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Abnormal Proteins Enhance Stress-Induced Cell Death

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The effect of abnormal proteins on cell viability was studied using artificially cleaved polypeptides. *Escherichia coli* methionyl-tRNA synthetase (MetRS) consists of two distinct domains and its activity is essential for cell viability. The polypeptide chain was split by linker insertion and expressed as two fragments. Two pairs of polypeptides, one split within the N-terminal domain and another at the junction of the two domains retained aminoacylation activity. The *in vitro* activities of these split mutants were enhanced by the presence of chaperonin, GroESL. However, cells containing these split polypeptides became sensitive to conditions that induce GroESL. The results of this work suggest that an abnormally generated protein can cause cell death under stressful conditions.

A vast array of biological machinery is involved in maintaining the fidelity of protein synthesis and conformational integrity. Nonetheless, some abnormal proteins are inevitably generated in the cell. Misfolded or truncated proteins are repaired and removed by chaperones and proteolytic enzymes (1, 2). However, the effect of abnormal proteins on cell viability and physiology is generally unknown although there are limited numbers of reports that some proteins of abnormal conformation or truncated peptides cause disease (3, 4) or give dominant negative lethality to the cell (5, 6). In this work, we expressed artificially cleaved proteins that still retained the active conformation and studied how these polypeptides influenced the cell viability.

The protein used in this work was *E. coli* methionyl-tRNA synthetase (MetRS), which catalyzes transfer of methionine to its cognate tRNA. The native polypeptide contains 676 amino acids and forms a homodimer in solution (7). The N-terminal domain consisting of about 360 amino acids is involved in enzyme catalysis (8, 9, 10) and the C-terminal domain is responsible for binding to the anticodon of $tRNA^{Met}$ (11, 12, 13, 14). Attached to the C-terminal domain is an about 100 amino acid peptide appendix required for protein dimerization. Removal of the C-terminal appendix results in an active monomeric protein (15), the X-ray structure of which has been solved at 2.5 Å (16) (Fig. 1). The polypeptide of this enzyme was split at various points and expressed as multiple fragments. A few pairs of the split polypeptides reassembled and retained the activity (17, 18). Although these split peptide variants retained aminoacylation activity, they may depend on molecular machinery such as chaperones and energy to maintain its structural integrity since they are expressed as multiple subunits.

It is known that polypeptide folding and assembly are facilitated by molecular chaperones (19, 20). Among the *E. coli* chaperones, multisubunit chaperonin including GroEL has been extensively studied on its structure and working mechanism. GroEL cooperates with its cofactor GroES and promotes protein folding and assembly in an ATP-dependent manner (21). We thus expected that GroESL complex would be involved in the stabilization of the split mutants. To our surprise, the results of this work suggested that conditions inducing this stress protein causes cell lethality when the cells contain these abnormally generated polypeptides.

MATERIALS AND METHODS

Materials. The wild type and mutant *E. coli* methionyl-tRNA synthetase proteins were purified using FPLC MonoQ HR10/10 column (Pharmacia) as described previously (18). The purified *E. coli* GroESL chaperonin complex was purchased from Sigma. The recombinant plasmid pCAYC133 encoding the *E. coli* chaperonin, GroESL, was obtained from Dr. K. H. Ryu (Yuhan Pharmaceticals). The recombinant phagemid pJB103 encoding the *E. coli* MetRS (17) was used to generate the split MetRS mutants. The bipartite split MetRS mutants R366//M (17) and S276//M (18) were generated by inserting oligonucleotide linkers containing the sequence of stop codon, ribosome binding site and start codon. The rabbit antibody raised against the *E. coli* GroEL was a kind gift from Dr. D. K. Lee (Sung Kyun Kwan University). The antibiotics were purchased from Sigma.

Aminoacylation assay. Aminoacylation activity of the *E. coli* methionyl-tRNA synthetase was determined by the method described previously (22). The reaction contained 20 mM HEPES buffer (pH 7.5), 0.1 mM EDTA, 0.15 M NH_4Cl, 100 ug/ml bovine serum

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albumin, 2 mM ATP, 4 mM $MgCl_2$, 20 uM [^{35}S] methionine. and 4 uM *E. coli* $tRNA^{fMet}$ (Sigma). 5 nM of purified *E. coli* MetRS wild type and mutants were used for the reactions. The reactions were quenched with 5% trichloroacetic acid containing 1 mM methionine on a filter disk (Whatman 3MM). After washing the filters, the charged tRNA was quantified by liquid scintillation counter (LKB). To determine the effect of the GroESL complex on the activity of E. coli MetRS, 400 nM of the purified GroESL complex was preincubated with 400 nM of the purified MetRS proteins in Tris-HCl buffer (pH 7.5), 20 mM KCl. 5mM dithiothreitol, 10 mM $MgCl_2$ and 5 mM ATP at 37 °C for 30 minutes. The enzymes were then transferred to the aminoacylation reaction at 2 nM final concentration. Also, MetRS proteins were added directly to aminoacylation reaction at 2 nM in the presence or absence of the same molar ratio of GroESL.

Measurement of cell growth. Growth of *E. coli* cells was measured by the absorbency at 599 nm. To measure the effect of heat shock on cell growth, cells at the log phase were transferred to fresh LB broth containing 50 ug/ml ampicillin preincubated at 37 or 42 °C. For the antibiotic sensitivity test, an equal number of log phase cells were transferred into LB broth containing different concentrations of antibiotics and cell growth was measured after 10 hours.

RESULTS

Stabilization of MetRS by GroESL in vitro.The polypeptide chain of the E. coli MetRS was previously split at various positions and expressed as multiple fragments. Among them, two split mutants that contained

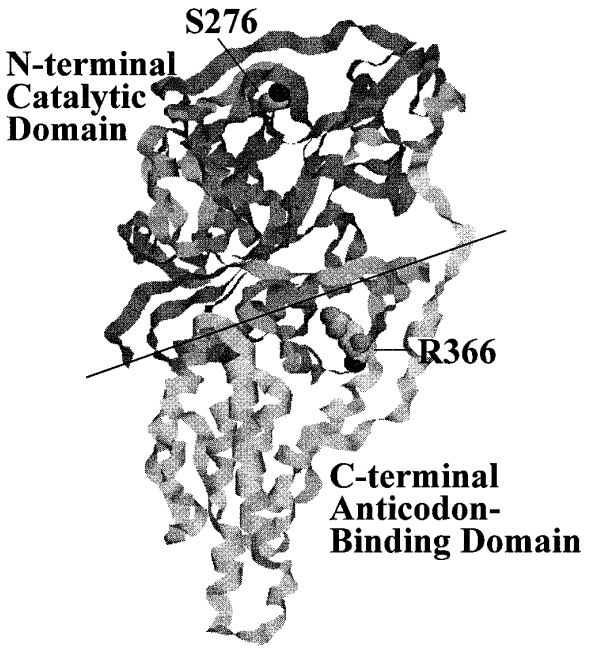

FIG. 1. Three-dimensional ribbon structure of active monomeric *E. coli* MetRS and the locations of polypeptide split sites. *E. coli* MetRS consists of the N-terminal catalytic domain (about 360 amino acids, dark region) and the C-terminal anticodon binding domains (about 190 amino acids, light region) which are essential for the enzyme activity. The C-terminal appendix is required for dimerization of the protomer but not essential for the activity. The split mutants S276//M and R366//M are expressed as two separate polypeptides cleaved at S276 and R366, respectively. The locations of the two residues at the cleaved locations were highlighted.

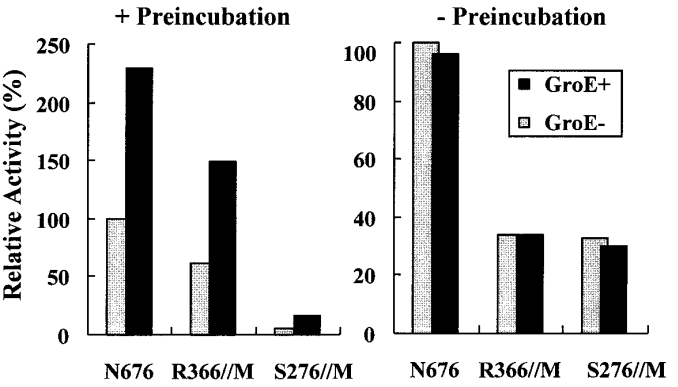

FIG. 2. Stabilization of *E. coli* MetRS by GroESL. (Left) The purified native and split MetRS proteins (400 nM) were preincubated with or without the purified GroESL complex (400 nM) at 37 °C for 30 minutes. The MetRS proteins were taken from the preincubation mixture and used for the aminoacylation reaction at the concentration of 2 nM as described in Materials and Methods. (Right) MetRS proteins were directly used for aminoacylation in the absence or presence of GroESL. The initial activity of the wild type MetRS without GroESL was taken as 100% activity in both cases.

polypeptide cleavages at S276 in the N-terminal catalytic domain and at R366 in the junction of the two domains retained aminoacylation activity (17, 18) (Fig. 1). Activity and circular dichroism analyses of the split mutants showed that these mutant retain the native-like conformation but they are thermally less stable than the wild type protein (data not shown).

R366//M and S276//M mutants showed 62 and 5 %, respectively of the *in vitro* aminoacylation activity of the wild type MetRS at the same concentration (Fig. 2 left, grey bars). Since both of the split polypeptides are required for the protein activity, the lower activity of the split mutants may result from the decreased stability of the three dimensional conformation and improper assembly of the split polypeptides. Among the two mutants, S276//M mutant was less stable than R366//M mutant based on its stability during the purification as well as temperature sensitivity of the activity (data not shown). We then investigated whether the split MetRS proteins are stabilized by incubation with one of the *E. coli* chaperones, GroESL. The wild-type and mutant MetRS proteins were mixed with GroESL at 37 °C for 30 minutes in the presence of ATP and Mg^{2+} ion and their aminoacylation activities were compared with those of the proteins incubated under identical conditions without GroESL. The enzyme activities were 2.3-3 fold enhanced by the preincubation with GroESL (Fig. 2 left, black bars), suggesting that the thermal denaturation of MetRS proteins were protected by GroESL. When MetRS proteins were added directly to the reaction mixture in the presence or absence of GroESL without preincubation, the activity enhancement by GroESL was not observed (Fig. 2 right). This excludes the possibility that the increase of activity may result from GroESL itself.

TABLE 1

Effect of GroESL Coexpression with MetRS on Cell Growth

Selection temperature	*E. coli* Host	MetRS		
		N676	R366//M	S276//M
25°C	MJR	+	+	+
	MJR/GroESL	+	+	+
37°C	MJR	+	+	+
	MJR/GroESL	+	−	−

Note: + indicates 300–400 transformants on the selection media while − indicates no colony formation. *E. coli* host strain MJR was transformed with the plasmid pACYC133 (tetracyclin resistance marker) encoding the *E. coli* chaperonin GroESL. The recombinant phagemids (ampicillin resistance marker) encoding various forms of *E. coli* MetRS were introduced into the MJR strain with or without pACYC133 and the double transformants were selected on LB plates containing ampicillin and tetracycline at 25 and 37°C.

Cell lethality caused by co-expression of split MetRS and GroE. We expected that the split MetRS mutant would be also stabilized by increasing the cellular level of GroESL based on the *in vitro* effect shown above. To test this possibility, We first introduced the plasmid encoding GroEL and ES into the *E. coli* cells. Cells containing this plasmid expressed about 3 fold more GroEL protein as determined by immunoblotting with a GroEL antibody (data not shown). Then, the recombinant plasmids encoding the *E. coli* MetRS wild type and each of the split mutant proteins were transformed into the normal and GroESL overexpressing *E. coli* MJR strain and the transformants were selected at 37 °C. The plasmid encoding the wild type MetRS was introduced into both the normal and GroESL overproducing cells with an equal transformation frequency. In contrast, the plasmids encoding the split MetRS mutants did not generate transformants from cells expressing the plasmid-encoded GroESL whereas they formed the usual number of transformants from the normal host (Table 1). To confirm these results, the transformation was conducted in the reverse order. Namely, the plasmid encoding GroESL was introduced into each of the cells expressing different MetRS proteins. Again, transformants of the GroESL-encoding plasmid were obtained from the cells expressing the wild type MetRS but not from those expressing the split MetRS mutants (data not shown). These results suggest that simultaneous expression of GroESL and the split MetRS at high level inhibit cell growth, although GroESL enhanced the aminoacylation activity *in vitro*.

We wondered whether the growth inhibition is related to the thermal instability of the split MetRS mutants in conjunction with the high cellular level of GroESL. Since the split MetRS mutants may be stable at lower temperature, the transformants containing the plasmids expressing GroESL and the split MetRS mutants were selected at 25 °C. As expected, the cells expressing the MetRS split mutants and GroESL grew at this temperature (Table 1). MetRS and GroEL expressions were about 2 fold decreased at 25 °C compared with that at 37 °C as determined by immunoblotting with anti-MetRS and -GroEL antibodies (data not shown). Thus, the cell growth observed at 25 °C may be due to either the decreased expression or stabilization of the split MetRS mutants or both. Since the expression of GroESL is under the control of *lac* promoter in the plasmid, the expression of GroESL was further induced by adding IPTG to see whether the higher level of GroESL can inhibit the cell growth at 25 °C. Although the GroESL itself level was increased to about 4 fold by IPTG, the growth of the cells containing the split MetRS mutants was not affected at 25 °C, suggesting that the overexpression of GroESL is not responsible for the cell growth inhibition at higher temperature (data not shown). The results suggest that the growth arrest may result from the thermal instability of the split mutant in conjunction with high level expression of GroESL.

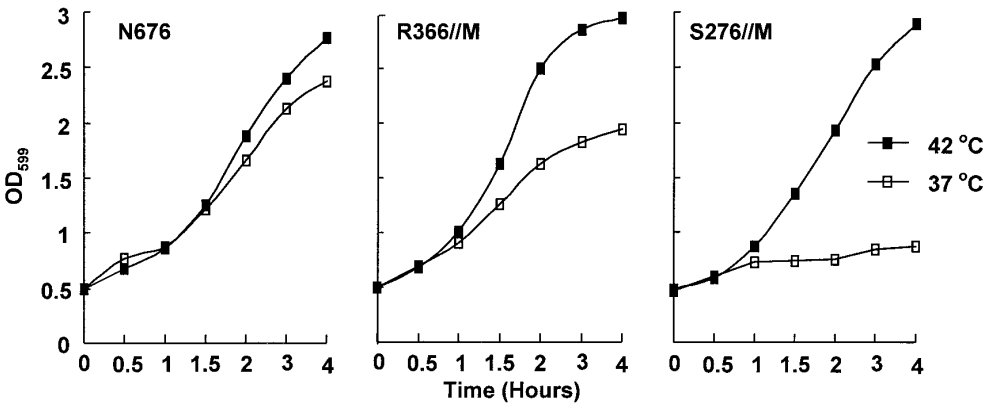

FIG. 3. Growth inhibition of *E. coli* cells containing the split MetRS mutants by temperature shift. Log phase cells expressing the wild type and split MetRS mutants were transferred to the fresh LB broth containing 50 ug/ml ampicillin preincubated at 37 or 42 °C and cultivated at each temperature. The growth was measured at 599 nm at 1 hour interval.

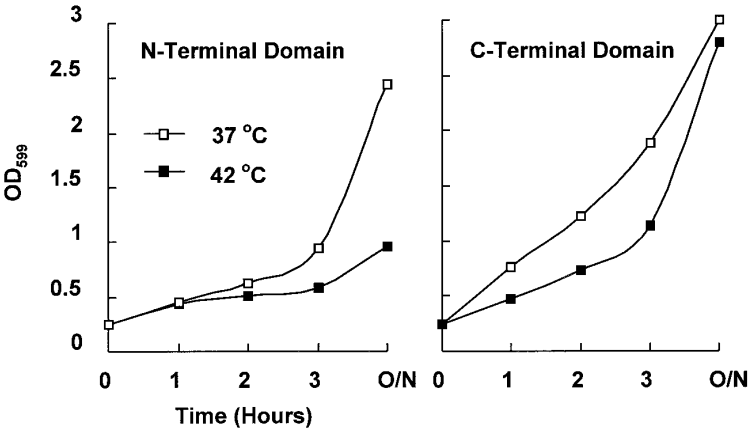

FIG. 4. Heat shock effect on growth of cells containing the N- or C-terminal domain. Log phase cells containing each of the N- and C-terminal domains of *E. coli* MetRS were transferred to the fresh LB broth preincubated at 37 or 47 °C and the cell growth was monitored as described above.

Growth inhibition by heat shock. The results above suggest that inducing chaperones like GroESL could be lethal to cells containing an abnormally generated polypeptides. Since GroESL is also induced by heat shock, we tested whether the cells containing the split mutants are also susceptible to heat shock. The cells expressing each of the wild type and split MetRS proteins were grown to log phase and then equal amount of the cells were transferred into fresh broth preincubated at 37 or 42 °C. Cells expressing the wild type MetRS grew at a comparable rate (Fig. 3, left). However, the growth of the cells expressing the split mutants was inhibited after 1 hour cultivation at 42 °C (Fig. 3, middle and right). The inhibition was more severe in the cells containing the S276//M mutant compared to those containing the R366//M mutant, suggesting that the growth inhibition is related to the instability of the split mutant.

To further address the relationship between the protein stability and cell death, we expressed the N- and C-terminal domains of *E. coli* MetRS separately. It was known that the separately expressed N-terminal domain does not fold properly (17) whereas the C-terminal domain alone makes a folded conformation (23). Cells containing each domain were subjected to heat shock to compare the effect of the two separate domains on cell growth. Cells containing the N-terminal domain showed higher sensitivity to heat shock (Fig. 4, left) while those containing the C-terminal domain did not (Fig. 4, right). This result further supports the correlation between the protein instability and its effect on cell viability. Cells overexpressing GroESL alone did not show sensitivity to heat shock, indicating that the overexpression of GroESL itself does not affect cell growth (data not shown).

Sensitivity to antibiotic treatment. We then tested whether the split MetRS polypeptides also enhance the sensitivity of the cells to chemical stress that can cause the induction of GroESL. It is known that the antibiotics of fluoroquinolone derivatives, ofloxacin and sparfloxacin, induce chaperones including GroEL (24). The growth of cells containing the split mutants showed higher sensitivity to both of the quinolone antibiotics than those containing wild type MetRS (Fig. 5). However, the mutant containing cells did not show the sensitivity to other antibiotics such as kanamycin and streptomycin (data not shown), suggesting that the growth inhibition requires a specific type of stress.

DISCUSSION

Conformational or chemical changes of cellular proteins can occur spontaneously or can be induced by

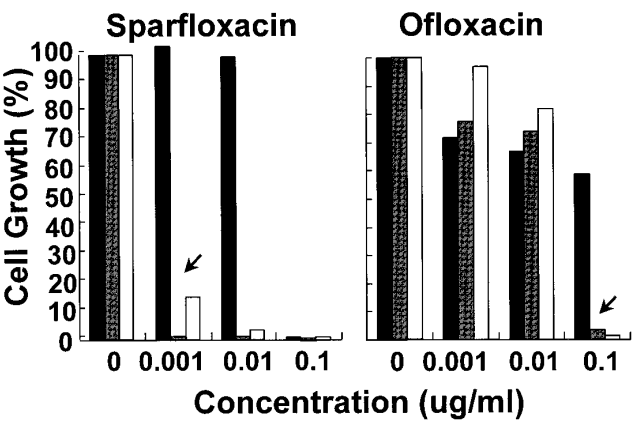

FIG. 5. Effect of antibiotic treatment on growth of cells expressing the wild type and split MetRS. The same numbers of cells were transferred to the fresh LB broth containing various concentrations of antibiotics. The cell growth was measured after 10 hour cultivation at OD_{599}. Arrows indicate the concentrations in which the growth of cells containing the split MetRS mutants was specifically inhibited.

stress (25). However, the effect of the modified proteins on cell physiology and viability has not been well understood. We introduced artificially cleaved but functional polypeptides into the cell and studied how these polypeptides affect cell propagation under stressful conditions. Both of the split MetRS mutants were active in *E. coli* but the intradomainal split mutant S276//M is less stable and has lower activity than the interdomainal split mutant R366//M (18) (Fig. 2). We showed that these abnormally generated polypeptides make the cells vulnerable to conditions that induce the expression of chaperonin GroESL. The growth of the cells inhibited by heat shock was not restored by decreasing the temperature back to 37 °C, suggesting that the heat shock stress was lethal to the cell (data not shown). The activity of the MetRS encoded by the plasmid is not a direct determinant for the cell viability because the cells contain a chromosomally encoded MetRS that is functional in the conditions used for the experiments. Therefore, the split mutants may influence other essential functions of the cell or result in a dominant negative effect on the chromosomally encoded native MetRS.

It is not known whether the stress-induced cell death is specific to the *E. coli* MetRS split polypeptides or if it can occur with other abnormally expressed polypeptides. At least, the same effect was found when we did the same experiments in other E. coli hosts, implying that the lethal effect is not specific to the host type (data not shown). Interactions of partial peptide fragments with their native proteins have been previously reported (26, 6). In these cases, partial peptide fragments inhibited the folding process of the native protein and thus inhibit the enzyme activity. Although we do not know whether a similar mechanism can be applied to the lethality of the split *E. coli* MetRS variants, the unique finding of this work is that a growth arrest by the split MetRS mutants appears to be mediated by stress proteins like GroESL.

Although the ordinary role of stress proteins is to repair or remove abnormal proteins, they can result in adverse effects depending on the conditions. *E. coli* heat shock protein, GroEL and the cold shock protein, trigger factor (TF) were toxic to cells when they were induced at their opposite temperatures (27). Another *E. coli* chaperone, DnaK, also gave a bacteriocidal effect on stationary phase cells (28, 29). Perhaps, they can mediate some fatal interactions between the abnormal proteins with other essential cellular components including their native proteins. This work thus gives an insight into how an abnormal protein in combination with a stress protein can be pathogenic to the cell.

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