Articles

Site-Specificity of the Second-Site Suppressor Mutation of the Asp-285 → Asn Mutant of Metal−Tetracycline/H^+ Antiporter of *Escherichia coli* and the Effects of Amino Acid Substitutions at the First and Second Sites†

Yuichi Someya, Akiko Niwa, Tetsuo Sawai, and Akihito Yamaguchi*

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan

Received September 23, 1994⊗

ABSTRACT: The deleterious effect of the mutation of Asp-285 to Asn of the metal−tetracycline/H^+ antiporter (TetA) of *Escherichia coli* is suppressed by the second-site mutation of Ala-220 to an acidic amino acid residue (Yamaguchi, A., O'yauchi, R., Someya, Y., & Sawai, T. (1993) *J. Biol. Chem. 268*, 26990−26995). In this study, site-specific second-site Glu mutants as to 11 different positions around position 220 were established from the Asp-285 → Asn mutant TetA protein. Among them, only the Ala-220 → Glu mutant completely suppressed the deleterious effect of the Asp-285 → Asn mutation, indicating that position 220 is highly specific for the suppression. Although *E. coli* cells producing second-site Glu mutants as to positions 213, 216, 217, 218, 219, 221, 222, and 223 of the Asn-285 mutant were as tetracycline sensitive as the host cells without TetA, Gly-224 → Glu and Pro-227 → Glu second-site mutants of the Asn-285 mutant conferred low-level tetracycline resistance, the levels decreasing in this order. These two positions and position 220 are on the same side of putative transmembrane helix VII. The Phe-289 → Asp mutation, which is located at a position one-α-helical-turn downstream from Asp-285 in the same putative helix, IX, did not suppress the Asn-285 mutation. The introduction of an acidic residue at the second site was essential for suppression of the Asn-285 mutation because Lys-220 and Gln-220 second-site mutants of the Asn-285 mutant showed very low tetracycline resistance. Interestingly, the second-site Glu or Asp mutation at position 220 showed no suppression of the Asp-285 → Lys mutation, indicating that the positive charge at position 285 inhibits the compensatory function of the carboxylic acid side chain at position 220, probably due to an ionic interaction. These results revealed that the essential function of Asp-285 is specifically compensated for by a newly introduced carboxylic acid side chain around position 220 on one side of putative helix VII, which is sterically close to position 285 on putative helix IX.

The metal−tetracycline/H^+ antiporter of *Escherichia coli* (TetA) (Yamaguchi et al., 1990a) confers high-level bacterial resistance to tetracyclines (McMurry et al., 1980) and is predicted to have the 12 membrane-spanning structure (Eckert & Beck, 1989; Allard & Bertrand, 1992) common to most secondary transporters, including not only antiporters (Yoshida et al., 1990; Neyfakh et al., 1991) but also symporters (Foster et al., 1983; Botfield et al., 1992; Seol & Shatkin, 1993) and uniporters (Henderson, 1990). There are only four charged residues, Asp-15, Asp-84, Asp-285, and His-257, conserved in the putative transmembrane regions of TetA proteins of Gram-negative bacteria (Waters et al., 1983). All of these four transmembrane-charged residues play important roles in the metal−tetracycline/H^+ antiport function (Yamaguchi et al., 1991, 1992). Among them, Asp-285 is especially essential for the function because no mutants of Asp-285 except for the Glu-285 one confer tetracycline transport activity (Yamaguchi et al., 1992). The Glu-285 mutant retains considerable tetracycline resistance but shows very low tetracycline transport activity (Yamaguchi et al., 1992). Asn-285 and Lys-285 mutant cells were as sensitive as host cells producing no TetA against tetracyclines (Yamaguchi et al., 1992).

In the *lac* permease of *E. coli*, which is a well-studied example of bacterial symporters, some of the transmembrane-charged residues form charge-neutralizing pairs (King et al., 1991). The deleterious effect of the mutation of one charged residue of such a pair to a neutral residue is suppressed by a mutation of the counterpart to a neutral residue (Lee et al., 1992). In one case, an exchange mutant of a residue pair retained significant transport activity (Dunten et al., 1993). Such a pair of charged residues may have a gating function, opening and closing the entrance or exit of the substrate translocation pathway (Lee et al., 1992) in addition to maintaining the protein structure (Dunten et al., 1993).

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan and a Grant-in-Aid from the Tokyo Biochemical Research Foundation. Y.S. was supported by a predoctoral fellowship from the Japan Society for the Promotion of Science.

* To whom correspondence should be addressed.

‡ Supported by a predoctoral fellowship from the Japan Society for the Promotion of Science.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1994.

0006-2960/95/0434-7$09.00/0

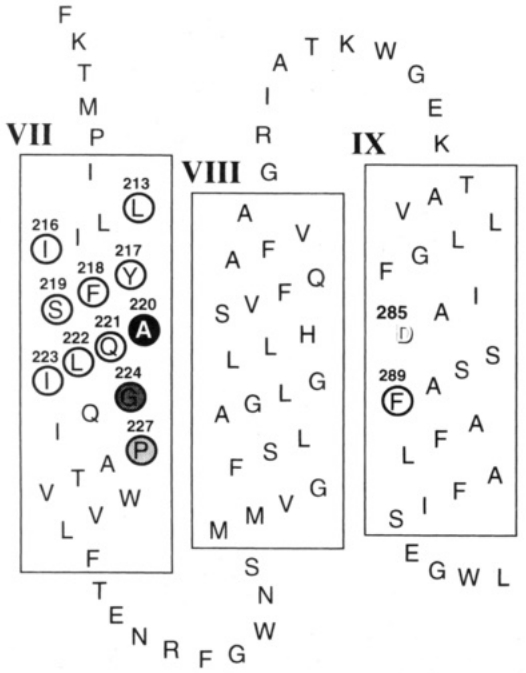

FIGURE 1: Schematic representation of the putative secondary structure of Tn10-TetA around transmembrane helices VII, VIII, and IX. Amino acid residues which underwent second-site mutations in this study are enclosed in circles, with the position numbers in the primary sequence. The residues of which Glu mutants showed any suppression to the D285N mutant are enclosed by shadowed circles. The primary mutation residue, Asp285, is denoted by an outlined letter.

TetA has unequal numbers of acidic and basic residues in its putative transmembrane region (Yamaguchi et al., 1992). Second-site suppressor mutants derived from the Asp-285 → Asn mutant of TetA were isolated (Yamaguchi et al., 1993). In contrast to *lac* permease, there was no suppressor mutant in which a charged residue was replaced by a neutral one. All the revertants showed the mutation of Ala-220 to Glu, that is, the introduction of a new carboxylate side chain can compensate for the function of the carboxyl side chain at position 285. Ala-220 is located in putative transmembrane α-helix VII, while Asp-285 is located at helix IX (Figure 1), indicating that these two helices are sterically close to each other or that these two positions are functionally equivalent. The Asp-220 mutant established by site-directed mutagenesis also suppressed the effect of the Asp-285 → Asn mutation. Therefore, the negative charge or carboxyl group at either position 285 or 220 must be essential for the transport function. Interestingly, although Asp is far better than Glu at position 285, the reverse is the case at position 220, suggesting that the active site requiring the carboxyl group is closer to position 285 than position 220. Since the K_m values of the tetracycline transport of the revertants were significantly higher than that of the wild-type, the carboxyl residue at position 285 or 220 is related to the substrate recognition in addition to its possible role in H^+ translocation (Yamaguchi et al., 1991).

The questions remaining are (1) whether the suppression of the Asp-285 → Asn mutation is specific to the mutation at position 220, (2) whether carboxylic acid at position 220 is essential for the suppression, and (3) whether a positive charge introduced at position 285 inhibits the suppression. In this study, we constructed 11 Glu-scanning second-site mutants around position 220 and a Phe-289 → Asp mutant established from the Asp-285 → Asn mutant TetA. In addition, an Ala-220 → Gln or Lys mutant from the Asp-285 → Asn mutant TetA and an Ala-220 → Glu or Asp mutant from the Asp-285 → Lys mutant TetA were established. These studies revealed that carboxylic acid at position 220 is highly essential for suppression of the Asp-285 → Asn mutation and the positive charge at position 285 inhibits the function of the carboxyl group at position 220.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]dCTP was purchased from Amersham. The Sequenase version 2.0 7-deaza-dGTP kit was obtained from U.S. Biochemicals. Restriction and modifying enzymes were obtained from Takara (Kyoto, Japan), TOYOBO (Osaka, Japan), Nippon Gene (Toyama, Japan), and New England Biolabs (Beverly, MA). A *Bgl*II linker was obtained form Takara. All other chemicals were of reagent grade and from commercial sources.

Bacterial Strains and Plasmids. All *E. coli* strains were derived from K-12. CJ236 (Kunkel, 1987) was used for the preparation of ssDNA containing deoxyuracil. BMH71-18 *mutS* (Kunkel, 1987) and TG1 (Taylor et al., 1985) were used for mutagenesis by the method of Kunkel (1987). TG1 was also used for transformation after the ligation reaction. W3104 (Yamamoto et al., 1981) were used for membrane preparation and measurement of tetracycline resistance.

pUC1183 was constructed by ligation of a *Bgl*II linker with a vector fragment from pUC118, which was purchased from TAKARA (Kyoto, Japan), after digestion with *Eco*RI followed by S1 nuclease digestion. A multicopy plasmid, pCT1183, was constructed by insertion of a 2.45-kg *Bgl*II-*Bam*HI fragment containing the entire *tetR* and *tetA* genes of transposon Tn10 from pCT1182 (Yamaguchi et al., 1992) into pUC1183. pCTD285N was constructed by changing the 870-bp *Eco*RI-*Bam*HI fragment from pLGD285N (Yamaguchi et al., 1992) with the corresponding region of pCT1183. pRHD285N was obtained by subcloning of the 1.36-kb *Eco*RV-*Bam*HI fragment of the mutant *tetA* gene prepared from pCTD285N into pUC118RV (Yamaguchi et al., 1992). pCTD285N and pRHD285N were used for mutagenesis. pLGT2 is a low-copy plasmid containing the *tetR* and *tetA* genes of transposon Tn10 (Yamaguchi et al., 1992).

Site-Directed Mutagenesis. Mutagenesis was performed by the method of Kunkel (1987) using pCTD285N or pRHD285N as a template. The mutagenic primers used were synthesized with a Cyclone Plus DNA/RNA synthesizer (MilliGen Biosearch Co.). Mutations Ile-216 → Glu (ATT → GAG) and Gln-221 → Glu (CAA → GAA) were introduced into pCTD285N. Other mutations were introduced into pRHD285N: Leu-213 → Glu (TTG → GAG), Tyr-217 → Glu (TAT → GAA), Phe-218 → Glu (TTT → GAA), Ser-219 → Glu (TCA → GAA), Ala-220 → Gln (GCG → CAG), Ala-220 → Lys (GCG → AAG), Leu-222 → Glu (TTG → GAG), Ile-223 → Glu (ATA → GAG), Gly 224 → Glu (GGC → GAA), Gly-224 → Asp (GGC → GAT), Pro-227 → Glu (CCC → GAA), and Phe-289 → Asp (TTT → GAT). Mutations were detected as the appearance of a newly introduced restriction site and then verified by DNA sequencing. The *Eco*RI-*Bam*HI fragment containing a double mutation was exchanged with the corresponding region of pLGT2 to reconstruct a low-copy double-mutant

plasmid. The low-copy single-mutant plasmids as to positions 213−227 were reconstructed by ligation of the 187-bp *Eco*RI-*Bgl*I fragment containing the mutations, the 680-bp *Bgl*I-*Bam*HI fragment in which the residue at position 285 was that of the wild-type, and vector fragment from pLGT2.

Measurement of Bacterial Resistance to Tetracycline. Bacterial resistance to tetracycline was measured by the agar dilution method (Yamaguchi et al., 1990c) and expressed as the minimum inhibitory concentration.

Preparation of Inverted Membrane Vesicles. Inverted membrane vesicles were prepared by passing cells through a French pressure cell at 5000 psi, suspended in 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl, and used for the tetracycline uptake assay as described previously (Yamaguchi et al., 1990a).

Transport Assay. Inverted membrane vesicles were energized with NADH, and then tetracycline uptake was assayed in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$, unless otherwise stated. The kinetic constants were determined in the presence of various concentrations of [^{3}H]tetracycline and 1 mM $CoCl_2$.

Immunoblot Analysis. For immunoblot analysis, membranes were prepared as follows. W3104 cells harboring the mutant plasmids were grown at 37 °C in 10 mL of the minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. At the middle of the logarithmic phase, 0.25 μg/mL heat-inactivated chlortetracycline was added and the *tetA* gene expression was induced for 2 h. Cells were harvested, resuspended in 13 mL of 50 mM MOPS-KOH (pH 7.0), and then sonicated at 4 °C. After removal of unbroken cells, the membrane fraction was collected by ultracentrifugation and suspended in the same buffer. Ten micrograms of membrane proteins was separated by SDS−polyacrylamide gel electrophoresis and then electrically blotted onto nitrocellulose membranes. The TetA proteins were visualized with anti-carboxyl-terminal peptide antiserum and alkaline phosphatase-linked goat anti-rabbit IgG, as described previously (Yamaguchi et al., 1990b).

RESULTS

Glu-Scanning Mutagenesis of the Asp-285 → Asn Mutant TetA Protein. In order to determine whether the suppression of the D285N mutation by a second-site mutation is specific to position 220, we establish 11 Glu-scanning second-site mutants as to Leu-213, Ile-216, Tyr-217, Phe-218, Ser-219, Ala-220, Gln-221, Leu-222, Ile-223, Gly-224, and Pro-227, on the basis of the D285N mutant TetA protein, by site-directed mutagenesis using synthetic oligonucleotides. All mutations were verified by DNA sequencing. Low-copy-number mutant pLG-plasmids were introduced into *E. coli* W3104 cells. Inverted membrane vesicles were prepared from the cells after induction of *tetA* gene expression, and the TetA protein was detected by Western Blotting using C-terminal-specific antiserum. The amounts of mutant TetA proteins were about the same as that of the wild-type TetA protein (data not shown).

The tetracycline resistance levels of *E. coli* W3104 cells harboring these second-site mutant plasmids were measured as the minimum inhibitory concentration (MIC) of tetracycline (Table 1). MIC for D285N mutant cells was 0.8 μg/mL, which was the same as that for the host cells without a plasmid. The second-site A220E mutation restored the resistance of the D285N mutant to the same level as in the wild-type (200 μg/mL). Among the other 10 second-site mutants, only the G224E/D285N and P227E/D285N ones showed significant tetracycline resistance (12.5 and 3.1 μg/mL, respectively). MICs for the other eight second-site Glu mutants were 0.8 μg/mL, which was exactly the same as the host cell level. Gly-224 and Pro-227 are located at positions one and two α helical turns downstream, respectively, from Ala-220 in putative transmembrane helix VII (Figure 1), indicating the presence of a suppression potential vector, that is, the suppression potential decreased with increasing distance from position 220, along one side of helix VII. This may be reasonable, for example, helix VII is parallel to the substrat translocation pathway. On the other hand, the Glu mutations of Ile-216 and Leu-213, which are located one and two α helical turns upstream, respectively, from Ala-220, showed no suppression (Table 1). Thus, the appropriate depth of the second-site Glu location in the membrane must be essential for the suppression function.

Table 1: Tetracycline Resistance Levels of *E. coli* W3104 Cells Harboring Mutant Plasmids[a]

second mutation	first mutation, minimum inhibitory concentration (μg/mL)	
	D285N (Asp-285 → Asn)	wild-type (Asp-285)
none	0.8	200
L213E (Leu-213 → Glu)	0.8	50
I216E (Ile-216 → Glu)	0.8	50
Y217E (Tyr-217 → Glu)	0.8	100
F218E (Phe-218 → Glu)	0.8	100
S219E (Ser-219 → Glu)	0.8	100
A220E (Ala-200 → Glu)[b]	200	100
A220D (Ala-220 → Asp)[b]	50	50
Q221E (Gln-221 → Glu)	0.8	100
L222E (Leu-222 → Glu)	0.8	25
I223E (Ile-223 → Glu)	0.8	100
G224E (Gly-224 → Glu)	12.5	25
G224D (Gly-224 → Asp)	12.5	100
P227E (Pro-227 → Glu)	3.1	3.1
F289D (Phe-289 → Asp)	0.8	NC[c]

[a] The resistance levels are expressed as minimum inhibitory concentrations. [b] Data cited from Yamaguchi et al. (1993). [c] NC, not constructed.

As to Ala-220 and Gly-224, second-site Asp mutants were also established. At position 220, the Asp mutation could suppress the D285N mutation; however, the efficiency was less than in the case of the Glu-220 mutation, as reported in our previous paper (Yamaguchi et al., 1993). On the other hand, there was no significant difference between the resistance levels of the G224E/D285N and G224D/D285N mutants (Table 1), probably due to their resistances being too low to detect a significant difference.

Tetracycline Transport Activity of the Second-Site Suppressor Mutants. [^{3}H]Tetracycline transport activity of the second-site Glu-scanning mutants which conferred the tetracycline resistance was measured (Figure 2). Inverted membrane vesicles containing these second-site mutant TetA proteins showed no detectable tetracycline uptake in the presence of 10 μM tetracycline except for the A220E/D285N mutant (Figure 2). In our previous paper (Yamaguchi et al., 1993) we reported that the K_m value of the second-site revertant derived from D285N was about 5-fold higher than that of the wild-type. However, significant transport activity of the second-site mutants, except for mutants as to position

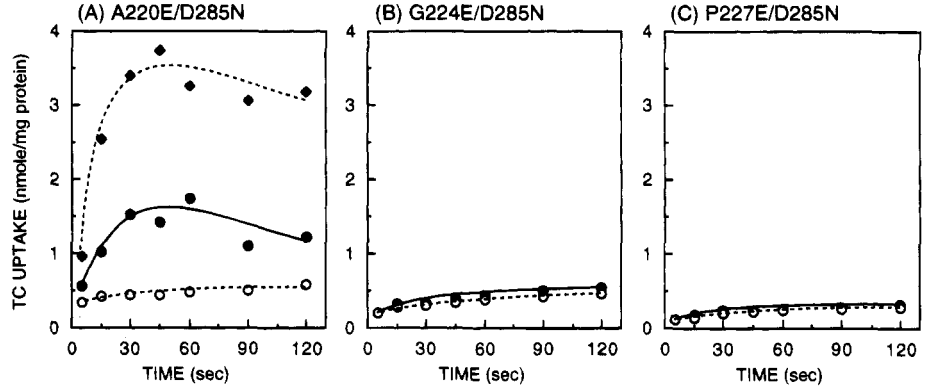

FIGURE 2: Tetracycline (TC) uptake by inverted membrane vesicles prepared from the A220E/D285N, G224E/D285N, and P227E/D285N double-mutant strains. Plain lines with closed circles and dashed lines with open circles indicate the uptake by the mutant vesicles in the presence and absence of NADH, respectively. The dashed line with diamonds indicates the uptake by the energized wild-type vesicles.

Table 2: Tetracycline Resistance Levels of *E. coli* W3104 Cells Harboring Mutant Plasmids[a]

mutants	amino acids at positions: 220	285	minimum inhibitory concentration (μg/mL)
wild-type	Ala	Asp	200
A220K	Lys	Asp	25
A220Q	Gln	Asp	50
D285N	Ala	Asn	0.8
A220K/D285N	Lys	Asn	4.7
A220Q/D285N	Gln	Asn	3.1
D285K	Ala	Lys	9.5
A220E/D285K	Glu	Lys	4.7
A220D/D285K	Asp	Lys	3.1

[a] The resistance levels are expressed as minimum inhibitory concentrations.

220, could not be detected, even in the presence of 100 μM tetracycline (data not shown).

Effect of the Replacement of Phe-289 with Asp on the D285N Mutant TetA Protein. Phe-289 is located one helical turn downstream from Asp-285 in the same putative α-helix, IX (Figure 1). A second-site mutation to Asp was introduced at Phe-289 of the D285N TetA protein by site-directed mutagenesis. The mutation was verified by DNA sequencing, and the normal production of the mutant TetA protein was confirmed by Western blotting using C-terminal-specific antiserum (data not shown). The tetracycline resistance level of the D285N/F289D mutant cells was 0.8 μg/mL, which was the same as that of tetracycline-sensitive host cells (Table 1). Therefore, it is clear that the essential carboxylic acid side chain must be at position 285 in helix IX, whereas there is some flexibility as to the positioning of the carboxyl group in helix VII.

A Carboxyl Group Is Critical for the Suppression. The results described above and in our previous paper (Yamaguchi et al., 1993) indicate that a properly-positioned acidic residue can compensate for the function of Asp-285. The second question is whether or not an acidic residue is essential for the function. Thus, Ala-220 of the D285N mutant TetA protein was replaced with a neutral residue, Gln, or a basic residue, Lys, by site-directed mutagenesis. The DNA sequences of the resultant double mutants were also verified, and the normal production of the mutant TetA proteins was confirmed. As judged from the MIC values (Tables 2), second-site mutations of Ala-220 to Gln or Lys hardly suppressed the first mutation at Asp-285 to Asn. The MICs for the A220Q/D285N and A220K/D285N mutants were 3.1 and 4.7 μg/mL, respectively, which were far lower than those for the A220E/D285N and A220D/D285N ones. Thus, an acidic residue is essential for the function. It is not clear why the MICs of the A220Q/D285N and A220K/D285N double mutants were a little higher than those of the D285N single mutant and the tetracycline-sensitive host cell (0.8 μg/mL). Inverted membrane vesicles containing two double mutants showed no detectable tetracycline transport activity (data not shown).

A Positively-Charged Residue Introduced at Position 285 Inhibits the Function of the Acidic Residue at Position 220. There are two possibilities for the suppression: (1) An acidic residue introduced at position 220 opens a new tetracycline transport pathway independent of the pathway conferred by Asp-285 and (2) the positions of the side chains of Asp-285 and Ala-220 are spatially close to each other, and the carboxylic acid group at position 220 can compensate for the function of that at position 285 in the same pathway. If the former is the case, the kind of residue replacing Asp-285 must hardly affect the suppression function of an acidic residue introduced at position 220. In order to determine which is the case, second-site A220E and A220D mutants were established from the D285K mutant TetA protein by site-directed mutagenesis. The DNA sequences were verified, and the normal production of the mutant TetA proteins was confirmed. To our surprise, these second-site mutations showed no increase in the drug resistance in comparison with D285K single-mutant cells (Table 2). D285K conferred very low tetracycline resistance (9.5 μg/mL); however, the A220E/D285K and A220D/D285K double mutants showed resistance levels lower than that of the D285K single mutant (4.7 and 3.1 μg/mL, respectively). Although it is not obvious why the D285K mutant retained a low-level resistance, it is clear that a positively-charged residue at position 285 interferes with the function of the acidic residue introduced at position 220. There must be some ionic interactions between the two positions; thus, the latter possibility is the case.

Effects of Single Mutations Introduced at Positions around Position 220. The effects of the Glu-scanning single mutations on the drug resistance level were determined. L213E, I216E, Y217E, F218E, S219E, A220E, Q221E, L222E, I223E, G224E, and P227E single mutants were constructed, as described under Experimental Procedures. All the mutants except for the P227E one retained significantly

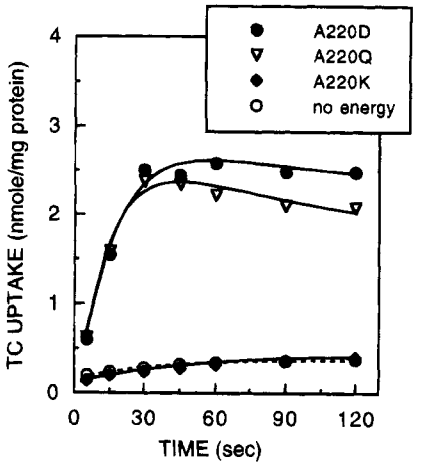

FIGURE 3: Tetracycline (TC) uptake by inverted membrane vesicles prepared from the A220D, A220K, and A220Q single-mutant strains. Inverted vesicles were energized with NADH and then exposed to the assay solution containing 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. The dashed line indicates the background uptake in the absence of NADH.

high tetracycline resistance, although the levels were 2- or 4-fold lower than that of the wild-type (Table 1). These results indicated that there is no essential residue around position 220 in helix VII except for Pro-227. The drastic decrease in the drug resistance on substitution of Pro-227 with Glu may be due to a conformational distortion. It should be noted that the introduction of a net negative charge in such a hydrophobic region did not cause crucial damage to the protein folding, suggesting that the formation of charge-neutralizing pairs in the transmembrane region is not always necessary for membrane protein folding. It is possible that these acidic residues are present in the transmembrane region as a protonated neutral form.

The introduction of a basic residue or neutral polar residue at position 220 of the wild-type TetA protein also caused a decrease in the drug resistance (Table 2). The A220Q mutant showed a similar phenotype to that of the A220D mutant, that is, the MIC of tetracycline was 50 μg/mL, and the transport activity of inverted membrane vesicles containing A220Q mutant TetA was somewhat less than that of the wild-type and similar to that of the A220D vesicles (Figure 3). The K_m value for the tetracycline transport of the A220Q mutant was 20 μM, which is slightly lower than that of the wild-type (36 μM) and comparable to that of the A220D mutant (16 μM). In contrast, the vesicles containing the A220K mutant showed almost no tetracycline transport activity (Figure 3), although cells producing the A220K mutant showed a low but significant level of tetracycline resistance (25 μg/mL). Thus, the introduction of a positively-charged residue at position 220 caused more severe interference with the transport process, probably through an ionic interaction with the negative charge of Asp-285.

DISCUSSION

The present work revealed that: (1) Only an acidic residue introduced at position 220 can fully compensate for the function of Asp-285. An acidic residue at no other position fully substituted for Asp-285, whereas an acidic residue introduced at positions one- or two-helical-turns downstream from position 220 can partially substitute for it, the degree of suppression decreasing with increasing distance from position 220. Such flexibility as to the position of the functional carboxyl group in the transmembrane helix was also observed for Glu-113 of bovine rhodopsin (Zhukovsky et al., 1992; Zvyaga et al., 1993). (2) The mutation of Ala220 to a basic or neutral residue does not suppress the Asp-285 → Asn mutation. (3) The Asp-285 → Lys mutation is not suppressed by a second-site mutation at position 220.

These results have many implications as to the relationship between the residues at positions 285 and 220 and the roles of these residues in the protein function. At first, the vertical arrangement of the second-site suppressive positions suggests the presence of a pathway along putative helix VII. The inhibition of the suppression by a basic residue introduced at position 285 suggests the occurrence of an ionic interaction between residues at positions 220 and 285; thus, the suppression is unlikely to be due to the formation of an alternative new pathway but due to the compensation for the function of Asp-285 by Glu-220 or Asp-220 in the same "active site". The "active site" requiring the carboxyl group may be closer to position 285 than position 220, since (1) a carboxyl group with a longer side chain is more effective than a shorter one at position 220, while the reverse is the case at position 285, and (2) the degree of the inhibitory effect of a positively-charged group at position 220 is lower than that in the case of position 285. The lack of suppression by a mutation of the residue one-helical-turn downstream from Asp-285 may also be explained in terms of the closeness of the "active site" to helix IX, that is, the carboxyl group at position 289 cannot be arranged in an appropriate angles as to the "active site".

Recently, Guay et al. (1994) reported that Trp-231 in the TetA protein was involved in the substrate recognition. It is interesting that Trp-231 is a residue three-herical-turns downstream from position 220 on the same side of putative transmembrane helix VII, supporting our idea presented in this paper that the substrate translocation pathway may be present along helix VII.

It is not obvious why the D285K mutant showed higher drug resistance than the D285N mutant. D285C and D285A mutants also showed a MIC value as low as the D285N mutant (data not shown). Thus, it is clear that a charged residue at position 285 is essential for the TetA function. The remaining of the low-level activity in Asp → Lys mutants was also observed in the mutants of Asp-15 and Asp-84 (Yamaguchi et al., 1992). This phenomenon may be related to the protonation/deprotonation ability of the residues in the substrate translocation pathway.

REFERENCES

Allard, J. D., & Bertrand, K. P. (1982) *J. Biol. Chem. 267*, 17809−17819.

Botfield, M. C., Naguchi, K., Tsuchiya, T., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 1818−1822.

Dunten, R. L., Sahin-toth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 3139−3145.

Eckert, B., & Beck, C. F. (1989) *J. Biol. Chem. 264*, 11663−11670.

Foster, D., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31−34.

Guay, G. G., Tuckman, M., & Rothstein, D. M. (1994) *Antimicrob. Agents Chemother. 38*, 857−860.

Henderson, P. J. F. (1990) *J. Bioenerg. Biomembr. 22*, 525−569.

King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177−186.

Kunkel, T. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Lee, J., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−20764.

McMurry, L., Petrucci, R. E., Jr., & Levy, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3974−3977.

Neyfakh, A. A., Bidenko, V. E., & Chen, L. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4781−4785.

Seol, W., & Shatkin, A. J. (1993) *J. Bacteriol. 175*, 565−567.

Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764−8785.

Waters, S. H., Rogowsky, P., Grinsted, J., Altenbuchner, J., & Schmitt, R. (1983) *Nucleic Acids Res. 11*, 6089−6105.

Yamaguchi, A., Udagawa, T., & Sawai, T. (1990a) *J. Biol. Chem. 265*, 4809−4813.

Yamaguchi, A., Adachi, K., & Sawai, T. (1990b) *FEBS Lett. 265*, 17−19.

Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T., & Sawai, T. (1990c) *J. Biol. Chem. 265*, 15525−15530.

Yamaguchi, A., Adachi, K., Akasaka, T., Ono, N., & Sawai, T. (1991) *J. Biol. Chem. 266*, 6045−6051.

Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., & Sawai, T. (1992) *J. Biol. Chem. 267*, 7490−7498.

Yamaguchi, A., O'yauchi, R., Someya, Y., Akasaka, T., & Sawai, T. (1993) *J. Biol. Chem. 268*, 26990−26995.

Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y., & Yamagishi, S. (1981) *J. Bacteriol. 145*, 808−813.

Yoshida, H., Bogaki, M., Nakamura, S., Ubukata, K., & Konno, M. (1990) *J. Bacteriol. 172*, 6942−6949.

Zhukovsky, E. A., Robinson, P. R., & Oprian, D. D. (1992) *Biochemistry 31*, 10400−10405.

Zvyaga, T. A., Min, K. C., Beck, M., & Sakmar, T. P. (1993) *J. Biol. Chem. 268*, 4661−4667.

BI942256L