Second-site Mutation of Ala-220 to Glu or Asp Suppresses the Mutation of Asp-285 to Asn in the Transposon Tn10-encoded Metal-Tetracycline/H+ Antiporter of Escherichia coli*

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A carboxyl group of Asp-285 is essential for tetracycline/H+ antiporter mediated by the transposon Tn10-encoded metal-tetracycline/H+ antiporter (TetA) of Escherichia coli (Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., and Sawai, T. (1992) J. Biol. Chem. 267, 7490-7498). Spontaneous tetracycline resistance revertants were isolated from E. coli cells carrying the Asn-285 mutant tetA gene. All of the revertants were due to the second-site mutation at codon 220 of GCG (Ala) to GAG (Glu). The Km value of the tetracycline transport mediated by the revertant TetA protein was about 4-fold higher than that of the wild-type, indicating that the revertant is a low affinity mutant. A Glu-220 and Asn-285 double mutant constructed by site-directed mutagenesis showed the same properties as the revertants, confirming that the mutation of Ala-220 is solely responsible for the suppression. The Asp-220 mutation of the Asn-285 mutant resulted in a lower level of restoration of the tetracycline resistance and the transport activity than in the case of the Glu-220 mutation. A single mutation replacing Ala-220 with Glu or Asp caused a 2-4-fold decrease in the tetracycline resistance, but no crucial change in the transport activity.

It is not likely that Glu-220 is required for a charge-neutralizing salt bridge because an unpaired negative charge in a Glu-220 or Asp-220 single mutant did not cause a serious change in the activity. An alternative explanation is reasonable; Asp-285 directly contributes to the binding of a cationic substrate, metal-tetracycline chelation complex, or proton, and an acidic residue at position 220 can take over the role of Asp-285.

The transposon Tn10-encoded tetracycline resistance protein (Tn10-TetA) of Escherichia coli is a well-studied example of a proton substrate antiport system (1, 2). The substrate exported by the antiporter is a monocationic metal-tetracycline chelation complex (3). According to the nucleotide sequence of the Tn10-tetA gene, the Tn10-TetA protein comprises 401 amino acid residues (4, 5). Eckert and Beck (6) proposed a secondary structure model comprising 12 transmembrane segments with the amino and carboxyl termini on the cytoplasmic side. We modified the model slightly, in which the charged residues at the boundaries between hydrophobic transmembrane helices and hydrophilic loops are placed in the loop region, on the basis of the results of our site-directed mutagenesis studies on the charged residues (7, 8). The general features of the model are supported by the results of protease digestion (6) and antibody binding (9). The same model was also supported, for the pBR322-encoded TetA protein, by the results of analysis of a series of TetA-alkaline phosphatase fusions (10). A 12-membrane-spanning structure is common not only in drug export proteins (11-15), but also proton substrate symporters and uniporters (14). The resemblance between antiporters and symporters is not restricted to the secondary structure. They have a commonly conserved sequence motif, GXXDRXGRR, and a derivative of it in the hydrophilic loop2,3 and loop6,9 regions, respectively (14, 15). However, the distributions of functionally or structurally important residues in the hydrophobic transmembrane regions are quite different from each other. According to the results of site-directed mutagenesis studies on lactose permease (16, 17), the important residues are located mainly in the carboxyl-terminal half of the protein, and a truncated mutant deleted from helices 2 to 6 showed downhill transport activity (18). In contrast, the functional residues in the transmembrane regions of the TetA protein show a symmetrical distribution between amino- and carboxyl-terminal halves (19, 20). TetA proteins having deletions in their carboxyl-terminal halves show functional complementation with ones having deletions in their amino-terminal halves (21), indicating that both halves contribute to the transport function. The Tn10-TetA protein contains only 4 putative transmembrane charged residues, 3 aspartate and 1 histidine (8, 22), and all these residues are conserved in at least class A, B, and C TetA proteins (23). Site-directed mutagenesis studies revealed that all of these charged residues are important for the tetracycline transport function (8, 22, 24), the three carboxyl side chains being especially essential (8). It is characteristic in TetA proteins that the number of transmembrane negatively charged residues is higher than the number of positively charged residues. This feature is very different from the distribution of transmembrane charged residues in lactose permease. In the putative secondary structure model of lactose permease modified by King et al. (25), the transmembrane regions contain the same numbers of negatively and positively charged residues, most of them forming charge-neutralizing pairs (25, 26). Such charge-neutralization is necessary for protein folding in a hydrophobic environment (27). The pairings of the charged residues were found on analysis of second-site suppressor mutants (25, 26). Namely, replacement of either charged residue with a neutral residue creates an unpaired charge causing a functional defect, while additional replacement of the unpaired residue with a neutral residue causes functional restoration. In the TetA protein, Asp-285 is located sterically close to His-257 in the putative structure (22); however, the occurrence of charge-pair neutralization between Asp-285 and His-257 is not likely because the replacement of these residues with neutral residues caused different results, that is, the replacement of Asp-285 with a neutral residue, Asn, resulted in the complete loss of the transport activity (8), while on the replacement of

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His-257 with a neutral residue, Tyr, significant activity re-
maind (20). In addition, there is no other positively charged
candidate for pairing with Asp-15, Asp-84, or Asp-285 in the
transmembrane region.

To examine the possibility of charge-pair neutralization in
the TetA protein, we isolated second-site suppressor mutants of
the Asn-285 mutant TetA protein. Unexpectedly, the revertants
showed no removal of the positively charged residue. All of
them showed the introduction of a new negatively charged
residue at a position distant from Asn-285 in the primary se-
quence. The results indicated that charged residues in the
transmembrane region are not always necessary to make charge-
neutralizing pairs. It is likely that the carboxyl group of
Asp-285 is protonated or neutralized on the binding of a cati-
onic substrate, or a negatively-charged group of Asp-285 may
be located in a hydrated transmembrane pathway involved in
substrate translocation.

EXPERIMENTAL PROCEDURES

Materials—[7-3H]Tetracycline and [5-3H]CTP were purchased from
Du Pont NEN and Amersham, respectively. A 7-deaza-dGTP Se-
quencing kit, S-1 nuclease, and DNA polymerase (version 2.0),
was purchased from Bioensors. An oligonucleotide-directed in vitro mutagenesis system
was purchased from Amersham. Lambda-Lift Expression Detection Kit
for immunoblotting was purchased from Bio-Rad. All other materials
were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids—E. coli MV1184 (28), TGl(29),
and W3104 (30) were used for single-strand DNA preparation, transforma-
tion, and expression of the mutant plasmids, respectively. pUC118 was
purchased from TAKARA (Kyoto, Japan). pLGT2 was constructed by clon-
ing of the tetR and tetA genes into a low-copy-number plasmid,
pLG339 (31), as described previously (8). pLGD285N is a derivative of
pLGT2, in which the Asp-285 codon was changed to an Asn one by
site-directed mutagenesis, as described previously (8).

Isolation of Spontaneous Revertants—E. coli W3104/pLGD285N
was grown overnight at 37 °C in 3 ml of 2 % YT medium (1.6% Bat-
cractopine, 1% yeast extract, and 0.5% NaCl) containing 50 μg/ml kana-
mycin. The cells were harvested from 1.5 ml of the medium and then
resuspended in 0.1 ml of fresh 2 % YT medium. The concentrated cell
suspension was poured on a YT-agar (0.8% Bacto-tryptone, 0.5% yeast
extract, 0.5% NaCl, and 1.5% agar) plate containing 50 μg/ml tetracy-
cline and then incubated at 37 °C. After a 5-h incubation, colonies were
isolated.

Site-directed Mutagenesis—At first, the EcoRI-BamHI (686
bp)1 of the D285N mutant tetA gene was subcloned into the EcoRI-
BamHI site of pUC118. The fragment contains the 3'-terminal part of
the tetA structure gene, which includes codons 220 and 285. The result-
ing plasmid was named pTBD285N. Site-directed mutagenesis of codon
220 was performed using the mutagenic primers listed in Table II
and pTBD285N as a template with an oligonucleotide-directed in vitro muta-
genesis system (version 2.1, Amersham). The mutation was first de-
ected as the appearance of a new PstI restriction site and then con-
formed by DNA sequencing. The resulting plasmids were
pTBA220ED285N and pTBA220DD285N. The double mutant tetA
genes at positions 220 and 285 were reconstructed by replacing the
EcoRI-BamHI region of the wild-type pLGT2 plasmid with the corre-
sponding fragment of pTBA220ED285N or pTBA220DD285N. The result-
ning plasmids were pLGA220ED285N and pLGA220DD285N, re-
spectively. A single mutant at position 220 was constructed by exchange of
the EcoRI-BglII fragment (187 bp), which includes codon 220 but not
codon 285, of pLGT2 with the corresponding fragment of
pTBA220ED285N or pTBA220DD285N. The resulting plasmids were
pLGA220E and pLGA220D, respectively.

Measurement of Bacterial Resistance to Tetracycline—Bacterial re-
sistance to tetracycline was measured by the agar dilution method (32)
and expressed as the minimum inhibitory concentration.

Preparation of Inverted Membrane Vesicles—Cells were grown in
1 liter of 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 0.25 μg/ml heat-inac-
tivated chlorotetracycline. Inverted vesicles were prepared by disruption

of the cells with a French press in 50 mm MOPS-KOH buffer (pH 6.6)
containing 0.1 M KCl and 10 mm EDTA. Then the vesicles were washed
once with 50 mm MOPS-KOH (pH 7.0) containing 0.1 M KCl (3).

Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis of
the inverted vesicles was followed by electrophoretic transfer of the
proteins. The TetA protein was detected by means of an enzyme-linked immunosor-
bent assay using an anti-carboxyl-terminal peptide antibody and an
Express blot assay kit (Bio-Rad), as described in the previous paper (9).

Tetracycline Transport Assaying of Inverted Vesicles—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, CoCl2 (final concentration, 50 μM), and [3H]teta-
cycline (final concentration, 10 μM), unless otherwise stated. After in-
cubation at 30 °C for the indicated periods, 2 ml of 5 mm MOPS-KOH
(pH 7.0) containing 0.15 μl LiCl was added, the mixture was immedi-
ately filtered through a Millipore filter (pore size, 0.45 μm) and washed
twice, and then the radioactivity of the filter was measured.

RESULTS

Isolation of Second-site Revertants from E. coli Strains Carrying
the D285N Mutant Plasmid—When concentrated cell suspension of E. coli
W3104/pLGD285N was spread on nutrient agar plates and incubated at 37 °C for 48 h, 10 colonies were
formed. Plasmids were prepared from 6 of these 10 revertant strains and transferred into E. coli
W3104. Table I shows the minimum inhibitory concentrations of tetracycline when E. coli
W3104 cells carrying these plasmids were grown on agar plates. The tetracycline resistance level (0.8 μg/ml) of the cells carrying
pLGD285N was the same as that of the host cells. In
contrast, the cells carrying the revertant plasmids showed the same resistance level (200 μg/ml) as the cells carrying the wild-
type pLG2 plasmid.

The nucleotide sequence, 1912GATAGT918 (the numbers
indicate the base positions from the HincII site located at 60 bp
upstream from the start codon of tetA), of the wild-type tetA
gene is changed to 1912AACTCG9218 in the D285N mutant tetA
gene. The former encodes Asp-285-Ser-286 and the latter Asn-
285-Ser-286. The latter sequence contains an additional XhoI
site (CTCGAG). All of the 6 revertant plasmids (pLGD285NB-1
to pLGD285NB-6) retained this XhoI site at the same position
as in pLGD285N. Then, the nucleotide sequence of about 150
bases around codon 285 was determined. All of the 6 revertant
plasmids showed the same sequence as pLGD285N in the
region from 850 to 900. The sequence from 913 to 918 of the
revertant genes was confirmed to be AACTCG. Thus, the res-
orption of the tetracycline resistance was not due to the back-
mutation from Asn-285 to Asp.

Determination of the Position of Second-site Mutation—In
order to limit the range of the location of the second-site mu-
tation, the revertant tetA gene of pLGD285NB-1 was cut at the
middle with EcoRI and at 262 bp downstream from the stop
codon with BamHI. The resultant EcoRI-BamHI fragment (868

| Plasmid | Minimum inhibitory concentrations μg/ml |
|---------|-----------------------------------------|
| No plasmid | 0.8 |
| pLGT2 (wild-type) | 200 |
| pLGD285N (Asp-285 → Asn) | 0.8 |
| pLGD285NB-1 (revertant 1) | 200 |
| pLGD285NB-2 (revertant 2) | 200 |
| pLGD285NB-3 (revertant 3) | 200 |
| pLGD285NB-4 (revertant 4) | 200 |
| pLGD285NB-5 (revertant 5) | 200 |
| pLGD285NB-6 (revertant 6) | 200 |
| pLpGbac (chimera) | 200 |

1 The abbreviations used are: bp, base pair(s); MOPS, 3-(N-morpho-
line)propanesulfonic acid.
whether positions may be sterically close to each other, although an than Glu-285, as described previously
responsible for suppression of Ala-220 was opened by Glu-220 cannot be ruled out.
compensating for the mutation at position 285. Asp-220 mutant TetA proteins were the same as that of the wild-type (Fig. 3). Thus, the differences in the tetracycline resistance between the wild-type and these mutant strains are not due to differences in the amount of the TetA protein.
Tetracycline uptake by inverted membrane vesicles was measured in the presence of 10 µM [3H]tetracycline and 50 µM CoCl_2 (Fig. 4, A and B). The initial rate of tetracycline uptake by wild-type vesicles was 2.1 nmol/mg of protein/30 s. D285N mutant vesicles showed no tetracycline transport, as reported in the previous paper (8). Single mutants A220E and A220D showed rates of about 4.0 (190% as to wild-type) and 2.1 (100%), respectively. On the other hand, the double mutants showed unexpectedly low tetracycline transport activity. The initial rates of the revertant (B-1), A220E/D285N, and A220D/D285N mutant vesicles were about 0.8, 0.9, and 0.2 nmol/mg of protein/30 s, respectively, which correspond to only 43%, 43%, and 10% as to the wild-type, respectively.
On the other hand, the revertant (B-1) vesicles showed high tetracycline transport activity, comparable to that of the wild-type, in the presence of 100 µM [3H]tetracycline (Fig. 4C). It is likely that the revertant and the double mutants may be low affinity mutants. Therefore, the kinetics of tetracycline transport in inverted membrane vesicles were measured in the presence of a high CoCl_2 concentration (1 mM). As shown in Table IV, the K_m values for tetracycline transport by the revertant and the A220E/D285N mutant were 158 µM and 145 µM, respectively, which are the same within experimental deviation. These K_m values were about 4.3-fold higher than the value in the case of the wild-type (36 µM), indicating that the A220E/D285N mutant has significantly lower substrate affinity than the wild-type. Similarly, the K_m value of the A220D/D285N mutant was also higher than that of the wild-type. The K_m values of the A220E/D285N and A220D/D285N mutants were very close to each other. This suggests that the decrease in the substrate affinity is mainly due to the change of the location of an essential negative charge from position 285 to 220.

**Second-site Revertants of the Asp-285 → Asn Mutant Tetracycline Carrier**

**Site-directed Mutagenesis of Ala-220**—In order to determine whether or not the mutation of Ala-220 is the only cause of suppression of the D285N mutation, site-directed mutagenesis of Ala-220 to Glu or Asp was performed using the mutagenic primers shown in Table II. As shown in Table III, the A220E/D285N (Glu-220-Asn-285) double mutant strain showed high tetracycline resistance as the wild-type and revertant strains (minimum inhibitory concentration, 200 µg/ml). Therefore, it is clear that the change from Ala-220 to Glu is solely responsible for suppression of the D285N mutation.

The A220D/D285N (Asp-220-Asn-285) double mutant strain showed a lower resistance level (50 µg/ml) than the A220E/D285N mutant. A longer spacer of the carboxyl side chain at position 220 seems more favorable for suppression of the D285N mutation than a shorter one. This is in contrast with the results as to position 285. Asp-285 is far more favorable than Glu-285, as described previously (8).

A single mutation of Ala-220 to Glu or Asp in the wild-type TetA causes a decrease in the resistance level (Table III), but the mutant strains retained significant tetracycline resistance. Thus, the introduction of an extra negative charge at position 220 did not cause crucial damage to the TetA function. The resistance level of the A220E mutant (100 µg/ml) was some what higher than that of the A220D mutant (50 µg/ml). This is very different from the result of the removal of a negative charge from position 285. The latter causes the complete loss of the function (8). If the second-site suppression is based on the charge neutralization of an unpaired positive charge in the D285N mutant, the insertion of an extra negative charge at position 220 in the wild-type TetA should cause crucial damage in the function through the generation of an unpaired charge, similar to that in the case of the negative-charge removal from position 285. This is clearly not the case.

**Determination of TetA Protein Production and Kinetics of Tetracycline Transport in Inverted Membrane Vesicles—SDS-polyacrylamide gel electrophoresis of the inverted vesicles (10 µg of membrane protein) was performed, followed by electrob-lotting onto a nitrocellulose filter. TetA proteins were detected by enzyme-linked immunosorbent assay using anti-carboxyl-terminal specific antiserum and alkaline phosphatase-linked goat anti-rabbit IgG.**

| 657 | 710 | 720 | 730 |
|-----|-----|-----|-----|
| wild | GAATT | . . . . . . . . . . | . . . . . . . . . . |
| D285N | GAATT | . . . . . . . . . . | . . . . . . . . . . |
| D285NB-1 | GAATT | . . . . . . . . . . | . . . . . . . . . . |
| 900 | 910 | 920 | 930 | 1290 |
| wild | . . . . . . . . . . | CGGATTATGCACGATCATTGATG | . . . . . . . . . . |
| D285N | . . . . . . . . . . | CGGATTATGCACGATCATTGCC . . | . . . . . . . . . . |
| D285NB-1 | . . . . . . . . . . | CGGATTATGCACGATCATTGCC . . | . . . . . . . . . . |

**Fig. 1.** The DNA sequences of the 3'-terminal halves of the wild-type, D285N, and revertant (B-1) tetA genes. The sequences determined, from 657 to 1290, were the same except for the bases depicted by bold letters. The stop codon is located at 1293. The sequence encodes from Asn-200 to Ala-401 of TetA.
shadowed letters in pop-up balloons acidic and basic residues in the transmembrane locus are depicted by bold letters. Asp-285 and Ala-220 are depicted by shadowed letters. The shadowed letters in pop-up balloons indicate the first site-directed mutation at position 285 and the second-site suppressor mutation at position 220.

**FIG. 2.** Secondary structure model of the Tn10-encoded TetA protein. Hydrophobic α-helical segments are enclosed by boxes. Conserved acidic and basic residues in the transmembrane locus are depicted by bold letters. Asp-285 and Ala-220 are depicted by shadowed letters. The shadowed letters in pop-up balloons indicate the first site-directed mutation at position 285 and the second-site suppressor mutation at position 220.

**TABLE II**

**Mutagenic primers used for site-directed mutagenesis**
The mutagenic primers contained two kinds of mismatches to cause amino acid replacements and silent mismatches to cause a new PvuII restriction site. Asterisks indicate the mismatches. The sequences of the primers correspond to the sequence of the antisense strand of tetA gene. Underlines indicate the new restriction site.

| Mutagenic primer | Primer sequence | Codon change | Amino acid substitution |
|------------------|-----------------|--------------|------------------------|
| A220E 5′-GGCCTATCAGCTGCT-3′ | GCG → GAG | Ala-220 → Glu |
| **PvuII** | **+** | **+** |
| A220D 5′-GGCCTATCAGCTGAT-3′ | GCG → GAT | Ala-220 → Asp |
| **PvuII** | **+** | **+** |

**TABLE III**

**Tetracycline resistance levels of E. coli W3104 cells harboring site-directed mutant plasmids**
The resistance levels are expressed as minimum inhibitory concentrations.

| Plasmid | Minimum inhibitory concentrations |
|---------|----------------------------------|
| pLGA220ED285N (Ala-220 → Glu, Asp-285 → Asn) | 200 μg/ml |
| pLGA220DD285N (Ala-220 → Asp, Asp-285 → Asn) | 50 |
| pLGA220E (Ala-220 → Glu) | 100 |
| pLGA220D (Ala-220 → Asp) | 50 |

D285N mutant are just the same, being about 2-fold higher than that of the wild-type. On the other hand, the $V_{max}$ value of the A220D/D285N mutant was about one-half that of the wild-type. Therefore, the length of the spacer at position 220 affects the turnover rate of the transporter rather than the substrate binding affinity.

**FIG. 3.** Immunoblot analysis of inverted membrane vesicles prepared from E. coli W3104 cells harboring the wild-type or a mutant plasmid. Each lane contained 10 μg of total protein. Lane 1, pLGT2 (wild-type); lane 2, no plasmid; lane 3, pLG285N; lane 4, pLG285N-1 (revertant-1); lane 5, pLGA220ED285N; lane 6, pLGA220E; lane 7, pLGA220DD285N; lane 8, pLGA220D.

The introduction of an extra negative charge, at position 220, into the wild-type TetA did not cause crucial change to the protein function. Instead, the $K_m$ values are decreased to about 2-fold that of the wild-type by this extra negative charge. The results may indicate that dual negative charges in the active site probably strengthen the binding of the cationic substrate. The $K_m$ values of the A220E and A220D mutants were similar to each other. In contrast, the $V_{max}$ value of the A220D mutant was lower than that of the A220E mutant. The latter was about the same as that of the wild-type. That is, the length of the side chain at position 220 has no effect on the affinity, but it affects the turnover rate. A longer chain is better than a shorter one. This result is the same as that in the case of the Asp-285-Ala-220 double mutants.

**DISCUSSION**

Second-site suppressor mutants of the Asp-285 → Asn mutant TetA protein were isolated. In all of the suppressor mutants, Ala-220 was replaced by Glu. Site-directed mutagenesis
FIG. 4. [3H]Tetracycline uptake by inverted membrane vesicles prepared from E. coli W3104 cells harboring the wild-type or a mutant plasmid. In A and B, tetracycline uptake was measured in the presence of 50 μM CoCl₂ and 10 μM [3H]tetracycline. In C, the uptake was measured in the presence of 100 μM CoCl₂ and 100 μM [3H]tetracycline. Solid lines indicate the uptake in the presence of 2.5 mM NADH. Broken lines indicate the average background uptake in the absence of NADH.

TABLE IV
The kinetic constants for tetracycline uptake by inverted membrane vesicles

| Plasmid         | Kₐ | Vₘₐₓ   |
|-----------------|----|--------|
|                 | μM | nmoi/mg protein/min |
| pLGT2 (wild-type) | 36 | 27     |
| pGB-1           | 158| 53     |
| pLGA220ED285N   | 145| 53     |
| pLGA220DD285N   | 141| 15     |
| pLGA220E        | 14 | 28     |
| pLGA220D        | 16 |        |

of Ala-220 revealed that Glu was preferable to Asp for the suppression of the Asp-285 → Asn mutation. A single mutation of Ala-220 to Glu or Asp did not cause a serious defect in the function. The results indicated that the second-site suppression is not due to the neutralization of an uncharged pair. It is based on the compensation of the Asp-285 function by an acidic residue introduced to position 220.

King et al. (25) first reported that melibiose-positive revertants derived from the Lys-358 → Thr mutant of lac permease contained a second-site mutation converting Asp-237 to a neutral amino acid. This indicates that Lys-358 and Asp-237 form a charge-neutralizing salt bridge. The second-site suppression is due to charge neutralization through removal of the unpaired negative charge. Lee et al. (26) found a similar charge-neutralizing pair, Lys-319 and Asp-240, in lac permease. According to the topology of lac permease reported by Foster et al. (33), Asp-237 and Asp-240 are located in the periplasmic loop region. On the basis of these observations, King et al. (25) modified the topology of lac permease. In the modified one, both Asp residues are located in the transmembrane region and, as a result, the numbers of transmembrane negative and positive charges are equal to each other. They suggested that most of the charged residues may form charge-neutralizing pairs (26), although, according to the results of recent site-directed mutagenesis studies by Sahin-Toth et al. (34), transmembrane charged residues other than the 2 pairs described above exhibited no such charge-neutralizing suppression. These charge-neutralizing salt bridges contribute to the protein folding (27) and/or may act as a gate opened/closed through protonation/deprotonation (26).

In the putative secondary structure of the TnlO-TetA protein (8), there are 3 aspartates and 1 histidine in the deep transmembrane locus. The numbers of transmembrane negative and positive charges are not equal, but a modification like that made by King et al. (25) is not possible in the central loop region of TnlO-TetA. The current topology of TetA has a large central loop common to the other 12-membrane-spanning transporters
(14) and the modified structure of lac permease according to
King et al. (25). In the present study, a charge-neutralizing-
type revertant was not obtained. It is certain that the presence
of an unpaired charge in the hydrophobic environment requires
high free energy (25). However, the location of unpaired
charges in the hydrated transmembrane channel does not re-
duire high free energy. Thus, charge-pair neutralization is not
essential in such a hydrated channel. The transmembrane
functional residues of Tn10-TetA including Asp-285 are distrib-
uted on the hydrophilic side of each amphiphilic transmem-
brane helix (20). This indicates that these helices form a hy-
drated transmembrane channel.

The predominance of transmembrane negative charges in
the TetA protein may be related to the positive charge of the
transported substrate, the metal-tetracycline chelation com-
plex (3). Unpaired negative charges in the transmembrane lo-
cus of the TetA protein probably contribute to the binding of a
positively charged substrate in the hydrated substrate-trans-
location channel. This prediction is supported by the fact that
an extra negative charge created by a single mutation of Ala-
220 increases the substrate binding affinity.

There is significant symmetry in the distribution of func-
tional residues of TetA (19, 20). Two sets of quartets of residues
symmetrically conserved in the amino- and carboxyl-terminal
transmembrane loci are important for the transport function
(20). Symmetrically conserved sequence motifs in the hydro-
philic loop region also play a functional role (19). The origin of
the symmetry may be a tandem duplication of the tetA gene
(36). As to the transmembrane negative charges, Asp-285 is
symmetrical to Asp-84. However, Asp-15 is an exception; there
is no corresponding acidic residue in the carboxyl-terminal
half. The position symmetrical to Asp-15 is occupied by Ala-220.
This is the position suppressing the mutation of Asp-285. It
seems not incidental that a 1-base-change of codon 220 gives a
Glu codon. Position 220 might have been originally occupied by
Glu in the ancestral TetA protein.

The revertant reported in this study is based on charge com-
penation. A similar type of revertant was recently reported in
a bacterial photosynthetic reaction center of Rhodobacter
sphaeroides (35). The removal of Asp-213 in the L-subunit was
suppressed by the mutation of Asp-44 to Asp in the M-subunit
(35). These Asp residues are predicted to act as a proton donor
in the proton transfer reaction. Previously, we pointed out the
possibility that Asp-285 of TetA plays dual roles in substrate
binding and proton transfer (8). According to the fact that the
double mutants of TetA showed low substrate affinity but high
V_{max} values, an acidic residue at position 220 may be fully able
to act as a proton donor/acceptor but less effective as a sub-
strate binding group.

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