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TETRACYCLINE/H^+ ANTIPORTER WAS DEGRADED RAPIDLY IN ESCHERICHIA COLI CELLS WHEN TRUNCATED AT LAST TRANSMEMBRANE HELIX AND THIS DEGRADATION WAS PROTECTED BY OVERPRODUCED GroEL/ES

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SUMMARY: The *in vivo* degradation of the plasmid-encoded tetracycline/H^+ antiporter (TET) in *Escherichia coli* cells was studied using three mutants with carboxyl-terminal truncation at the positions in the hydrophilic carboxyl-terminal tail (TET388), in the last putative transmembrane helix XII (TET382), and immediately before the helix XII (TET365). All the mutant TET proteins were localized in the membrane. Expressed TET388 was active in transport and stable against proteolysis. However, TET382 and TET365 were inactive and proteolyzed rapidly. Thus, the importance of the helix XII for protease-resistant proper folding of TET is obvious. Interestingly, overproduced chaperonin (GroEL and GroES) partly prevented degradation of TET365.

Protein degradation in the cell has important roles in the regulation of the amount of specific proteins and in the exclusion of abnormal proteins. Early investigations suggested that most proteins in bacterial cells are stable or turn over extremely slowly. In growing bacteria, about 1-2% of the cell proteins are degraded to amino acid per hour [1, 2]. The bacterial cell, however, has a system for eliminating abnormal proteins rapidly, such as nonsense peptide fragments, peptidyl puromycin, missense mutant proteins, and proteins containing amino acid analogs [3, 4]. On the other hand, while our knowledge of protein degradation in cytosol has been relatively well-developed, degradation process of membrane proteins is only poorly understood. Roepe *et al* demonstrated that truncation of the carboxyl terminus at the last transmembrane helix of the lactose permease of *E. coli* leads to a decrease in the lifetime of this membrane protein [5]. Their result implies the presence of an yet-unidentified proteolytic system for the degradation of improperly folded membrane proteins. We have studied the *in*

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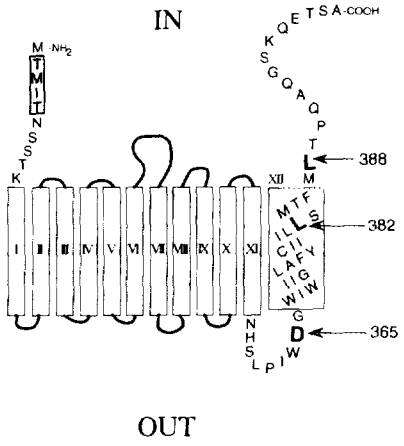


Fig. 1. A model of secondary structure of TET protein based on the hydropathy plot [8]. Positions of amino acid residues 365, 382 and 388 are indicated. Hydrophobic α-helical segments are enclosed by boxes and the amino acids residues derived from LacZ are shown in a shaded box.

vivo degradation of plasmid-encoded tetracycline/H^+ antiporter (TET). TET is a protein consisting of 401 amino acid residues and is predicted to be composed of 12 transmembrane helices connected by hydrophilic loops [6] (Fig. 1). Carboxyl-terminal tail of TET is on the cytoplasmic surface of the plasma membrane. Overexpression system for TET was constructed by Yamaguchi and his colleagues, and functions and structure of this protein have been extensively studied [7, 8]. Here we report that, while the truncated TET protein at the position the helix XII were degraded rapidly. This degradation was partly prevented by overproduced *E. coli* chaperonin, GroEL/ES.

MATERIALS AND METHODS

Bacterial strains. The following strains of *E.coli* K-12 were used: JM109[*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)*/F' (*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)][9]; CJ236[*dut1, ung1, thi-1, relA1 / pCJ*105(F' *cam*^{r}) [10].

Site-directed mutagenesis. All site-specific mutations were directed by synthetic oligodeoxynucleotide primers corresponding to the antisense strand of the TET gene cloned into pUC118. Deoxyoligonucleotide primers containing given mismatches were synthesized with a DNA synthesizer (Applied Biosystems) and purified by gel filtration. Amino acid codons 365, 382 or 388 were substituted for premature stop codons (TAA), and the region of the mismatch was put in the center of the sequence. Sequences of the mutagenic primers used were; 5'GCTTGAGGGGTTTACATGAAGGTCA3' (pSY388), 5'TGAAGGTCATATGTTACAG GATAATA3' (pSY382), and 5'AAATCCAGCCTTACCATATGGGTAGT3' (pSY365). Changed bases are indicated by bold letters. Underlines indicate the new (pSY382 and pSY365, NdeI) or deleted (pSY388, HincII) restriction sites. Mutations were detected as appearance or disappearance of the restriction sites.

Plasmid construction. The plasmid pKSELS was a derivative of pACYC177; A 5.9kb BamHI-EcoRI (partly digested) fragment of pKY206 (a pACYC188-based plasmid bearing the groES-groEL genes which was a generous gift from Dr. K. Ito [11]) containing groES-groEL genes was ligated with pACYC177 that had been cut with BamHI-HincII, creating pKSELS. The TET-containing plasmid pSYTET was derived from pUC118 as previously described [8]. As a result, the amino-terminal methionine residue of *tet*A was replaced by the amino-terminal five residues of *lac*Z, MTMIT. *E.coli* JM109 was used for expression of the *tet*A gene from the *lac* promoter and for preparation of inverted membrane vesicles.

Growth of cells and *in vivo* pulse-labeling with [^{35}S]methionine. Unless otherwise stated, *E. coli* cells were grown at 37 °C on the synthetic minimal medium M9 [12] supplemented with 0.2 % glucose, 0.01 % thiamine, 0.1 % casamino acids, and 50 μg/ml of ampicillin. Kanamycin was added in the culture medium for the cells bearing plasmid pKSELS. Tetracycline was not included in all culture medium. Induction of the *lac* promoter was initiated by addition of 1 mM (final concentration) isopropyl β-D-thiogalactoside (IPTG) at a middle phase of the logarithmic growth (OD_{600} = 0.5).
For pulse-labeling by [^{35}S]methionine, induction by IPTG was continued for 15 min and then [^{35}S]methionine (1000 Ci/mmol, American Radiolabeled Chemicals) was added to a final concentration 100 μCi/ml. Cells were incubated for another 3 min and incorporation of [^{35}S]methionine was chased by the addition of an excess amount of unlabeled methionine (2 mM, final concentration). At the indicated times, portions were removed into a tube containing NaN_3 (50 mM, final concentration) and were rapidly frozen. Radioactively labeled proteins were analyzed with 12 % polyacrylamide gel electrophoresis in the presence of 0.1 % sodium dodecylsulfate (SDS-PAGE) [13]. The quantitative analysis of the radioactive bands was performed by Bioimaging Analyzer BAS2000 (FUJI FILM Co.,Tokyo). Only in the case when the culture was continued for a long period up to 20 hrs after the chase, a procedure to remove IPTG by washing cells twice with M9 medium at 4 °C was inserted immediately after the chase addition of unlabeled methionine. Then, the cells were resuspended in the medium supplemented with 2 mM unlabeled methionine and culture was continued at 37 °C.
Alkali fractionation. Alkali fractionation of total *E.coli* proteins were carried out to know the distrubution of TET proteins between cytosolic and membrane fractions according to the method by Ito and Akiyama [11]. Briefly, the cells were subjected to lysozyme treatment, freezing-thawing, and alkali fractionation. The resulting supernatant and pellet were concentrated by acid precipitation (10 % trichloroacetic acid), solubilized with SDS, and analyzed with SDS-PAGE.
Assays. Uptake of [^{3}H]tetracycline (American Radiolabeled Chemicals) into the inverted membrane vesicles, driven by the addition of 2.5 mM NADH, was assayed as described previously [7] in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ in 50 mM 3-(N-morpholino)propanesulfonic acid-KOH buffer (pH 7.0). Protein was assayed as described [14] with bovine serum albumin as a standard.

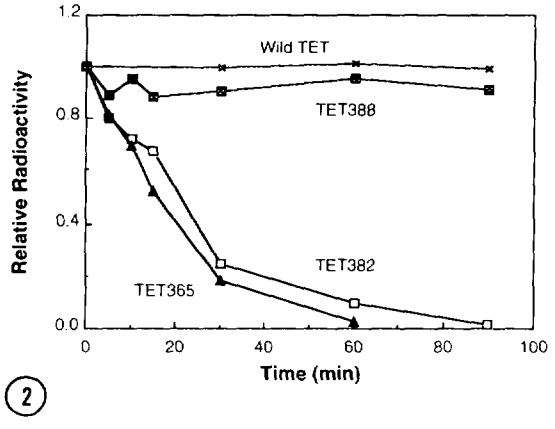
RESULTS

Stability of wild-type and truncated TET proteins in E. coli cells. When membrane fractions were analyzed with SDS-PAGE after IPTG-induced expression of wild-type TET was continued for 2 hrs, the band corresponding to wild-type TET protein was densely stained even by Coomassie Brilliant Blue, indicating that the wild-type TET protein was overproduced (data not shown) [8]. The intensity of the band did not change over a 20 hr culture in the medium without IPTG. When *E. coli* cells were pulse-labeled with [^{35}S]methionine and radioactivity of the TET band in SDS-PAGE was measured over 20 hrs, the amount of pulse-labeled wild-type TET protein contained in an unit volume of culture medium was not changed for the entire period of the experiment. Thus, wild-type TET protein is extremely stable in *E. coli* cells even under the conditions where it is not necessary.
Although the amount of the truncated TET proteins expressed in the presence of IPTG was much less than the wild-type TET protein, they were well detectable by pulse-labeling with [^{35}S]methionine. Fluorographic intensities of the corresponding radioactive bands in SDS-PAGE were measured over 90 min after pulse-labeling. As shown in Fig. 2, TET388 protein was stable for the entire period of the experiment while TET382 and TET365 proteins decreased rapidly and disappeared completely after 90 min. The half-times for disappearance of TET382 and TET365 proteins were almost the same and were calculated to be about 15 min.
Transport activity of truncated TET proteins. Time courses of [^{3}H]tetracycline uptake by inverted membrane vesicles mediated by wild-type TET and each mutant, TET365, TET382

or TET388, are presented in Fig. 3. As shown, truncation at position 388 had only little effect on transport activity. This indicates that the carboxyl-terminal hydrophilic tail of TET is dispensable without loss of activity. On the other hand, the inverted vesicles prepared from cells expressing TET382 or TET365 exhibited essentially no transport activity. Since the amount of expressed TET365 or TET382 in the cells used for preparation of vesicles was roughly the same or 20 %, respectively, as that of TET388, failure of transport by TET382 or TET365 might not be due to insufficient amount of TET proteins in the vesicles. Based on predicted topology of TET protein in the membrane, the transmembrane helix XII should be completely lacked in the case of TET365 and it may not be properly formed in the case of TET382 (see Fig. 1). Indication of these results is that the carboxyl-terminal transmembrane helix is essential for the tetracycline/H^+ antiporter to be functional.

Membrane localization of truncated TET proteins. As demonstrated above, the TET382 and TET365 proteins are degraded at a significant rate, and both of them retain no activity as a tetracycline/H^+ antiporter. One might think that this is just due to failure of proper localization of truncated TET proteins. Then, we applied alkali fractionation technique [11] to examine whether truncated TET proteins were integrated into membrane or existed in cytosol as inclusion bodies. As shown in Fig. 4, lane 3 and 4, the majority of pulse-labeled TET365 protein was found in the alkali pellet fraction, that is, in the membrane fraction. Similar results were also obtained for the TET 388 and TET382 proteins (data not shown). Thus, truncated mutant TET proteins were integrated into *E. coli* cytoplasmic membrane.

Stability of TET365 protein in E. coli cells overproducing GroEL/ES. The GroEL/ES, *E. coli* chaperonin, assists in the cellular folding of cytosolic proteins by interacting with proteins in non-native state [15]. Although the mechanism of chaperonin function has not



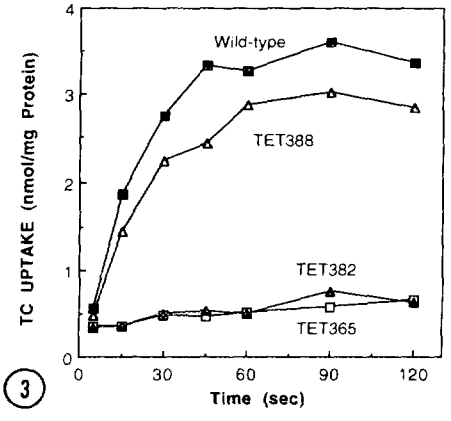


Fig. 2. Stability of wild-type TET and truncated TET proteins during pulse-chase experiments with [^{35}S]methionine. The radioactive bands of [^{35}S]methionine-labeled TET proteins in SDS-PAGE were analyzed. The data were normalized to the zero time point in each set of samples, which was taken as 1.0. All the experiments were carried out more than three times and typical data were shown. Other experimental details are given under "Materials and methods".

Fig. 3. Tetracycline uptake by inverted membrane vesicles prepared from *E. coli* cells expressing the wild-type or truncated TET prtoeins. [^{3}H]Tetracycline uptake was measured in the presence of 50 μM $CoCl_2$ and 10 μM [^{3}H]tetracycline and the reactions were initiated by the addition of 2.5 mM NADH. The data were normalized to the background uptake in the absence of NADH in each sample. Other experimental details are given under "Materials and methods".

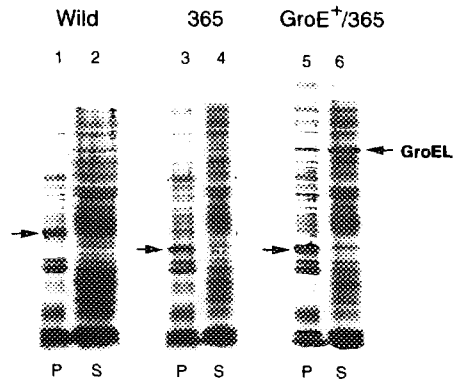


Fig. 4. Membrane localization of newly-synthesized TET proteins judged by alkali fractionation. *E.coli* JM109 cells harboring the wild-type TET plasmid (lane 1 and 2), the TET365 plasmid (lane 3 and 4), or the TET365 plasmid and the GroE$^+$ plasmid (lane 5 and 6) were grown at 37°C and expression was induced by IPTG for 15 min. Then, cells were pulse-labeled with [^{35}S]methionine for 3 min and chased with unlabeled methionine. A portion of the culture was treated with lysozyme and freezing-thawing, and mixed with an equal volume of 0.2 N NaOH as described in the "Materials and Methods". Lanes 1, 3 and 5, pellet fraction (P); lanes 2, 4 and 6, supernatant fraction (S). "GroE$^+$" signifies the cells overproducing GroEL/ES. Arrows indicate the bands of TET proteins.

been well understood, it is thought that GroEL/ES recognizes hydrophobic stretch or surface of non-native proteins. If so, it is possible to think that GroEL/ES can also interact with newly-synthesized or improperly-folded membrane proteins and we measured the effect of the presence of large amount of GroEL/ES proteins on degradation of TET365 protein. Synthesis of excess GroEL/ES proteins in addition to TET365 protein in the same cell was accomplished by transforming *E. coli* cells with an expression vector pACYC177 which is compatible with pUC118-based pSY365. Judging from fluorographic intensity, the amount of pulse-labeled TET365 protein was not significantly changed by overproducing GroEL/ES but the degradation of TET365 protein was slowed down in the cells overproducing GroEL/ES (Fig. 5). The half

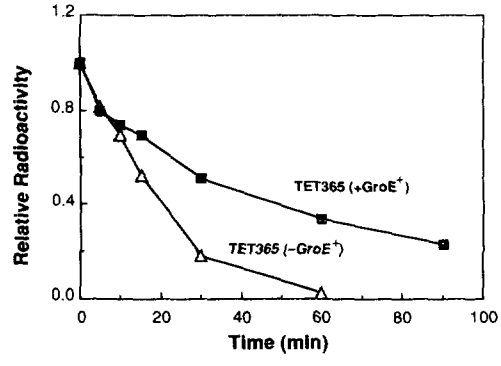


Fig. 5. Degradation of [^{35}S]methionine pulse-labeled TET365 expressed in *E. coli* cells with (+GroE$^+$) or without (-GroE$^+$) overproducing GroEL/ES. Both experiments were carried out more than three times and typical data were shown. Experimental details are given under "Materials and methods".

time of the degradation in the presence of overproduced GroEL/ES was about double (30 min) of that in the absence of overproduced GroEL/ES. Majority of TET protein was properly localized at membrane even though slight increase of cytosolic distribution was noticed (Fig. 4, lane 5 and 6).

DISCUSSION

Several aspects of factors determining stability of the tetracycline/H^+ antiporter in *E. coli* cells have been revealed from this study. First of all, the essential role of the transmembrane helix XII of the tetracycline/H^+ antiporter in its functional expression becomes evident. TET388 is fully active but TET382 is totally inactive (Fig. 3). The difference is only 6 amino acid residues which are supposed to be located at carboxyl-terminal edge of the helix XII from prediction based on hydropathy plot. Without these 6 residues, TET protein cannot fold into an active form even though it is transported to the membrane (Fig. 4). The importance of the carboxyl-terminal transmembrane helix have also been pointed out for bacteriorhodopsin [16], melibiose permease [17] and lactose permease [5]. The overproduced pulse-labeled wild-type TET protein was not degraded over 20 hrs after IPTG induction was ceased, even though it was not necessary, or even harmful [18], for the growth of the cells under the condition without tetracycline in the culture medium. This stability was not restricted in TET protein; we observed that patterns and intensities of various pulse-labeled protein bands in SDS-PAGE of the membrane fraction obtained from a certain volume of the culture was almost unchanged over 20 hrs. The population of TET protein in individual cells decreases since the number of the cells in the medium increases several fold during 20 hrs, but the total amount of [^{35}S]methionine-labeled TET protein in the culture medium remains unchanged. Kaback and his colleagues observed that the lactose permease, a membrane protein with 12 transmembrane helices, was also very stable against proteolytic degradation [5]. Although it is premature to draw a conclusion from a limited number of examples, it seems like at present that *E. coli* cell does not have a degradation system for intact membrane proteins even in the case when they are not necessary for viability or growth of the cell.

In contrast to wild-type TET protein, truncated TET proteins (TET365 and TET382), which are unable to form the helix XII and thereby inactive in tetracycline transport, are rapidly proteolyzed even though they are present in the membrane (Fig. 2). Another truncated TET mutant (TET388), in which the helix XII can be formed, is as stable as the wild-type TET against proteolysis. Similar observations have been reported for lactose permease and bacteriorhodopsin [5, 16, 19]. These observations indicate strongly the presence of a proteolytic system for improperly folded, abnormal membrane proteins. This system can somehow distinguish membrane proteins with abnormal structure from those with native structure.

Finally, we found a protective effect of overproduced GroEL/ES on degradation of truncated TET protein (Fig. 5). At this stage of study, the mechanism of this protection is not known. One might think of several possibilities such as; the improperly folded TET365 protein interacts directly with GroEL/ES in some manners, or the responsible proteolytic machinery is less efficient in the presence of overproduced GroEL/ES. Also, it should be pointed that slight

inhibition of membrane localization of β-lactamase by overproduced GroEL/ES [11] could be relevant to our observations.

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