Mechanism of methionine synthase overexpression in chaperonin-depleted Escherichia coli

Kei Fujiwara¹ and Hideki Taguchi²

¹Department of Bioengineering and Robotics, Tohoku University, Japan
²Department of Biomolecular Engineering, Tokyo Institute of Technology, Japan

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.

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).

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...
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 (Fujiiwara & 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 the 3 bp before the metE first codon (AAA) was replaced with CAT. pMetEp240-EcMetK was constructed by replacing the parM-FLAG gene with the metK gene of E. coli K-12 MG1655.

![Image of Fig. 1](https://example.com/fig1.png)

**Fig. 1.** The promoter and operator of the *metE* gene and reconstitution of MetE overexpression by GroE depletion. (a) Schematic representation of the promoter and operators for the *metE* and *metR* genes. TSP, transcriptional start point of *metE* and of *metR%; MetR-Bd with filled grey box, MetR-binding region; MetJ-Bd with open grey boxes, MetJ-binding regions. Double-ended arrows indicate (top) the 240 bp region upstream from the MetE first codon (MetEp240), (bottom) the 210 bp region upstream from the MetE first codon (MetEp240-dR), (middle) the 240 bp region upstream from the MetE first codon without MetJ-binding regions (MetEp240-dJ). The dotted line indicates the region deleted from metEp240. These annotations refer to Maxon et al. (1989). (b) Reporter plasmids used for *metE* promoter activity measurement. The 240 bp region upstream from the metE first codon was fused with the parM-FLAG gene, encoding C-terminal flag-tagged ParM, or with the metK gene. (c) Overexpression of genes fused with the metE promoter in GroE-depleted cells. Cell extracts under GroE-normal and GroE-depleted conditions with each plasmid were loaded onto SDS gels and stained by CBB. T, total lysate; S, supernatant; P, precipitate. Arrows indicate the bands corresponding to MetE, EcMetK and ParM-FLAG. metE-parM-FLAG and metE-EcMetK indicate cells harbouring pMetE240-ParM-FLAG and pMetE240-EcMetK, respectively.
Mechanism of MetE overexpression by GroE depletion

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.

Strain. E. coli MGM100 cells [MG1655 groE::araC-PBAD::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 DAP in GroE-depleted cells, the growth medium was supplemented with 1 mM DAP diaminopimelate (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 an ~2200 bp fragment, which was from 600 bp upstream of the MetR first codon to 600 bp downstream from 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 566 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, pMetE240dJ-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 and 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 20 000 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 dJ 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 detected 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.

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-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
Deletion analysis of the metE promoter reveals that both the repressor MetJ and the activator MetR are associated with MetE overexpression

To investigate how MetK contributes to MetE overexpression, we analysed the metE promoter in detail. Previous studies have revealed that both the repressor MetJ and the activator MetR regulate the metE operator (Cai et al., 1989a; Maxon et al., 1989) (Fig. 1a). MetJ requires S-adenosylmethionine (SAM), the product of the MetK reaction, for the efficient repression of target promoters (Shoeman et al., 1985a, b). MetR requires homocysteine, an indirect substrate of the MetF enzyme, to strengthen the activation (Cai et al., 1989a; Urbanowski & Stauffer, 1989). The MetJ-binding sites are located around the regions 160 and 55 bp upstream from the metE first codon, and the MetR-binding site is located at around 230 bp upstream of the metE first codon (Fig. 1a). The start point of the metE transcript is single, and is located at 169 bp upstream from the metE first codon (Cai et al., 1989b). Thus, if MetK is the only class IV protein responsible for the overexpression of MetE, the deletion of MetJ-binding sites would be expected to negate UuMetK suppression, and deletion of the MetR-binding site would not cancel the induction of MetE by GroE depletion. No factors other than absence of MetJ binding are expected to be associated with the overexpression of MetE.

A deletion series of the metE promoter was constructed to estimate the contributions of MetJ and MetR to the overexpression of MetE (Fig. 1a). As a promoter with no MetR-binding sites, the 210 bp region upstream from the metE first codon was used (Fig. 1a, MetEp240-dR). As a promoter with no MetJ-binding sites, the region from −160 to −16 of the metE first codon was deleted from the 240 bp metE promoter (Fig. 1a, MetEp240-dJ). Deletion of MetJ-binding regions led to 92-fold higher expression under GroE-normal conditions, and similar expression levels (4459 vs 5288) under GroE-depleted conditions compared with that of the 240 bp promoter under the respective conditions (Table 1). If the absence of MetJ binding is the only factor that is responsible for the overexpression of MetE, the expression of metE from the dJ reporter should be high under all conditions. The 92-fold increase in expression between p240 and dJ when GroE is present can thus be attributed to the lack of binding sites. The further 50-fold increase in expression under GroE-depleted conditions must be attributed to something else. Since the exogenous MetK plasmid lowers expression 26-fold (Table 1), the logical explanation is that a separate SAM-dependent process is responsible for the further derepression.

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 MetEp240-dR in GroE-normal cells, and between MetEp240 in GroE-depleted cells expressing the UuMetK gene in the presence of 50 μM IPTG and MetEp240-dJ 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 μM 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 located in the opposite direction to the metE operator in an overlapping manner (Fig. 1a). A start point of the metR transcript is located at 39 bp upstream from the metR first codon (Cai et al., 1989b), and a putative −35 promoter region of the start points of the metR transcript overlaps the MetJ-binding region. Additionally, MetJ has actually been shown to be involved in the regulation of the metR promoter in an in vitro study, and also in Salmonella (Maxon et al., 1989; Urbanowski & Stauffer, 1987). To confirm this assumption, the 240 bp region upstream from the MetR first codon, which is the reversed metE promoter, was fused with ParM-FLAG. With this promoter, GroE depletion increased the expression of ParM-FLAG by 2-fold, from a relative activity of 8±4 (±SD) to 209±53, and this increase was suppressed by the expression of UuMetK (relative activity 21±2). All these activities were significantly different (P<0.05). These results indicated that MetJ regulates the levels of both MetE and MetR, and suggested that a shortage of SAM through MetK dysfunction in GroE-depleted cells increases the levels of the activator MetR. A similar overexpression of MetK has been observed when SAM is depleted through the strong SAM hydrolyase of a phage (LaMonte & Hughes, 2006). Since the metE 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
Mechanism of MetE overexpression by GroE depletion

![Diagram](https://via.placeholder.com/150)

**Fig. 4.** Summary of the mechanism of MetE overexpression in GroE-depleted *E. coli.*

the *metE* promoter was responsible for the overexpression of MetE in GroE-depleted cells (Fig. 1c), and that it was suppressed by *Ureaplasma* MetK, which folds to a functional state even under GroE-depleted conditions (Table 1). Deletion mutants of the *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 *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 *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 *metE* and *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 *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 (Fujisawa et al., 2010). Studies of the mechanism of protein overexpression in GroE-depleted cells such as the present study would further clarify *in vivo* obligate substrates of GroE.

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