Metal-Tetracycline/H⁺ Antiporter of Escherichia coli Encoded by a Transposon Tn10

HISTIDINE 257 PLAYS AN ESSENTIAL ROLE IN H⁺ TRANSLLOCATION*

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The transposon Tn10-encoded tetA gene product is a metal-tetracycline/proton antiporter (Yamaguchi, A., Udagawa, T., and Sawai, T. (1990) J. Biol. Chem. 265, 4809-4813). Its tetracycline transport activity was inhibited by a histidine-specific reagent, diethyl pyrocarbonate. Among five histidine residues in this antiporter, only His⁶⁷ is located in the putative transmembrane helices. Thus, His⁶⁷ was replaced by Glu or Asp. Inverted vesicles containing the Glu⁶⁷ and Asp⁶⁷ mutants showed only 20 and 10% of the tetracycline uptake of wild-type vesicles, respectively. In contrast to wild-type vesicles, the mutant vesicles showed no tetracycline-dependent proton translocation, indicating that the mutant proteins had lost the tetracycline/H⁺ antiporter activity. The significant ⁶⁶Co⁺ uptake without proton translocation by the mutant vesicles also confirmed that the mutant carriers act as uniporters of a metal-tetracycline complex. The metal-tetracycline uniport by the mutant proteins was not inhibited by diethyl pyrocarbonate, indicating that His⁶⁷ is the only histidine residue essential for proton translocation. These mutant proteins conferred about half-level resistance to tetracycline, probably due to their catalyzing downhill efflux of a metal-tetracycline complex out of the cells.

The transposon Tn10-encoded tetA gene product is responsible for the highest resistance to tetracycline among bacterial tet¹ determinants (1). The resistance mechanism is based on the active efflux of the drug out of the cells driven by a proton gradient (2, 3). We found that a metal-tetracycline chelate complex was excluded by the TetA protein (4) and designated this protein as a metal-tetracycline/H⁺ antiporter. The efflux was electrically neutral (3), indicating that the 1:1 stoichiometric exchange of a metal-tetracycline complex and a proton. However, no direct evidence had been obtained that the TetA protein carries protons. In the previous paper (5), we first showed the tetracycline-dependent H⁺ translocation mediated by the Tn10-TetA protein.

A substrate/H⁺ antiporter principally requires at least three features: 1) a substrate binding site(s) which fluctuates between high and low affinity states; 2) at least two gating sites, one on the cytoplasmic side and the other on the periplasmic side, which open and close together with the affinity change of the binding site; and 3) a H⁺ transfer site(s) of which protonation and deprotonation affect the affinity of the binding site and probably opening and closing of the gates. According to the putative secondary structure of the Tn10-TetA protein (Fig. 1), the protein is composed of 12 membrane-spanning helices and 11 inter-helix loops, 5 on the cytoplasmic side and 6 on the periplasmic side. Among these loops, the first cytoplasmic loop from the NH₂ terminus has a highly homologous sequence with other TetA proteins (10). In the previous paper (5), we revealed that Asp⁶⁶ in this loop is essential for tetracycline transport. The properties of Asp⁶⁶ and Ser⁶⁶ substitution mutants indicate that this loop probably acts as a cytoplasmic gate (5).

Although many charged residues are located in the loop regions, there are only five charged amino acid residues in the putative membrane-spanning α-helices (Fig. 1). Among these, three aspartate (Asp¹⁵, Asp⁶⁶, and Asp⁶⁷) and one histidine (His²⁵⁷) residues are conserved in the TetA proteins so far sequenced (11), whereas one aspartate residue (Asp⁶⁶) is not conserved. We found that a histidine-specific reagent, DEPC, inhibits the antiporter, suggesting the participation of histidine residue(s) in the tetracycline transport. The only histidine residue (His²⁵⁷) is located in the transmembrane region (helix 8) (Fig. 1). Although there is no other charged residue in helix 8, there is a negatively charged residue, Asp⁶⁶, in the adjacent helix 9, at a similar depth in the hydrophobic core (Fig. 1). This residue could be sterically located at a position very close to His²⁵⁷ and may form a charge-relay system with His²⁵⁷, as reported for other H⁺-translocating membrane proteins (12-14).

In this study, we revealed the role of His²⁵⁷ in the transport function of the antiporter by means of site-specific mutagenesis. We measured H⁺ translocation coupled with tetracycline transport mediated by mutant antiporters (His²⁵⁷ → Glu, Asp). They showed no H⁺ translocation, although they retained the uniport activity for metal-tetracycline complexes. In contrast to the wild-type antiporter, the mutant antiporters were resistant to the histidine-specific reagent, DEPC. We conclude that His²⁵⁷ is the only histidine residue essential for H⁺ translocation, probably as a component of a charge-relay system.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, the Klenow fragment, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co. (Kyoto, Japan), Nippon Gene Co. (Toyama, Japan), and Toyobo Co. (Osaka, Japan).

[⁷-³H]Tetracycline and [α-²³P]dCTP were obtained from Du Pont-New England Nuclear and Amersham Corp., respectively. Diethyl pyrocarbonate and quinacrine were purchased from Sigma. Other chemicals were commercial products of the highest grade available.

Bacterial Strains and Plasmids—Escherichia coli MV1184 (15),

1 The abbreviations used are: DEPC, diethyl pyrocarbonate; MOPS, 3-(morpholino)propanesulfonic acid.

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The radiolabeled compound was added, and then the mixture was immediately passed through a French press in 50 mM MOPS-KOH buffer (pH 6.6) containing 0.1 M KCl as described previously (5).

Site-directed Mutagenesis—The EcoRI-HindIII fragment (688 base pairs) of the tetA gene was subcloned into the EcoRI-HindIII site of pUC19 (18) for mutagenesis. The resulting plasmid, pCT118e, was used as a template for the mutagenesis (Fig. 2).

Mutagenesis was performed (16) with an oligonucleotide-directed in vitro mutagenesis system (version 2, Amersham Corp.). The mutagenic primers (Table I) were designed with an Applied Biosystems DNA synthesizer, model 380B. The mutant plasmids, pTE257 (His<sup>257</sup> → Glu) and pTD257 (His<sup>257</sup> → Asp), were detected on the basis of the appearance of a new StuI restriction site and confirmed by DNA sequencing using a Sequenase kit (version 2, Toyobo Co.). The mutations introduced were found in all cases, and no other base change was found in the vicinity of the mutation site corresponding to the 657-777 base pairs downstream from the first base of the tetA coding sequence.

The EcoRI-HindIII fragments containing these mutations were then transferred to pCT118e through fragment exchange to reconstitute the mutant tetA gene (resulting plasmids, pCTE257 and pCTD257). Finally, the mutant tetA genes were inserted into pLGE259 BE (K<sup>m</sup>, T<sup>c</sup>) (5), which is a derivative of pLG339 (K<sup>m</sup>, T<sup>c</sup>) (19), following the procedure for the construction of pLGT (resulting plasmids, pLGE257 and pLGD257) (Fig. 2).

Preparation of Inverted Membrane Vesicles—Cells were grown on the minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. At the middle of the logarithmic phase, tetA gene expression was induced for 2 h by incubation with 1 µg/ml tetracycline. Inverted vesicles were prepared after the disruption of cells with a French press in 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl as described (4).

Immunoblot Analysis—Immunoblot analysis of inverted membrane vesicles was performed according to Carrasco et al. (20). Anti-carboxy-peptide antibodies were raised by using a synthetic oligopeptide corresponding to the COOH-terminal 14-amino acid sequence of the Tn10::TetA protein as an epitope, as described in the previous paper (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the inverted vesicles (10 µg of membrane protein) was followed by electroblotting of the proteins, and the bands corresponding to TetA were detected by enzyme-linked immunosorbent assay using anti-carboxy-peptide antibodies and an Express blot assay kit (Bio-Rad) as described in the previous paper (5).

Transport Assays—A mixture of 10 µl of the vesicle suspension (3.5 mg of protein/ml) and 0.5 µl of 250 mM NADH was preincubated at 30 °C for 1 min. The tetracycline uptake was initiated by the addition of 40 µl of MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, MgSO<sub>4</sub>, (final concentration, 5 mM), and [3H]tetracycline (final concentration, 10 µM), unless otherwise stated. After incubation at 30 °C, 2 ml of 5 mM MOPS-KOH (pH 7.0) containing 0.15 M LiCl was added, and then the mixture was immediately passed through a Millipore filter (0.45 µm). The filter was washed twice with the same buffer, and then the radioactivity was measured with a liquid scintillation counter.

The <sup>3</sup>H<sub>3</sub>CO<sup>2+</sup> uptake was measured similar to the tetracycline uptake except that the <sup>3</sup>H<sub>3</sub>CO<sup>2+</sup> uptake was initiated by the addition of 40 µl of MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, 10 µM (final) tetracycline, and 10 µM (final) <sup>3</sup>CoCl<sub>2</sub>.

Measurement of the Change in Fluorescence of Quinacrine—Proton translocation across inverted vesicles was measured as the change in fluorescence of quinacrine (21). A suspension (50 µl) of inverted vesicles (2 mg of protein/ml) was diluted with 2 ml of 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl and 10 mM MgSO<sub>4</sub>. After the addition of 10 µl of a quinacrine solution (160 µM), the emission (500 nm/excitation (440 nm)) ratio was monitored. Five µl of 250 mM NADH, 5 µl of 2 mM tetracycline, and 2 µl of 10 mM CCCP were successively added at about 2-min intervals.

Measurement of Bacterial Resistance to Antibiotics—Bacterial resistance to antibiotics was measured by the agar dilution method (22) and expressed as the minimum inhibitory concentration.

The effect of tetracycline on the growth rate of the cells was automatically monitored with a Bio-Log II photometer (Jobin-Yvon, France).

RESULTS

The Effect of Replacement of His<sup>257</sup> by Glu or Asp on Tetracycline Transport—Among five histidine residues of the Tn10-encoded tetracycline/H<sup>+</sup> antiporter, only His<sup>257</sup> is located in the putative transmembrane helices (Fig. 1), and this residue is conserved in other TetA proteins (11). His<sup>257</sup> was replaced by an acidic amino acid residue, Glu or Asp, by means of site-directed mutagenesis. The resulting plasmids containing the mutant tetA genes were named pLGE257 (Glu<sup>257</sup>) and pLGD257 (Asp<sup>257</sup>), respectively (Fig. 2).

Inverted membrane vesicles were prepared from E. coli W3104 cells harboring a plasmid, pLGE257, pLGD257, or pLGT (wild, His<sup>257</sup>), after 2-h induction of the tetA gene expression in midlog phase cells. Fig. 3 shows the typical time course of the tetracycline uptake by these inverted vesicles in the presence of 10 µM tetracycline and 5 mM MgSO<sub>4</sub>. The vesicles derived from cells containing the wild-type antiporter showed tetracycline uptake of about 2.0 nmol/mg of membrane protein for the initial 30 s when the no-energy, back-ground influx of tetracycline was subtracted (Fig. 3). Under the same conditions, the rates of tetracycline uptake by the Glu<sup>257</sup> and Asp<sup>257</sup> vesicles were 0.47 and 0.20 nmol/mg mem-

brane protein/30 s, respectively. The relative activities of the Glu<sup>257</sup> and Asp<sup>257</sup> vesicles were reproducibly about 20 and 10%, respectively, of the activity of wild type vesicles. Vesicles prepared from the cells containing no plasmid showed no significant tetracycline uptake in either the presence or the absence of NADH (data not shown).

These mutant vesicles contained similar amounts of the antiporter proteins to the wild-type vesicles, as judged on immunoblot analysis with the carboxyl terminus-specific antibody (Fig. 4). Thus, the defect in the tetracycline transport of the mutant vesicles reflects the defect in the transport activity of the mutant antiporters, indicating an important role of His<sup>257</sup> in the transport process.

### TABLE I

DNA sequence analysis of mutants of tetA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutagenic primer</th>
<th>DNA sequence codons</th>
<th>tetA amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLGT</td>
<td></td>
<td>253-257</td>
<td>Leu-Gly-Leu-Leu-His</td>
</tr>
<tr>
<td>pLGE257</td>
<td>5'-TATGAGGCCTCTAGGGCCTTTTAGAGTCTTAGTCTCA-A-3'</td>
<td>StuI</td>
<td>Leu-Gly-Leu-Leu-Glu</td>
</tr>
<tr>
<td>pLGD257</td>
<td>5'-TATGAGGCCTCTAGGGCCTTTTAGAGTCTTAGTCTCA-A-3'</td>
<td>StuI</td>
<td>Leu-Gly-Leu-Leu-Asp</td>
</tr>
</tbody>
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Asterisks indicate the base or amino acid changed. Underlines with the names of restriction enzymes indicate the new restriction sites appearing in the mutated sequences.
Role of His\(^{357}\) in a Tetracycline Carrier

**H\(^+\) Translocation Coupled with Tetracycline Transport**—Tetracycline-dependent H\(^+\) translocation is one of the important criteria for metal-tetracycline/H\(^+\) antiport activity (5). The H\(^+\) translocation across inverted membrane vesicles was measured by assaying the fluorescence of quinacrine (Fig. 5). When 5 \(\mu\)M tetracycline was added to energized, wild-type vesicles, a significant increase in quinacrine fluorescence, which corresponds to H\(^+\) efflux coupled with the antiporter-mediated tetracycline influx, was observed. In contrast, when tetracycline was added to vesicles lacking the antiporter, no significant change in fluorescence was observed. Although a very small energy-independent fluorescence change was observed when more than 10 \(\mu\)M tetracycline was added to the vesicles lacking the antiporter, the change was due to the intrinsic fluorescence of tetracycline molecules (data not shown).

Interestingly, when 5 \(\mu\)M tetracycline was added to the energized mutant vesicles, no significant fluorescence change was observed at all (Fig. 5). The fluorescence traces of the mutant vesicles after the tetracycline addition were just the same as those of the vesicles lacking the antiporter even when more than 10 \(\mu\)M tetracycline was added (data not shown). Thus, the mutant antiporters had clearly lost the tetracycline/H\(^+\) antiport activity, indicating the essential role of His\(^{357}\) in the H\(^+\) translocation process.

**\(^{60}\)Co\(^{2+}\) Transport Mediated by the Mutant Antiporters**—The results described above suggested that the mutant antiporters mediate tetracycline transport without proton translocation. As shown in Fig. 6, even the downhill influx of a metal-tetracycline complex mediated by such uncoupled carriers can result in the energy-dependent accumulation of tetracycline in the form of a protonated molecule (TH\(_3\)), because the metal-tetracycline complex probably dissociates into a divalent cation and TH\(_2\) in the energized vesicles due to the low \(pH_{in}\). If this is the case, the accumulation of the divalent cation should be greater than that of tetracycline because, in contrast to membrane-impermeable divalent cations, the protonated tetracycline slowly but significantly diffuses through the lipid bilayer membrane (4, 23, 24).

![Diagram of secondary structure model for the Tn10-TetA protein based on the hydropathy profile.](image)

**FIG. 1.** Secondary structure model for the Tn10-TetA protein based on the hydropathy profile. The amino acid sequence of the Tn10 TetA protein was cited from Nguyen et al. (6) and Hillen and Schollmeier (7). Hydrophobic segments are shown in boxes as symbols with their positions in the primary sequence. Residues enclosed by broken lines are those important for tetracycline transport, transmembrane helices connected by hydrophilic loops. The charged residues in the transmembrane segments are indicated by one-letter symbols with their positions in the primary sequence. Residues extruded by solid lines.

![Diagram showing translocation coupled with tetracycline transport.](image)

**FIG. 2.** Subcloning of the Tn10-encoded tet genes and reconstruction of the mutant tet genes. Open bars indicate the structural genes for tetA and tetR. Boldface lines represent the DNA sequence originating from Tn10. Open wedges represent the distance from the top of the tetA structural gene. In the plasmids, pLGT, pLG257, and pLG257, the tet genes are inserted in the reverse orientation. The numbers in parentheses represent the distance from the top of the tetA structural gene. In the plasmids, pLGT, pLG257, and pLG257, the tet genes are inserted in the reverse orientation.

![Diagram showing \(^{60}\)Co\(^{2+}\) uptake by inverted vesicles.](image)

**Fig. 7** shows the \(^{60}\)Co\(^{2+}\) uptake by inverted vesicles. The rates of \(^{60}\)Co\(^{2+}\) uptake by the wild-type, Glu\(^{357}\), and Asp\(^{357}\) vesicles were 2.6 (100%), 0.93 (36%), and 0.48 (18%) nmol/mg membrane protein/30 s, respectively, when the background influx into vesicles without NADH was subtracted. There was no significant difference between the no-energy, background infuxes into the mutant and wild-type vesicles. Thus, the Co\(^{2+}\)/tetracycline transport rate ratios were at least 1.3, 2.0, and 2.4 for the wild-type, Glu\(^{357}\), and Asp\(^{357}\) vesicles, respectively. The fact that the Co\(^{2+}\)/tetracycline ratios of the mutant vesicles were about 2-fold higher than that of the wild-type vesicles confirmed the assumption that tetracycline in the mutant vesicles was accumulated as a membrane-permeable protonated form. The significant uptake of \(^{60}\)Co\(^{2+}\) by the mutant antiporters without proton translocation clearly indicates that these mutants act as uniporters of a metal-tetracycline complex.

**Kinetics of Tetracycline Transport by the Mutant Antiporters**—The \(K_m\) and \(V_{max}\) values for tetracycline uptake by in-
Role of His$^{257}$ in a Tetracycline Carrier

FIG. 3. Tetracycline (TC) uptake by inverted membrane vesicles prepared from E. coli W3104 harboring the wild type or a mutant plasmid. Symbols: O, pLGT (wild); ●, pLGE257 (Glu$^{257}$); ▲, pLDG257 (Asp$^{257}$); Δ, no NADH. The no NADH data represent the averages of the data for the three different types of vesicles, which are very close to each other.

FIG. 4. Immunoblot analysis of inverted membrane vesicles prepared from E. coli W3104 cells harboring the wild type or a mutant plasmid. Each lane contained about 10 μg of total protein. Lane 1, no plasmid; lane 2, pLGT (wild); lane 3, pLGE257 (Glu$^{257}$); lane 4, pLDG257 (Asp$^{257}$). The arrow indicates the TetA protein.

verted membrane vesicles were obtained from Lineweaver-Burk plots of the initial rate of the tetracycline uptake versus the tetracycline concentration in the presence of excess divalent cations (10 mM MgSO$_4$). The $K_m$ value of the wild-type vesicles was 19 μM, which was in good agreement with the $K_m$ value (17 μM) in the presence of 1 mM CoCl$_2$, as reported in the previous paper (5). On the other hand, the Glu$^{257}$ and Asp$^{257}$ mutant vesicles showed $K_m$ values of 93 and 62 μM, respectively, indicating that the affinities of the mutant proteins to the substrate were decreased. The $V_{max}$ values of the Glu$^{257}$ and Asp$^{257}$ vesicles were 30 and 10% that of the wild-type vesicles, respectively.

Inhibition of the Tetracycline Transport by Diethyl Pyrocarbonate—DEPC is a reagent which specifically modifies histidine residues below pH 6.5 (25). Fig. 8 shows that DEPC inhibited the tetracycline uptake by the wild-type vesicles. About 90% of the activity was lost in the presence of 1 mM DEPC for 10 min at room temperature, although no further inhibition was not observed in the presence of more than 1 mM DEPC. Since DEPC inhibited only about 40% of the respiration of inverted membrane vesicles under the same conditions (Fig. 8, inset), it is clear that the modification of a histidine residue(s) of the wild-type antipporter was responsible for the loss of the tetracycline transport activity.

On the other hand, the degree of the apparent inhibition of the mutant vesicles by DEPC was far less than that of the wild-type vesicles, and the inhibition was due to the inhibition of respiration, indicating that the uniport activity itself was not inhibited by DEPC. As a result, the activity of the Glu$^{257}$ mutant vesicles was similar to that of the wild-type vesicles when the vesicles were treated with more than 1 mM DEPC. These results indicate that 1) His$^{257}$ is the only histidine residue responsible for the DEPC inhibition and 2) both the replacement of His$^{257}$ by an acidic residue and the modification of His$^{257}$ with DEPC result in the similar uncoupled state of the antipporter.

Resistance of Cells Harboring the Mutant Plasmids to Tetracycline—The minimum inhibitory concentration of E. coli...
W3104 cells containing pLGT (wild) for tetracycline was 200 μg/ml, which was 250 times higher than that for the sensitive cells lacking plasmids (MIC, 0.8 μg/ml). As judged from the minimum inhibitory concentration, the cells containing the mutant plasmids, pLGE257 (Glu257) and pLGD257 (Asp257), showed half-level resistance to tetracycline (MIC, 100 μg/ml). The growth of the cells containing the mutant plasmids was significantly retarded by only 5 μg/ml tetracycline, whereas the growth of the cells containing pLGT was not affected by such a low concentration of the drug (Fig. 9B). With 50 μg/ml tetracycline, the growth rate of the mutant cells was about half that of the wild type cells (Fig. 9C). Interestingly, with tetracycline concentrations higher than 100 μg/ml, the growth of the wild type cells was also retarded, and the rate became comparable with that of the mutant cells (Fig. 9D). The resistance of the cells containing pLGD257, as judged from the growth rate, was not less than that of the cells containing pLGE257 (data not shown), although the tetracycline transport activity of the Asp257 mutant was about one-half that of the Glu257 mutant, as described above.

It is very interesting that these uncoupled carriers can mediate fairly high resistance to tetracycline. It is known that tetracycline is accumulated in sensitive cells through simple diffusion of TH2 through the lipid bilayer membrane followed by dissociation into a proton and the anionic form of tetracycline (TH–), which may be converted to a magnesium-tetracycline complex in the cell interior. Thus, uniporters of metal-tetracycline complexes may confer tetracycline resistance because of the reduction in the metal-tetracycline complex concentration in cells through carrier-mediated downhill efflux of the metal-tetracycline complex out of the cells.

**DISCUSSION**

This work revealed that His257 is essential in H+ translocation during metal-tetracycline/H+ antiport by Tns11-TetA proteins. The mutant proteins in which His257 was replaced by Glu and Asp, respectively, lost the ability of tetracycline-dependent H+ translocation, indicating that they lost the metal-tetracycline/H+ antiport activity. Instead, they acted as uniporters of a metal-tetracycline complex without H+.
translocation. In contrast to the wild-type antiporter, the uniprot activity of the mutant proteins was not inhibited by a histidine-specific reagent, DEPC, in spite of the fact that 4 histidine residues remained in the hydrophilic regions of the mutant proteins.

The importance of a histidine residue(s) at the transmembrane locus has been reported in the cases of H+ -ATPase (12) and lactose permease (26, 27). In lac permease, His272 forms a charge-transfer triad with charged amino acid residues, Arg202 (28) and Glu256 (29), which are postulated to be sterically close to His272. When His272 of the lac permease was replaced by Arg, the mutant permease lost the ability of H+ translocation but retained the ability to mediate downhill efflux with high concentrations of lactose (30). His272 substitution mutants of the Tn10-TetA protein also mediated metal-tetracycline complex transport without H+ translocation, with decreased affinity for the substrate; thus, the mutants are "uncoupled" mutants fundamentally similar to the His272 substitution mutants of the lac permease. If His257 in the TetA protein forms a charge-relay system similar to the one postulated for the lac permease, which residue(s) conjugate with His257? There is no other charged residue in helix 8 than His257, but in the adjacent helix 9, there is a negatively charged residue, Asp256, which could be located sterically close to His257 (Fig. 1). This residue is conserved in the TetA proteins belonging to other classes (11) and, as observed in our preliminary experiment, replacement of Asp256 by Asn resulted in the loss of the tetracycline/H+ antiport activity. Therefore, there is a possibility that His257 and Asp256 form a charge-transfer pair.

In contrast to lac permease (30), the His257 substitution mutants of the Tn10-TetA protein can apparently mediate active transport. This phenomenon is based on the fact that the TetA protein is a carrier for a divalent cation-week acid chelate complex. Namely, the respiration-driven pH shift across a membrane can cause the dissociation of a metal-tetracycline complex into a divalent cation and protonated tetracycline (TH+), resulting in the respiration-dependent accumulation of the divalent cation and tetracycline in the lower pH compartment. A concentration gradient of a divalent cation also facilitates the apparent active transport. Thus, the uncoupled mutants of Tn10-TetA are expected to confer fairly high level resistance to tetracycline, because a high concentration of magnesium ions and a higher cell interior pH than that of the medium should facilitate such apparent active efflux of tetracycline out of cells. This assumption was supported by the fact that cells producing the Glu257 or Asp257 mutant protein showed almost half of the level of resistance to tetracycline as cells producing wild type TetA.

Unfortunately, direct measurement of the energy-independent efflux, exchange, or countertransport of metal-tetracycline complexes has not been successful in either wild-type or mutant inverted membrane vesicles. The difficulty of such measurements is mainly due to the simple diffusion of tetracycline through the lipid bilayer region (23, 24) and adsorption of divalent cations on the internal surface of the inverted membrane (4).

King and Wilson (31) reported that lac permease, of which His272 was replaced by Tyr or Phe, retained lactose-H+ symport activity. Brooker (32) also claimed that a Val177/Asn72 double mutant of lac permease can mediate H+ coupled lactose transport although it did not mediate active lactose transport against a concentration gradient. These observations may suggest that a H+ bypass arose with such mutations (31). As to the postulated charge-transfer pair of the TetA protein, such a bypass may be difficult because the system lacks a basic residue corresponding to Arg202 of lac permease. It may be interesting to examine whether a "bypass" mutation occurs in TetA or not.

It is known that the TetA protein is separated into two regions, α and β, by a central large hydrophilic loop (33). Each region contains six transmembrane helices. Since mutations in both regions show intracistronic complementation (34), it is postulated that the two regions play different roles in tetracycline transport. In the previous paper (5), we reported mutants as to the α region. In contrast to the His257 substitution mutants, the Asp256 substitution mutants showed decreased tetracycline-dependent H+ translocation in proportion to the tetracycline transport (5), indicating that the defect occurred in the substrate translocation pathway. These observations seem to support the hypothesized work sharing between the two regions, that is the α region is involved in substrate recognition and translocation and the β region is involved in energy coupling. However, this assumption does not exclude the possibility that any residue(s) in the β region may play a direct role in substrate binding.

Recently, Rubin et al. (35) proposed that the tetA gene was formed through tandem duplication of two homologous halves, each half probably being composed of 201 amino acids (Tn10-TetA:401 amino acids). This assumption is very interesting because the essential Asp256, which is the 84th residue of the β half, on the third helix of the β region (helix 9) has a counterpart, Asp44, on the third helix of the α region (helix 3). There is also a possible role for tetracycline transport. It is assumed that the single substrate-binding site is composed of these two negatively charged residues, Asp44 and Asp256, which form two symmetrical coordinate bonds with a cationic metal-tetracycline complex if the two aspartate residues are sterically close to each other. His257 might catalyze the change between the high affinity and low affinity states of the binding site through proton transfer to Asp256. Since there is no histidine residue in the transmembrane α region, the postulated ancestor of the TetA protein formed from gene duplication might not have contained His257, and, therefore, it may have mediated only the downhill efflux of tetracycline without proton translocation. According to the results of this work, such a hypothetical ancient TetA protein could also have mediated fairly high resistance to tetracycline. Thus, the Tn10-TetA protein might have acquired the active transport activity through the evolutionary addition of His257. This attractive hypothesis is presently under examination.

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