleted cells. This result raised the possibility that UuMetK also suppresses the increase in MetR activity, although MetR uses homocysteine as a co-activator, not SAM. We could not exclude the possibility of some unknown function of UuMetK, such as an interaction between UuMetK and MetR, catalysis of homocysteine by UuMetK, or indirect effects. However, one plausible explanation is that MetJ also regulates the expression of MetR, since the MetR promoter is regulated by MetR and UuMetK suppresses overexpression of the metE promoter lacking MetJ-binding regions, it is plausible that the increased level of MetR contributes to the overexpression of MetE in GroE-depleted cells.

**Effect of homocysteine metabolism on the overexpression of MetE by GroE depletion**

Chapman and co-workers assumed that homocysteine, the co-activator of MetR, accumulates in GroE-depleted cells, since MetF, a class IV substrate, is associated with homocysteine metabolism (Fig. 2; Chapman et al., 2006). Earlier studies of methionine synthesis have shown that homocysteine levels affect MetR activity in vitro and in vivo (Cai et al., 1989a; Urbanowski & Stauffer, 1989). To examine the effect of possible homocysteine accumulation in GroE-depleted cells, a synthetic pathway to decrease homocysteine was introduced (Fig. 2). We induced a cloned E. coli MmuM, which converts homocysteine and SMM, which were added to the medium, to methylhomocysteine (Thanbichler et al., 1999). The induction of MmuM and supplementation with SMM reduced the activity of the metE promoter by twofold and that of the promoter without MetJ-binding regions by threefold in GroE-depleted cells (Table 2). The reductions in the rate of MetR activation according to the homocysteine level were similar to those of an earlier report (four- to fivefold increase in MetE expression under supplementation with homocysteine) (Urbanowski & Stauffer, 1989). On the other hand, the introduced homocysteine metabolic pathway did not affect the activity of the promoter without MetR-binding regions (Table 2). It should be noted that in vitro translation has indicated that MetR works as an activator without homocysteine supplementation, although the activity decreases ~20% in comparison with homocysteine-supplementation (Cai et al., 1989b). These results support the notion that the assumed overaccumulation of homocysteine in
GroE-depleted cells affects the overexpression of MetE to an extent, although the effect is very small (two- to threefold increase) compared with the total overexpression of MetE (4459-fold increase) in GroE-depleted cells. These results and the full suppression caused by UuMetK expression indicated that MetK dysfunction caused by GroE depletion is mainly responsible for the overexpression of MetE, and MetF dysfunction caused by GroE depletion contributes slightly to the overexpression of MetE.

**Effect of MetR overexpression and metR disruption on MetE overexpression**

For further support of the notion that MetR overexpression is a trigger of MetE overexpression in GroE-depleted cells, we evaluated the effects of MetR overexpression and metR disruption. MetR was overexpressed via a tac promoter, and the 240 and dJ reporters were used to evaluate the effect of MetR overexpression under GroE-normal conditions. When MetR was at normal levels, ParM-FLAG expression was not visible on CBB-stained gels, irrespective of the reporter (Fig. 3a). On the other hand, when MetR was overexpressed, the dJ reporter showed expression levels of ParM-FLAG high enough to be visible on CBB-stained gels, and these levels were ~30% of those found with the mimicked overexpression of MetE by the 240 reporter in GroE-depleted cells (Fig. 3a). The 240 reporter did not show such high levels of expression (Fig. 3a). It should be noted that the growth of MetR-overexpressed cells with the dJ reporter was much slower than that with the 240 reporter or that of MetR-normal cells.

Next, we analysed the effect of disruption of the metR gene in MGM100 cells. The metR gene was disrupted by insertion of a chloramphenicol-resistance gene through homologous recombination. The growth of the metR disruptant was similar to that of metR-normal cells, and stopped after 5 h of cultivation under GroE-depleted conditions, in the same manner as MGM100. However, even under GroE-depleted conditions, the overexpression of chromosomal MetE was not observed in the metR disruptant (Fig. 3b). Similar suppression of MetE overexpression by metR disruption was observed when the 240 reporter was used (Fig. 3c). These results strongly support the notion that MetR overexpression in the absence of MetJ binding is the trigger for the overexpression of MetE.

**DISCUSSION**

**Mechanism of overexpression of MetE in GroE-depleted cells**

In this study, we analysed in detail how GroE depletion affects the metE promoter. We showed that activation of
the \textit{metE} promoter was responsible for the overexpression of MetE in GroE-depleted cells (Fig. 1c), and that it was suppressed by \textit{Ureaplasma} MetK, which folds to a functional state even under GroE-depleted conditions (Table 1). Deletion mutants of the \textit{metE} promoter revealed that both the repressor MetJ and the activator MetR are involved in the overexpression of MetE under GroE depletion (Table 1). In addition, GroE depletion raised the activity of the \textit{metR} promoter through the dysfunction of MetK, a class IV substrate protein. Reporter assays and suppression by UuMetK suggested that the increased expression of MetR in GroE-depleted cells contributes substantially to the overexpression of MetE, as has been suggested elsewhere (LaMonte & Hughes, 2006). Accumulation of the MetR co-activator homocysteine caused by MetE dysfunction contributed little to the overexpression of MetE (Table 2). Finally, we showed that MetE overexpression could be mimicked by MetR overexpression in the absence of MetJ binding, even under GroE-normal conditions, and was suppressed by \textit{metR} disruption. Taken together, these results suggest that the mechanism of MetE overexpression caused by GroE depletion can be outlined as follows (Fig. 4): (i) GroE deletion reduces the concentration of functional MetK; (ii) reduction of the concentration of functional MetK decreases the levels of intracellular SAM, which is the product of MetK and is the co-repressor of MetJ; (iii) the deficiency of SAM reduces the activity of the repressor MetJ; (iv) reduction of MetJ binding increases the activities of the \textit{metE} and \textit{metR} promoters, and the levels of MetE and MetR increase; and (v) the increased level of MetR further promotes the overexpression of MetE. Thus, we conclude that MetK is the class IV substrate that is responsible for the overexpression of MetE in GroE-depleted \textit{E. coli}. This study is believed to be the first to provide direct evidence that dysfunction of a class IV protein can induce the overexpression of proteins in GroE-depleted cells. MetE is not the only protein which shows overexpression in GroE-depleted cells (Fujiiwara et al., 2010). Studies of the mechanism of protein overexpression in GroE-depleted cells such as the present study would further clarify \textit{in vivo} obligate substrates of GroE.

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