Cysteine-scanning Mutagenesis of Transmembrane Segments 4 and 5 of the Tn10-encoded Metal-Tetracycline/H⁺ Antiporter Reveals a Permeability Barrier in the Middle of a Transmembrane Water-filled Channel*

Received for publication, December 29, 1999, and in revised form, April 21, 2000
Published, JBC Papers in Press, May 4, 2000, DOI 10.1074/jbc.M910354199

Shinobu Iwaki‡‡, Norihsa Tamura‡‡, Tomomi Kimura-Someya‡‡, Shigeyuki Nada¶, and Akihito Yamaguchi‡‡‡¶¶ From the Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki-shi, Osaka 567-0047, the Faculty of Pharmaceutical Science, Osaka University, Suita, Osaka 565-0871, and CREST, Japan Science and Technology Corp., Osaka 567-0047, Japan

Cysteine-scanning mutants as to putative transmembrane segments 4 and 5 and the flanking regions of Tn10-encoded metal-tetracycline/H⁺ antiporter (TetA(B)) were constructed. All mutants were normally expressed. Among the 57 mutants (L99C to I155C), nine conserved arginine-, aspartate-, and glycine-replaced ones exhibited greatly reduced tetracycline resistance and almost no transport activity, and five conserved glycine- and proline-replaced mutants exhibited greatly reduced tetracycline transport activity in inverted membrane vesicles despite their high or moderate drug resistance. All other cysteine-scanning mutants retained normal drug resistance and normal tetracycline transport activity except for the L142C and I143C mutants. The transmembrane (TM) regions TM4 and TM5 were determined to comprise 20 amino acid residues, Leu-99 to Ile-118, and 17 amino acid residues, Ala-136 to Ala-152, respectively, on the basis of N-[14C]ethylmaleimide (NEM) reactivity. The NEM reactivity patterns of the TM4 and TM5 mutants were quite different from each other. TM4 could be divided into two halves, that is, a NEM nonreactive periplasmic half and a periodically reactive cytoplasmic half, indicating that TM4 is tilted toward a water-filled transmembrane channel and that only its cytoplasmic half faces the channel. On the other hand, NEM-reactive mutations were observed periodically (every two residues) along the whole length of TM5. A permeability barrier for a membrane-impermeable sulfhydryl reagent, 4-acetamido-4- maleimidylstibene-2,2'-disulfonic acid, was present in the middle of TM5 between Leu-142 and Gly-145, whereas all the NEM-reactive mutants as to TM4 were not accessible to 4-acetamido-4- maleimidylstibene-2,2'-disulfonic acid, indicating that the channel-facing side of TM4 is located inside the permeability barrier. Tetracycline protected the G141C mutant from the NEM binding, whereas the other mutants in TM4 and TM5 were not protected by tetracycline.

* This work was supported by grants-in-aid from the Ministry of Education and the Ministry of Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Postdoctoral research fellow of the Japan Society for the Promotion of Science.

¶ To whom correspondence should be addressed. Tel.: 81-6-6879-8545; Fax: 81-6-6879-8549; E-mail: akihito@sanken.osaka-u.ac.jp.

The transposon Tn10-encoded metal-tetracycline/H⁺ antiporter (TetA(B))1 was the earliest found bacterial drug exporter (2), and it has been studied as a paradigm of bacterial drug efflux proteins (3). It belongs to a major facilitator superfamily (MFS) (4), and its 12-membrane-spanning structure (Fig. 1) was established by site-directed competitive chemical modification of cysteine-replaced mutants of a cysteine-free TetA(B) (5). We reported that putative transmembrane helices 3 (6), 6 (7), and 9 (8) are totally embedded in a highly hydrophobic environment because none of the cysteine-scanning mutants as to these transmembrane helices reacted with a maleimide derivative, N-ethylmaleimide (NEM), whereas cysteine mutants as to putative loop regions are generally highly reactive with NEM, except for those that are mutants as to a small number of nonreactive positions (9). Similar transmembrane segments totally embedded in a hydrophobic interior are known for the erythrocyte anion exchanger (10) and for the bacterial small multidrug efflux protein EmR (11). On the other hand, some transmembrane cysteine mutants of MFS transporters, such as lactose permease (12–18) and UhpT (19), are certainly inactivated by SH reagents, indicating that in these transporters, SH reagents can possibly gain access to some of the residues located in the interior of the transmembrane region.

Recently, we found that some cysteine-scanning mutants as to putative transmembrane helix 2 of TetA(B) are reactive with NEM (20). NEM-reactive and nonreactive positions were periodically observed in TM2, indicating that one side of the transmembrane helix faces a water-filled transmembrane channel. It is impossible that such a water-filled channel is composed of only one amphiphilic helix. Therefore, at least some other counterparts should be present in the transmembrane region of TetA(B). Considering the amphiphilic nature of the metal-tetracycline chelation complex (1), the water-filled channel may be at least a part of the substrate translocation pathway. The fact that the NEM-reactive cysteine-scanning mutants as to TM2 were all inactivated by NEM except for P59C, whereas the mutants as to the loop region were generally less affected by NEM, supporting the idea that the NEM-reactive positions in TM2 may face a substrate translocation pathway (20).

In this study, TM4, TM5, and the flanking loop regions were

1 The abbreviations used are: TetA(B), transposon Tn10-encoded metal-tetracycline/H⁺ antiporter; AMS, 4-acetamido-4-maleimidylstibene-2,2'-disulfonic acid; MFS, major facilitator superfamily; MIC, minimum inhibitory concentration; MOPS, 4-morpholinepropanesulfonic acid; NEM, N-ethylmaleimide; TM, transmembrane segment.
subjected to cysteine-scanning mutagenesis and chemical modification. The TM4 region shares features with MFS members in that arginine and glycine residues are conserved (Fig. 2), suggesting a common role of this region in the structure and function of MFS transporters. On the other hand, the TM5 region contains a glycine-rich motif, 

\[
\text{GG150, which is a characteristic sequence conserved in bacterial drug efflux proteins. (Fig. 2) (21–24), suggesting that TM5 possibly plays an important role in the drug recognition or translocation function. In previous topological studies, we found that mutants S92C to Y98C (6) and S156C to H158C (7) were reactive with NEM and thus were located in periplasmic loop 3–4 and loop 5–6, respectively. Thus, in this study, we analyzed the site-directed chemical modification of 57 cysteine-scanning mutants, L99C to I155C. As a result, we found that TM5 also faces a transmembrane water-filled channel, as does TM2, and that the channel has a permeability barrier for hydrophilic compounds in the middle of the membrane, which is necessary to avoid uncoupling. On the other hand, only the cytoplasmic half of TM4 faces the channel; that is, the transmembrane segment is tilted toward the channel. The blocking of NEM binding to the G141C mutant by tetracycline suggested that TM4 comprises a part of the substrate binding site or a site sensitive to a substrate-induced conformational change.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-[Ethyl-1-14C]maleimide (1.5 GBq/mmol) and [3H]tetracycline (3.7 GBq/mmol) were purchased from NEN Life Science Products. All other materials were of reagent grade and were obtained from commercial sources.

**Site-directed Mutagenesis—**Cysteine-scanning mutants were constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (25) using synthetic oligonucleotides. For the mutagenesis, plasmid pCT1183 (26), which carries the 2.45 kb Tn10 amber gene fragments, was used as a template. The I118C mutant was constructed by the oligonucleotide-directed dual amber mutagenesis, plasmid pLGC377A, which encodes a cysteine-free mutant TetA(B) (8), and then used for tetA(B) gene expression.

**Prevention of [14C]NEM Binding with 4-Acetamido-4-stilbene-2,2’-disulfonic acid (AMS)—**Five μl of 100 μM AMS (final concentration, 5 μM) or the same volume of distilled water was added to 100 μl of a suspension of E. coli W3104 cells expressing mutant TetA(B) proteins. The [14C]NEM binding experiment was performed as described previously (9). In brief, a membrane suspension prepared by brief sonication of Escherichia coli W3104 cells carrying pLGC377A or one of its derivatives was incubated with 0.5 μM [14C]NEM in 50 mM MOPS-KOH buffer (pH 7.0) including 0.1 mM KCl for 5 min at 30 °C. The membrane protein was solubilized with 1% Triton X-100 and 0.1% SDS in phosphate-buffered saline containing 5 mM unlabeled NEM, and then TetA(B) proteins were immunoprecipitated with anti-TetA(B) C-terminal peptide antiserum (30) and Pansorbin Staphylococcus aureus cells (31). The TetA(B) protein was separated by SDS-polyacylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. The dried gel was exposed to an imaging plate for visualization with a BAS-1000 Bioimaging Analyzer (Fuji Film Co., Tokyo).

**Assaying of the Reaction of N-[14C]Ethylmaleimide with TetA(B) Proteins—**The [14C]NEM binding experiment was performed as described previously (9). In brief, a membrane suspension prepared by brief sonication of Escherichia coli W3104 cells expressing mutant TetA(B) proteins was incubated with 0.5 μM [14C]NEM in 50 mM MOPS-KOH buffer (pH 7.0) including 0.1 mM KCl for 5 min at 30 °C. The membrane protein was solubilized with 1% Triton X-100 and 0.1% SDS in phosphate-buffered saline containing 5 mM unlabeled NEM, and then TetA(B) proteins were immunoprecipitated with anti-TetA(B) C-terminal peptide antiserum (30) and Pansorbin Staphylococcus aureus cells (31). The TetA(B) protein was separated by SDS-polyacylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. The dried gel was exposed to an imaging plate for visualization with a BAS-1000 Bioimaging Analyzer (Fuji Film Co., Tokyo).

**Determination of Tetracycline Resistance—**Tetracycline resistance was determined by means of a 2-fold agar dilution method as described previously (29) and expressed as the minimum inhibitory concentration.

**FIG. 1. Putative membrane topology of TetA(B).** This structure was constructed on the basis of our previous results of site-directed chemical modification of Cys mutants of TetA(B) (5). Putative transmembrane segments are enclosed in squares. Gray squares denote the transmembrane segments of which the regions were precisely determined by site-directed chemical modification of cysteine-scanning mutants (6, 7, 8, 20). Residues of the cysteine-scanning mutants analyzed in this study are depicted as circled boldface letters.
**RESULTS**

**Drug Resistance Levels and Tetracycline Transport Activities of Cysteine-scanning Mutants**—Of the 57 cysteine-scanning mutants, mutants L99C to L103C (32), D120C (5), and R127C (32) were constructed previously. The other 50 cysteine-scanning mutants were constructed in this study. First of all, the tetracycline resistance levels of these mutants were measured by means of a 2-fold agar dilution method (29) and expressed as the minimum inhibitory concentration (MIC). As shown in Table 1, the MIC values of *E. coli* W3104 cells carrying pLG2 encoding wild-type TetA(B) and the host cells without the plasmid were 200 and 1.6 μg/ml, respectively. Out of the 57 cysteine-scanning mutants, R101C, D120C, R127C, G135C, G139C, and G141C completely lost the drug resistance (MIC, 1.6 μg/ml). The possible roles of Arg-101 and Asp-120 in the tetracycline transport function were reported previously (32, 33). The G105C, G111C, G132C, I143C, and G150C mutants exhibited very low resistance (3.1, 6.3, or 12.5 μg/ml). The G145C and P146C mutants exhibited moderate resistance (25 and 50 μg/ml, respectively). The other 44 mutants retained the wild-type resistance level (100 or 200 μg/ml). Although Gly-100, Gly-108, and Gly-149 are conserved in MFS transporters and/or bacterial drug exporters (Fig. 2), their mutation did not affect the drug resistance.

Tetracycline transport activity was measured in inverted membrane vesicles as proton motive force-driven uptake. As shown in Fig. 3, four resistanceless mutants, R127C, G135C, G139C, and G141C, had completely lost the tetracycline transport activity. As for the other two resistanceless mutants, R101C and D120C, R101C showed no transport activity (32), and the apparently contradictory behavior of the D120C mutant was discussed in detail previously (33). With respect to the five low resistance mutants, four of them, G105C, G111C, G132C, and G150C, exhibited very low transport activity, consistent with their resistance level, whereas the I143C mutant retained moderate transport activity, i.e. about 40% of the wild-type level. On the other hand, the G145C and P146C mutants exhibited no transport activity despite their moderate drug resistance. In addition, the G100C, L142C, and G149C mutants showed greatly reduced transport activity despite their wild-type level drug resistance (200 μg/ml). These high or moderate resistance mutants with low transport activity seem to be unstable and partially denatured during the preparation of membrane vesicles, except for the L142C mutant, which is a low affinity and high V_{max}-type mutant (data not shown). In brief, all of the conserved glycine and proline residues are functionally or structurally important except for Gly-108. In addition to conserved glycine, proline, and charged residues, Leu-142 and Ile-143 participate in the function and/or maintenance of the structure of TetAB.

[4]C/NEM Binding to Cysteine-scanning Mutants—A maleimide derivative reacts with a deprotonated form of a sulfhydryl group, and this reaction requires a water molecule as a proton acceptor (20); thus, the degree of NEM binding will depend on the presence of a water molecule. Therefore, a region embedded in a hydrophobic environment can be distinguished on the basis of the NEM reactivity of a cysteine mutant (6, 7, 8, 33). The G100C, L142C, and G149C mutants showed greatly reduced transport activity despite their wild-type level drug resistance (200 μg/ml). These high or moderate resistance mutants with low transport activity seem to be unstable and partially denatured during the preparation of membrane vesicles, except for the L142C mutant, which is a low affinity and high V_{max}-type mutant (data not shown). In brief, all of the conserved glycine and proline residues are functionally or structurally important except for Gly-108. In addition to conserved glycine, proline, and charged residues, Leu-142 and Ile-143 participate in the function and/or maintenance of the structure of TetAB.
than 50% of the S123C mutant (Fig. 4B). We previously reported that Cys mutants S92C to Y98C were all reactive with NEM (6). Therefore, it can be concluded that the former 20 residues, Leu-99 to Ile-118, and the latter 17 residues, Ala-136 to Ala-152, comprise TM4 and TM5, respectively.

In the former 20-amino acid transmembrane region, Cys mutants as to the first 10 residues, Leu-99 to Gly-108, were all NEM nonreactive or low reactive. As shown in Fig. 4B, most of the Cys mutants as to the first 10-amino acid region showed 10% or less reactivity with NEM compared with the S123C mutant. The reason why the S123C mutant was chosen as a control was that NEM reactivity of the mutant was about the S123C level, except for Ala-136, of which the reactivity was around 25–30%. Thus, the first half of TM4 seems to be totally embedded in the hydrophobic environment. On the other hand, as to the latter 10 amino acid residues, Leu-109 to Ala-119, NEM binding was not affected by AMS in intact cells, whereas mutants as to upstream from L142C were totally nonreactive (Fig. 5), indicating that the latter half of TM4 faces a water-filled cavity or channel. This labeling pattern with NEM was different from the pattern in the case of TM2 (20) and indicates that TM4 is tilted away from the water-filled channel if it crosses through the membrane. This conclusion is contradicted by the facts that three mutants, R101C, G105C, and G111C, were inactive and an additional one, G100C, lost the transport activity during the membrane preparation. However, it should be noted that there was no relationship between NEM reactivity and their residual activity, i.e., R101C and G105C were nonreactive, whereas G111C was highly reactive with NEM. Thus, it is not likely that the totally nonreactive nature of the first half of TM4 is due to the conformational change caused by a mutation of a structurally essential residue.

The NEM binding pattern of the TM5 region was different from that of the TM4 region. Between Ala-136 and Ala-152, Cys mutations highly reactive with NEM (more than 50% of the S123C level) appeared almost every two residues (Fig. 4B). The other mutants showed very low reactivity, i.e., less than 15% of the S123C level, except for Ala-136, of which the reactivity was about 40%. The high reactive mutations were also located on one side of the α-helical wheel projection of TM5 (Fig. 5), indicating that TM5 faces a water-filled channel along its whole length. The 17-amino acid length is shorter than that required for a polypeptide chain to cross the membrane as an α-helical form. Because the NEM reactivities of W133C and G132C (50–60%) were somewhat lower than those of their flanking mutants, the TM5 helix may extend to Gly-132. As to TM5, some of the NEM-reactive mutants lost the drug resistance (G139C, G141C, and G150C), or lost the transport activity (G145C and P146C). However, because the other NEM-reactive mutants, A136C, F138C, and G149C, showed wild-type drug resistance and retained the transport activity, the high NEM reactivity of TM5 mutants was not likely to be due to mutational denaturation.

Accessibility of a Membrane-impermeable Maleimide Derivative, AMS, to Cysteine-scanning Mutants—The results of an NEM binding experiment on cysteine-scanning mutants as to TM5 as well as TM2 strongly indicated the presence of a water-filled transmembrane channel. However, if such a channel crosses the membrane, water molecules with protons would freely permeate through the channel, and as a result, the membrane would be unfavorably uncoupled. A neck or permeability barrier for solute molecules must be present in the water-filled channel to avoid uncoupling. In order to detect such a permeability barrier, we investigated the accessibility of a membrane-impermeable maleimide derivative, AMS (34), to NEM-reactive cysteine residues in the transmembrane region, as determined from the blocking of [14C]NEM binding with AMS.

Fig. 6 shows the effect of AMS pretreatment of intact cells and sonicated membranes on the NEM binding to NEM-reactive Cys mutants as to TM4 (Fig. 6A) and TM5 (Fig. 6B). As to TM4, NEM binding was not affected by AMS in intact cells, whereas the NEM binding was almost completely blocked by AMS in sonicated membranes (Fig. 6A), indicating that the NEM-reactive positions in TM4 were located inside the permeability barrier. Among the mutants, the A109C one was not reactive with NEM in intact cells despite its high reactivity in sonicated membranes.

As to TM5, NEM binding to the NEM-reactive mutants as to downstream from P146C was completely blocked and the binding to the G145C mutant was largely prevented by AMS in intact cells, whereas mutants as to upstream from L142C were not affected by AMS, except for the G141C one. These results indicated that the permeability barrier for AMS is present between Leu-142 and Gly-145. Although the G145C, P146C, and G150C mutations are located in the putative transmembrane region, AMS molecules had access to cysteine residues at these positions. Thus, AMS molecules can penetrate to the middle of the transmembrane water-filled channel. On the other hand, with respect to sonicated membranes, the NEM
binding of all the NEM-reactive Cys mutants as to TM5 was significantly prevented by AMS, indicating that the water-filled channel is accessible from both sides of the membrane and the thickness of the permeability barrier is within one turn of the α-helix.

The prevention of the NEM binding to some of Cys mutants, G141C, L142C, G145C, P146C, G149C, and G150C, by AMS was not complete even in sonicated membranes (Fig. 6B). We previously found in the case of the S65C and L97C mutants (5) that a small portion of sonicated membranes was sealed, and
Thus, AMS did not completely prevent the NEM binding to these mutants in sonicated membranes despite their locations in hydrophilic loop regions. Although it is not clear why the degree of the sealing of sonicated membranes depends on the mutations, the incompleteness of the AMS prevention of the NEM binding in sonicated membranes seems to be due to
the presence of sealed vesicles. However, considering the fact that the mutations exhibiting incomplete accessibility of AMS in sonicated membranes are mainly located in the middle of TM5, the possibility that the small structural distortion during membrane preparation may affect the accessibility of AMS cannot be excluded.

The reason why the G141C and A109C mutants were inert as to NEM in intact cells despite their high reactivity in sonicated membranes is not clear. Because the A109C mutant exhibited normal drug resistance and transport activity in inverted vesicles whereas the G141C mutant was inactive, the alteration in the NEM reactivity was not due to denaturation during preparation of the membranes. It should be noted that Ala-109 and Gly-141 are both located close to the permeability barrier in TM4 and TM5, respectively. The small structural distortion near the barrier during membrane preparation may increase the accessibility of NEM without affecting the transport activity.

Protective Effect of Tetracycline on NEM Binding—We previously observed tetracycline-induced acceleration and inhibition of NEM binding to hot spot Cys mutants as to a conformationally sensitive loop region (6, 35). If the water-filled channel is a substrate translocation pathway, the NEM binding to Cys mutants facing the channel may be affected by tetracycline. Fig. 7 shows the NEM binding in sonicated membranes in the presence or absence of 1 mM tetracycline. As to TM4, almost all of the NEM-reactive mutants as to the transmembrane region were severely inactivated more than 70% by NEM, whereas only a few hot spot mutants as to the loop regions were sensitive to NEM (6). As to TM4, almost all of the NEM-reactive mutants as to the transmembrane region were severely inactivated more than 70% by NEM, whereas only a few hot spot mutants as to the loop regions were sensitive to NEM. However, considering the fact that the mutations exhibiting incomplete accessibility of AMS in sonicated membranes are mainly located in the middle of TM5, the possibility that the small structural distortion during membrane preparation may affect the accessibility of AMS cannot be excluded.

Effect of NEM on Tetracycline Transport Activity—The effect of 2 mM NEM preincubation on the tetracycline transport activity of inverted membrane vesicles was measured (Fig. 8). As reported previously, most NEM-reactive Cys mutants as to the transmembrane region of TM2 were inactivated by NEM, whereas only a few hot spot mutants as to the loop regions were sensitive to NEM (6). As to TM4, almost all of the NEM-reactive mutants as to the transmembrane region were severely inactivated more than 70% by NEM, the exception being the G108C mutant, which was moderately inactivated, i.e. about 35%. In contrast, as to the loop 4–5 region, most of the mutants were not affected or only moderately inactivated by NEM, the exceptions being the A119C and V128C mutants.

Effect of NEM on Tetracycline Transport Activity—The effect of 2 mM NEM preincubation on the tetracycline transport activity of inverted membrane vesicles was measured (Fig. 8). As reported previously, most NEM-reactive Cys mutants as to the transmembrane region of TM2 were inactivated by NEM, whereas only a few hot spot mutants as to the loop regions were sensitive to NEM (6). As to TM4, almost all of the NEM-reactive mutants as to the transmembrane region were severely inactivated more than 70% by NEM, the exception being the G108C mutant, which was moderately inactivated, i.e. about 35%. In contrast, as to the loop 4–5 region, most of the mutants were not affected or only moderately inactivated by NEM, the exceptions being the A119C and V128C mutants.
which were hot spot mutants inactivated more than 90% by NEM. These properties of Cys mutants were similar to in the case of TM2 and its flanking loops (20). It seems that the cytoplasmic half of TM4 comprises a part of the wall of the substrate translocation pathway and that chemical modification at these positions sterically prevents the substrate translocation even if the residues themselves are not essential for the function.

With respect to TM5, it is difficult to make a general rule for the NEM effect on the transport activity because most of the NEM-reactive positions in TM5 are critical for the transport function and the Cys mutants lost the transport activity. Only four NEM-reactive mutants, A136C, F138C, L142C, and G149C, retained significant tetracycline transport activity (Fig. 3). Among them, the G149C mutant was almost completely inactivated, and the activity of A136C was greatly reduced by NEM, whereas the L142C mutant was rather stimulated by NEM, i.e. about 3-fold, and the F138C mutant was not affected at all. Because the activity of the L142C mutant was one-third that of the wild-type, the level of activity of NEM-modified L142C was the same as that of the wild-type. Leu-142 is the residue just before the barrier. The volume and shape of the side chain at this position may be deeply related to the transport function through the local conformation, and a maleimidy1 side chain may be better than a sulfhydryl side chain for the local conformation.

**DISCUSSION**

In this study, we observed two different NEM binding patterns for the transmembrane regions of TetA(B). TM5 faces a water-filled channel along its whole length, as does TM2, whereas only the cytoplasmic half of TM4 faces the channel. This is a new pattern indicating that TM4 is tilted away from a water-filled transmembrane channel. In addition, the reason that the transmembrane water-filled channel does not cause uncoupling was determined in this study. The channel has a permeability barrier for hydrophilic molecules in the middle, which helps avoid uncoupling. Finally, we showed that only NEM binding at position 141 was prevented by tetracycline; however, it is not clear whether this position is a substrate binding site or only a site sensitive to a substrate-induced conformational change. On the basis of these results, we present the detailed topology around TM4 and TM5 with the intervening loop region (Fig. 9).

On comparison with the transmembrane regions estimated on hydropathy analysis (5, 36), TM4 determined through the NEM binding experiment (Leu-99 to Ileu-118) (Fig. 9) was in good agreement with the estimate (Leu-99 to Ala-119) (Fig. 1), whereas TM5 determined through the experiment (Ala-136 to Ala-152) was greatly different from the estimate (Trp-130 to Ala-152). The experimentally determined TM5 region only comprises 17 amino acid residues, which seems shorter than the number required for crossing of the membrane as an α-helix. Loop 5–6 comprises only six residues (Fig. 1), and the region of TM6 is known because it is a transmembrane segment entirely not reactive with NEM (7). Thus, the region of TM5 may be expanded upstream to Gly-132. The degree of NEM binding to the W133C and G132C mutants was between 50 and 60%, which was lower than in the case of the flanking mutants,
and the periodical appearance of NEM low reactive mutations every two residues in TM5 was maintained until the GI32C mutant. Therefore, at the cytoplasmic end of TM5, a water-filled channel may widen like a funnel, and the end of TM5 is exposed to the aqueous phase.

Despite its drastically reduced transport activity, the L142C mutant retained full resistance, probably due to the high $V_{\text{max}}$ value with high drug concentrations. Interestingly, the transport activity of the L142C mutant was significantly (about 3.3-fold) increased when it was modified with NEM (Fig. 8). This phenomenon is similar to in the case of the P59C mutant as to TM2 (20), which is stimulated about 2.1-fold by NEM. This may be due to the requirement of a hydrophobic bulky side-chain at position 142 in order to maintain the high affinity conformation of TetA(B). In contrast, the I143C mutant exhibited drastically reduced drug resistance (6.3 µg/ml), whereas it retained moderate transport activity (about one-third of the wild-type level), probably reflecting the low $V_{\text{max}}$ value. In any case, there are no functionally essential residues in TM5 except for the conserved glycines and prolines. Therefore, the permeability barrier and the substrate binding site or substrate-induced conformationally sensitive site in TM5 may be formed through a specific conformation of the glycine- and proline-rich polypeptide chain. Glycine residues in the transmembrane region are oriented toward helix-helix interfaces and exhibit a high occurrence at helix crossing points (37). This suggests that transmembrane glycine residues in TM5 comprise a critical structure functioning as a permeability barrier and a substrate binding site through helix-helix interaction with other transmembrane helices. The glycine-rich sequence and the importance of glycine residues are common to TM4, confirming the critical role of glycine residues in the TM4 and TM5 regions.

Analysis of cysteine-scanning mutants of putative TM5 of 12-membrane-spanning MFS transporters has been extensively performed for lactose permease (15). The authors concluded that TM5 of lactose permease does not contain functionally essential residues except for glycines (15, 38, 39). In the case of TM5 of lactose permease, only three Cys mutants, in which the mutations are located on one side of the helix, were inactivated by NEM (15). The authors of that study did not determine whether other mutants were accessible to NEM or not. In our experiment, it was revealed that some NEM-reactive mutants as to TM5 of TetA(B), such as F138C and L142C, were not inactivated or were, on the contrary, stimulated by NEM. Therefore, the accessibility of NEM to Cys residues and the inactivation by NEM should be independently determined through a topological study on polytopic membrane proteins.

On the other hand, TM4 contains the functionally essential residue Arg-101, which is conserved in MFS transporters (Fig. 2) and is the unique charged residue in TM4 (32). Arg-101 is located on the putative channel-facing side of the helix, although it exists in the N-terminal hydrophobic half of TM4. A conformational change during the transport process may expose this residue to the substrate translocation pathway. Another possibility is that it plays some role in the proton translocation or energy coupling in combination with Asp-54 and Asp-285, which are negatively charged residues located in TM3 and TM9, respectively. TM3 and TM9 are the transmembrane segments entirely embedded in the hydrophobic interior of the membrane (6, 8). It is interesting that Arg-101 may coordinate with these functionally essential aspartic acid residues in the transport function, whereas it seems not likely that Arg-101 forms a charge-neutralizing pair with one of these transmembrane aspartic acid residues (32).

The current study revealed that the chemical modification of cysteine-scanning mutants is very useful for determining the detailed topology of membrane transporters.

REFERENCES

1. Yamaguchi, A., Udagawa, T., and Sawai, T. (1990) J. Biol. Chem. 265, 4809–4813
2. McMurry, L., Petrucci, R. E., Jr., and Levy, S. B. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5397–5397
3. Levy, S. B. (1992) Antimicrob. Agents Chemother. 36, 695–703
4. Henderson, P. J. F. (1990) J. Bioenerg. Biomembr. 22, 525–569
5. Kimura, T., Ohnuma, M., Sawai, T., and Yamaguchi, A. (1997) J. Biol. Chem. 272, 580–585
6. Kimura, T., Shinya, Y., Sawai, T., and Yamaguchi, A. (1998) J. Biol. Chem. 273, 5245–5249
7. Konishi, S., Iwaki, S., Kimura-Someya, T., and Yamaguchi, A. (1999) FEBS Lett. 461, 315–318
8. Kimura, T., Suzuki, M., Sawai, T., and Yamaguchi, A. (1996) Biochemistry 35, 15896–15899
9. Yamaguchi, A., Someya, Y., and Sawai, T. (1992) J. Biol. Chem. 267, 19153–19162
10. Tang, X.-O., Fujinaga, J., Kopito, R., and Casey, J. R. (1998) J. Biol. Chem. 273, 22545–22553
11. Mordoch, S. S., Granot, D., Lebendiker, M., and Schuldiner, S. (1999) J. Biol. Chem. 274, 19480–19486
12. Sahin-Toth, M., and Kaback, H. R. (1993) Protein Sci. 2, 1024–1033
13. Dunte, R. L., Sahin-Toth, M., and Kaback, H. R. (1993) Biochemistry 32, 12644–12650
14. Flirrings, S., Sahin-Toth, M., Persson, B., and Kaback, H. R. (1994) Biochemistry 33, 8074–8081
15. Weitzman, C., and Kaback, H. R. (1995) Biochemistry 34, 9374–9379
16. He, M. M., Sun, J., and Kaback, H. R. (1996) Biochemistry 35, 12909–12914
17. Flirrings, S., Sun, J., Gonzalez, A., and Kaback, H. R. (1997) Biochemistry 36, 269–273
18. Flirrings, S., Ujwal, M. L., Sun, J., and Kaback, H. R. (1997) Protein Sci. 6, 431–437
19. Yan, R.-T., and Maloney, P. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5973–5976
20. Kimura-Someya, T., Iwaki, S., and Yamaguchi, A. (1998) J. Biol. Chem. 273, 32806–32811
21. Varela, M. F., Sansom, C. E., and Griffith, J. K. (1995) Molecular Membrane Biol. 12, 313–319
22. Hansen, T., McNulty, L. M., Levy, S. B., and Hirah, D. C. (1993) Antimicrob. Agents Chemother. 37, 2699–2705
23. Tercero, J. A., Lacalle, R. A., and Jimenez, A. (1993) Eur. J. Biochem. 218, 563–571
24. Coppe, J. J., Liras, P., and Martin, J. F. (1995) EMBO J. 14, 631–639
25. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
26. Someya, Y., Niwa, A., Sawai, T., and Yamaguchi, A. (1995) Biochemistry 34, 7–12
27. Hashimoto-Gotoh, T., Mizuno, T., Ogashara, Y., and Nakagawa, M. (1995) Gene 152, 271–275
28. Hashimoto-Gotoh, T., Yasojima, K., and Tsujimura, A. (1995) Gene 167, 333–334
29. Yamaguchi, A., Ono, N., Akasaka, T., Nouni, T., and Sawai, T. (1999) J. Biol. Chem. 265, 15525–15530
30. Yamaguchi, A., Adachi, K., and Sawai, T. (1990) FEBS Lett. 165, 17–19
31. Philipson, L., Anderson, P., Oslebysk, U., Weinberg, R., and Baltimore, D. (1978) Cell 13, 189–199
32. Kimura, T., Nakatani, M., Kawabe, T., and Yamaguchi, A. (1998) Biochemistry 37, 5475–5480
33. Someya, Y., Kimura-Someya, T., and Yamaguchi, A. (2000) J. Biol. Chem. 275, 210–214
34. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
35. Kimura, T., Inagaki, Y., Sawai, T., and Yamaguchi, A. (1995) FEBS Lett. 362, 47–49
36. Eckert, B., and Beck, C. F. (1989) J. Biol. Chem. 264, 11663–11670
37. Javadpour, M. M., Eilers, M., Groesbeek, M., and Smith, S. O. (1999) Biophys. J. 77, 1609–1618
38. Menezes, M. E., Rose, P. D., and Kaback, H. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1638–1642
39. Jung, K., Jung, H., Colacurcio, P., and Kaback, H. R. (1995) Biochemistry 34, 1030–1039