Complete Cysteine-Scanning Mutagenesis and Site-Directed Chemical Modification of The Tn10-Encoded Metal-Tetracycline/H⁺ Antiporter

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ABSTRACT

Bacterial metal-tetracycline/H\(^+\) antiporter (TetA(B)) was the first found drug exporter and has been studied as a paradigm of antiporter-type major-facilitator-superfamily transporters. Here the 400 amino acid residues of this protein were individually replaced by cysteine except for the initial methionine. As a result, we could obtain a complete map of the functionally or structurally important residues. In addition, we could determine the precise boundaries of all the transmembrane segments on the basis of the reactivity with \(N\)-ethylmaleimide (NEM). The NEM-binding results indicated the presence of a transmembrane water-filled channel in the transporter. The twelve transmembrane segments can be divided into three groups; four are totally embedded in the hydrophobic interior, four face a putative water-filled channel along their full length, and the remaining four face the channel for half their length, the other halves being embedded in the hydrophobic interior. These three types of transmembrane segments are mutually arranged with a four-fold symmetry. The competitive binding of membrane-permeable and -impermeable SH reagents in intact cells indicates that the transmembrane water-filled channel has a thin barrier against hydrophilic molecules in the middle of the transmembrane region. Inhibition and stimulation of NEM binding in the presence of tetracycline reflects the substrate-induced protection or conformational change of TetA(B). The mutations protected from NEM binding by tetracycline were mainly located around the permeability barrier in the N-terminal half, suggesting the location of substrate binding site.
INTRODUCTION

The transposon Tn10-encoded metal-tetracycline/H⁺ antiporter (TetA(B)) (1) was the first found bacterial drug exporter (2), and has been studied as a paradigm of bacterial drug efflux proteins. It belongs to a major facilitator superfamily (MFS) (3), and its 12-membrane-spanning structure (Fig. 1) was established by site-directed competitive chemical modification of cysteine mutants of a cysteine-free TetA(B) (5). We previously reported that putative transmembrane helices (TM) 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 as to a small number of non-reactive 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 EmrE (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.

Afterwards, we found that some cysteine-scanning mutants as to TM2 of TetA(B) are reactive with NEM (20). The NEM-reactive positions are periodically appeared along the full length of TM2, indicating that one side of this transmembrane helix faces a water-filled transmembrane channel. It is impossible that such a water-filled channel is composed of only one amphiphilic helix. Some counterparts should be present in the
transmembrane region of TetA(B). Very recently, we analyzed cysteine-scanning mutants of TM1 and TM11 (21), and TM4 and TM5 (22), and found that TM5 and TM11 also face the channel along their full length, while only the periplasmic (C-terminal) and cytoplasmic (C-terminal) halves of TM1 and TM4 face the channel, respectively. Considering the amphiphilic nature of the metal-tetracycline chelation complex (1), the water-filled channel may be at least a part of a substrate translocation pathway.

In this study, we constructed cysteine-scanning mutants as to the remaining four putative transmembrane segments, TM7, TM8, TM10 and TM12, and the central large loop region, loop6-7. Now, we have obtained a complete set of the 400 cysteine-scanning mutants of TetA(B), except for the mutant as to the initial methionine. In this manuscript, we report the results of NEM binding, and competitive binding of NEM and a membrane-impermeable sulfhydryl reagent, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), in comparison with the results for the other eight transmembrane helices. We first drew a complete map of the wall of the transmembrane water-filled channel and demonstrated the presence of a permeability barrier for hydrophilic molecules in the middle of the channel.

**EXPERIMENTAL PROCEDURES**

*Materials* — $N$-[Ethyl-1-$^{14}$C]maleimide (1.5 GBq/mmol) was purchased from DuPont-New England Nuclear. All other materials were of reagent grade and obtained from commercial sources.

*Site-directed mutagenesis* — Cysteine-scanning mutants were constructed by
oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (23) using synthetic oligonucleotides. For the mutagenesis, plasmid pCTC377A (8), which carries the 2.45 kb Tn10 tetA and tetR gene fragments, was used as a template. All of the mutations were detected as the appearance of a newly introduced restriction site and then verified by DNA sequencing.

Low-copy-number mutant plasmids were constructed through exchange of EcoRV-BamHI fragments (R186C to S201C) or EcoRI-BamHI fragments (V202C to A401C) of cysteine-scanning mutant tetA(B) genes with the corresponding fragments of low-copy-number plasmid pLGC377A, which encodes a cysteine-free mutant TetA(B) (8), and were then used for tetA(B) gene expression.

**Determination of tetracycline resistance** — Tetracycline resistance was determined by means of the two-fold agar dilution method as described previously (24), and expressed as the minimum inhibitory concentration.

**Assaying of the reaction of $^{[14]C}$N-ethylmaleimide with TetA(B) proteins** — The $^{[14]C}$NEM-binding experiment was performed as described previously (9). In brief, a membrane suspension prepared by brief sonication of *E. coli* W3104 cells carrying pLGC377A or one of its derivatives was incubated with 0.5 mM $^{[14]C}$NEM in 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M 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 (25) and Pansorbin *Staphylococcus aureus* cells (26). The TetA(B) protein was separated by SDS-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining. The dried gel was exposed to an imaging plate for visualization with a BAS-1000 Bio-
Imaging Analyzer (Fuji Film Co., Tokyo).

Prevention of $[^14\text{C}]$N-ethylnmaleimide (NEM) binding with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) - Five µl of 100 mM AMS (final conc., 5 mM) or the same volume of distilled water was added to 100 µl of a suspension of *E. coli* W3104 cells expressing a mutant TetA(B) or a sonicated membrane suspension containing a mutant TetA(B), followed by incubation for 30 min at 30 °C. Subsequently, 2 µl of 25 mM $[^14\text{C}]$NEM (final conc., 0.5 mM) was added to the reaction mixture, followed by incubation for 5 min, and then the mixture was diluted with 900 µl of the same buffer containing 5 mM unlabeled NEM. Immediately after the dilution, the cells were collected and washed once with the same buffer. Then the cells were disrupted by brief sonication and membranes were collected. The membranes were solubilized and TetA(B) proteins were immunoprecipitated as described above. The radioactive band densities on pretreatment with AMS were compared with those without AMS treatment.

RESULTS

Construction of Cysteine-scanning Mutants and Their Drug Resistance – We have hitherto constructed 260 cysteine-scanning mutants as to TM1 to TM6, TM9 and TM11, and the connecting loop regions of TetA(B), and reported their reactivity with sulfhydryl reagents (6, 7, 8, 20, 21, 22). In this study, we constructed 140 cysteine-scanning mutants as to TM7, TM8, TM10 and TM12, and their connecting loop regions. As a result, we have now obtained a complete set of cysteine-scanning mutants of TetA(B) comprising 400 cysteine mutants except for one as to the initial methionine.
Three of these 140 cysteine mutants, S199C, S201C and R238C, had been previously constructed (5), therefore, the number of newly constructed mutants was 137. With respect to the 11 residues, Leu213, Ile216 to Gly224, and Pro227, we had constructed glutamic acid and/or aspartic acid mutants (27), and alanine mutants of the other two residues, His257 and Gln261, were also reported previously (28).

The tetracycline resistance levels of these 140 cysteine-scanning mutants are shown in Table I. Surprisingly, the essential residues of which cysteine mutants showed host-level resistance (0.8 µg/ml) numbered only one (G247C). The cysteine mutants which showed drastically-reduced drug resistance of no more than 50 µg/ml were D190C, E192C, S201C and M210C for the putative central loop region, F234C and R238C for putative loop 7-8, G254C, H257C and S258C for putative TM8, L305C and A317C for putative TM10, Q319C for putative loop 10-11, and Y376C, L382C and T385C for putative TM12. In summary, the number of mutants which showed reduced or no resistance was 16 (11 %). The other 124 mutants (89 %) retained almost full resistance comparable to that of the wild type.

[^14]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, 20).

Fig. 2 shows the[^14]NEM binding to the cysteine-scanning mutants. There were no significant differences in the expression levels of these 138 cysteine-scanning mutants (data not shown). The NEM binding results were confirmed to be reproducible.
On the basis of the hydropathy profiles, the boundary between the central loop region and TM7 was predicted to be Pro211 (Fig. 1), however, the I212C and L213C mutants showed significantly high reactivity with NEM (Fig. 2). The reactivity of mutants R186C to L213C with NEM was generally high except in the case of the four scattered mutants, S201C, Y203C, I204C and P211C. In comparison with the results of NEM binding to the mutants as to around TM6 (7), it can be concluded that the central hydrophilic loop region comprises the 35 residues between Phe179 and Leu213. Similarly, although the periplasmic boundary of TM7 was predicted to be Phe234 (Fig. 1), it should be shifted to Val232 because the NEM reactivity of L233C and F234C was very high (Fig. 2), and that of the following mutants was also high. As a result, TM7 comprises 19 residues, this length being four residues shorter than the original prediction (Fig. 1), while the central position of TM7 was not shifted. TM7 included two highly reactive positions as to NEM, G224C and A228C. These positions were clustered in the periplasmic (C-terminal) half of TM7 and located on one side of the helical wheel (Fig. 3). This pattern is similar to that of TM1 (21). Although the number of NEM-reactive positions in TM1 was greater than that in TM7, the reactive positions were also concentrated on one side of the periplasmic half of TM1. These observations indicated that TM7 is tilted away from a water-filled channel and only its periplasmic half faces the channel, similar to in the case of TM1.

The mutants as to the putative loop7-8 region, T235C to M245C, were all highly or moderately reactive with NEM (Fig. 2). Thus, there is no doubt that the N-terminal boundary of TM8 is Val246 (Fig. 5). On the other hand, it is difficult to determine the precise boundary between TM8 and loop8-9 because the N-terminal half of TM8 includes many NEM-reactive positions. Two alternative boundaries are possible, that is,
Leu256 and Phe260. On the basis of only the NEM binding results, Leu256 seems to be more natural than Phe260 as a boundary because the following three positions (H257C, S258C and V259C) are highly reactive with NEM. However, if Val246 and Leu256 are at the two ends of TM8, it comprises only 11 residues, which is far less than the length required for a polypeptide chain to cross the membrane once in an $\alpha$-helical form. Such a situation is similar to in the case of TM5 (22), which also includes many NEM-reactive positions, and determination of the boundary is difficult. In the three-dimensional structure of TetA(B), the cytoplasmic entrance of the water-filled channel may resemble a funnel, and the cytoplasmic halves of TM8 and TM5 may be exposed to the aqueous phase on the funnel wall. Therefore, the cytoplasmic end of TM8 may be extended to Phe260 or even to Val264. In the former and latter cases, TM8 comprises 15 and 19 amino acid residues, respectively. In this manuscript, we tentatively adopted Phe260 as the boundary (Fig. 5). Anyway, the NEM-reactive positions were distributed along the full length of TM8 and they were located on one side of the helical wheel (Fig. 3), indicating that TM8 faces the water-filled channel along its full length.

We previously reported that the periplasmic boundary of TM10 is undoubtedly Trp299 (8). In this study, we constructed cysteine mutants as to TM10 downstream from P303C. As shown in Fig. 2 and by our previous results (8), TM10 can be clearly divided into two parts. The periplasmic (N-terminal) half is highly hydrophobic and includes no NEM reactive position. In contrast, the cytoplasmic half includes some NEM-reactive positions (Fig. 2). The NEM-reactive positions are also distributed periodically and, as a result, they are located on one side of the wheel (Fig. 3). Therefore, the cytoplasmic half of TM10 seems to face the water-filled channel. This situation is similar to that of TM4 (22). These two helices seem to be tilted away from
the channel in opposite directions to in TM1 and TM7. On the basis of the NEM-binding results, Leu318 seems to be the most natural boundary between TM10 and loop10-11. If so, TM10 comprises just 20 amino acid residues, whereas the possibility cannot be excluded that the C-terminal boundary is Met322 or Thr326. However, if the former or latter is the case, TM10 comprises 24 or 28 amino acid residues, respectively.

In our previous paper, we reported that the mutants as to L361C to G366C were all reactive with NEM (22). In this study, we observed that the 19 mutants as to W367C to T385C were all not or only slightly reactive with NEM, and the following mutants, as to F386C to the C-terminal end of TetA(B), were highly reactive with NEM, except for that as to T389C. Therefore, it is clear that the boundaries of TM12 are Trp367 and Thr385 (Fig. 5). This conclusion is almost the same as the original prediction (Fig. 1) except that one end was shortened by one residue (Phe386). TM12 was revealed to be a highly hydrophobic transmembrane segment similar to TM3, TM6 and TM9.

Prevention of $[^{14}\text{C}]$NEM Binding by Membrane-impermeable Maleimide Derivative AMS in Intact Cells — The results of $[^{14}\text{C}]$NEM binding indicated that three of the four transmembrane segments examined in this study face the water-filled channel. One of them (TM8) faces the channel along its full length, indicating that the channel completely crosses the membrane. If such a water-filled channel crosses the membrane, TetA(B) must cause uncoupling. We previously showed that a permeability barrier for hydrophilic molecules exists in the middle of the channel (21, 22). Thus, we examined whether or not the permeability barrier is in TM7, TM8 or TM10. Fig. 4 shows the effect of pretreatment of intact cells and sonicated membranes with AMS on the $[^{14}\text{C}]$NEM binding to the NEM-reactive cysteine mutants. Since AMS molecules can not pass through the cytoplasmic membrane, they should not have access to
cysteine residues located inside the permeability barrier in intact cells, and therefore they should not prevent $[^{14}\text{C}]\text{NEM}$ binding in intact cells. In contrast, the NEM binding to cysteine residues located outside the permeability barrier should be prevented by AMS in intact cells. In the case of unsealed sonicated membranes, AMS molecules prevent NEM binding to cysteine residues regardless of their location inside or outside the permeability barrier, because AMS molecules can gain access from both sides to TetA(B). If a cysteine residue is embedded in the narrow gap to which NEM molecules can gain access but AMS molecules cannot, AMS molecules should not prevent NEM binding to cysteine residues in either intact cells or sonicated membranes.

With respect to TM7, NEM binding to G224C was not affected by AMS in intact cells (Fig. 4A, upper panel). The binding to A228C was significantly prevented, and the binding to V232C and L233C was completely blocked. On the other hand, NEM binding to all of these mutants was completely prevented by AMS in sonicated membranes (Fig. 4A, lower panel). Thus, the permeability barrier seems to exist between Gly224 and Ala228.

In TM8, NEM binding to the NEM-reactive mutants as to upstream from L250C was completely prevented by AMS, whereas that to the mutants as to downstream from L253C was not affected in intact cells (Fig. 4B, upper panel). Therefore, it can be concluded that the permeability barrier is located between Leu250 and Leu253. Interestingly, the NEM binding to the G254C mutant was not affected by AMS even in sonicated membranes, while that to the other mutants was completely prevented in sonicated membranes (Fig. 4B, lower panel), indicating that Gly254 may be embedded in the narrow gap in the channel wall. For TM10, NEM binding to all the NEM-reactive mutants was not affected by AMS in intact cells and completely prevented in sonicated
membranes (Fig. 4C). These results indicated that TM10 faces the channel only inside the permeability barrier.

In Fig. 5, we depict the permeability barrier (green line) in TM7, TM8 or TM10. They are located at almost the same depth in the membrane. We also revealed the presence of a permeability barrier in TM2 (data not shown). Therefore, in comparison with the previously reported results (21, 22), all of the channel-facing transmembrane segments revealed the presence of a permeability barrier. These observations strongly indicated that these helices face the same unique water-filled channel with a single permeability barrier.

The Effect of Tetracycline on NEM Binding – First of all, we investigated the effect of tetracycline on the [14C]NEM binding to 76 NEM-reactive cysteine-scanning mutants as to loop6-7, TM7, loop7-8, TM10, TM12, C-terminus and their flanking regions. To be surprised, there was no significant effect on the NEM binding to the Cys mutants except for the mutants as to TM8 and its flanking regions. Fig. 6 shows only the results as to TM8 and the flanking regions. Out of 76 Cys mutants, the NEM binding only to the S258C and V259C mutants was inhibited and that to the S243C mutant was stimulated (Fig. 6A). The degree of NEM binding to the S258C and V259C mutants in the presence of 1 mM tetracycline was decreased to 40 % and 50 %, respectively, in compared with those in the absence of tetracycline. In contrast, the binding to the S243C mutant was increased to 230 % in the presence of tetracycline. The former two mutations are located in the cytoplasmic half of TM8 and the latter mutant is in periplasmic loop7-8 (Fig. 7).

Then, we re-investigated the effect of tetracycline on the NEM binding to all of the Cys mutants of TetA(B). As a result, the binding to the eight Cys mutants including
S258C and V259C was prevented by tetracycline and that to five Cys mutants was stimulated including S243C (Fig. 7). Among them, we previously reported the prevention of the NEM binding to the L97C (periplasmic loop3-4) (6), A109C (TM4), and G141C (TM5) (22) mutants and the stimulation of that to the S65C mutant (cytoplasmic loop2-3) (39). In the current study, we found that the NEM binding to the G20C (TM1), M23C (TM1), and A51C (TM2) was inhibited to 60 %, 60 %, and 50 %, respectively, in the presence of 1 mM tetracycline, while that to the L47C (TM2), L351C (TM11), and A354C (TM11) was stimulated to 180 %, 230 %, and 220 %, respectively (Fig. 6B). Since all these Cys mutants retained their drug resistance activity except the G141C mutant, the alterations in their NEM accessibility must reflect the tetracycline-induced conformational change or the protection by tetracycline binding during the substrate transport process. Five prevented mutations were concentrated in the vicinity of the permeability barrier in the N-terminal half, probably suggesting that the region involves the substrate binding site.

DISCUSSION

In this study, we first accomplished the construction of a complete set of cysteine-scanning mutants of a membrane transporter. To our surprise, only a small number of the mutants (around 14 %) showed reduced drug resistance. The majority of the mutants retained full level resistance. This is very advantageous for topology determination by means of the site-directed chemical labeling method. Fig. 7 shows a summary of the detailed topology of TetA(B) determined on the basis of the results of this study and our previous studies (6, 7, 8, 21, 22). Each transmembrane segment comprises a number of
amino acid residues of between 24 (TM11) and 15 (TM8), however, it should be noted that this method tends to underestimate the numbers of amino acid residues in "hydrophilic" or "amphiphilic" transmembrane segments. Highly "hydrophilic" segments such as TM5 and TM8 are followed by entirely hydrophobic segments, TM6 and TM9, respectively, indicating that the membrane insertion of these "hydrophilic" segments may be promoted by insertion of the following hydrophobic segments during topogenesis. The topology shown in Fig. 7 clearly reveals the presence of a striking four-fold symmetry in TetA(B). That is, the totally embedded segments, TM3, TM6, TM9 and TM12, are separated by two other segments, respectively. The “totally amphiphilic” segments, TM2, TM5, TM8 and TM11, and the “partly amphiphilic” segments, TM1, TM4, TM7 and TM10, are also separated by two other segments, respectively. All of the “partly amphiphilic” segments exhibit an amphiphilic nature in their C-terminal halves, while their N-terminal halves are entirely hydrophobic. The amphiphilic parts of the odd and even numbered “partly amphiphilic” segments are at the periplasmic and cytoplasmic ends, respectively. We previously reported that the periplasmic ends of TM1 and TM2 are close to that of TM11 (30, 31). Fig. 8 shows a hypothetical model of the helix arrangement of TetA(B) based on our previous and current results. There is a water-filled channel at the center of the molecule. The four “amphiphilic” helices, TM2, TM5, TM8 and TM11, comprise the wall of the channel. The “partly amphiphilic” helices, TM1, TM4, TM7 and TM10, are tilted away from the channel, only their cytoplasmic or periplasmic halves facing it. The four “hydrophobic” helices, TM3, TM6, TM9 and TM12, are probably located at the periphery of the helix bundle. This model is fundamentally similar to the model for lactose permease presented by Goswitz and Brooker (32). Recently, Yin, C. C. et al. reported the low-
resolution two-dimensional image of TetA(B) was reported by electron microscopic analysis (33), which also could be superimposed with the ring-like structure such as that of lactose permease.

The green lines in Fig. 7 indicate the location of the permeability barrier in the channel wall-forming helices. All wall-forming helices are involved in the barrier, which means that there is no uncoupling bypass in the water-filled channel. The barrier is located in the middle of the transmembrane segment a little to the periplasmic end, except for that of TM1. In TM1, the barrier is very close to the periplasmic end. There are two possibilities, i.e., 1) the TM1 helix may extend toward loop1-2 and form a funnel-like exit of the channel or 2) the TetA(B) molecule is dented on the periplasmic surface at TM1.

The effects of tetracycline on the NEM binding are indicated by downward (inhibitory) and upward (stimulatory) arrows in Fig. 7. In our previous paper (6), we supposed that tetracycline may cause the TetA(B) conformational change from the inside-closed/outside-open form to the inside-open/outside-closed form since the NEM binding to the S65C (inside) and L97C (outside) mutants was inhibited and stimulated by tetracycline (6). However, on the basis of our current results, the substrate-induced conformational change may be complicated. To be surprised, there was no region in which NEM binding to Cys mutants were continuously inhibited or stimulated by tetracycline, indicating that the substrate-induced occlusion or exposure of any domain(s) of TetA(B) did not occur. It should be noted that the mutations exhibiting inhibitory effect of tetracycline are mainly located around the permeability barrier in the N-terminal half, suggesting the presence of the substrate binding site.
Fig. 7 also shows the distribution of the functionally and/or structurally important residues of which cysteine mutants exhibit reduced drug resistance. Among them, the number of essential residues, the MIC of cysteine mutants of which were less than 10 \( \mu g/ml \), is only 17 (4 %) and that of moderately important residues (MIC < 50 \( \mu g/ml \)) is 41 (10 %). In total, 58 (14 %) of the 401 amino acid residues of TetA(B) contribute to the structure and/or function. The remaining 343 residues (86 %) are apparently related to neither the structure nor function. Out of the 17 essential residues, 13 are located in the transmembrane region. In addition, out of these 13 essential residues, eight are glycines and two are prolines, confirming the importance of glycines and prolines in the transmembrane regions of transporter molecules. The other essential residues in the transmembrane regions number only three, Arg101, Asp285 and Tyr357. Essential residues are concentrated in TM4, TM5, TM8 and TM11. Among the loop regions, only three loops, loop2-3, loop4-5 and loop10-11 include essential residues, all of which are cytoplasmic ones. However, it should not be forgotten that the residues of which cysteine mutants exhibit moderately reduced resistance also play significant roles in the transport process, such as Asp84 (34), Arg70 (35) and His257 (36).

We first established a complete set of cysteine-scanning mutants of a membrane transporter, which will be very useful for future studies on the molecular structures and mechanisms of efflux transporters.

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Table I.

The tetracycline resistance levels of E. coli W3104 cells harboring plasmids encoding cysteine-scanning mutant TetA(B)s. The resistance levels are expressed as the MICs of tetracycline.

| Mutation | MIC (µg/ml) | Mutation | MIC (µg/ml) | Mutation | MIC (µg/ml) | Mutation | MIC (µg/ml) | Mutation | MIC (µg/ml) |
|----------|-------------|----------|-------------|----------|-------------|----------|-------------|----------|-------------|
| wild-type no plasmid | 400 | L213C | 200 | N237C | 200 | P303C | 200 | W367C | 400 | Q397C | 200 |
| R186C | 200 | I215C | 100 | F239C | 400 | L305C | 12.5 | W369C | 200 | T399C | 200 |
| D187C | 400 | I216C | 200 | G240C | 400 | L306C | 200 | I370C | 400 | S400C | 200 |
| N188C | 400 | Y217C | 200 | W241C | 400 | L307C | 200 | I371C | 200 | A401C | 200 |
| T189C | 400 | F218C | 200 | N242C | 400 | L308C | 100 | G372C | 200 | |
| D190C | 25 | S219C | 400 | S243C | 200 | A309C | 200 | L373C | 400 | |
| T191C | 400 | A220C | 200 | M244C | 400 | G310C | 400 | A374C | 200 | |
| E192C | 12.5 | Q221C | 400 | M245C | 400 | G311C | 200 | F375C | 400 | |
| V193C | 400 | L222C | 400 | V246C | 200 | G312C | 400 | Y376C | 50 | |
| G194C | 100 | I223C | 400 | G247C | 0.8 | I313C | 400 | I378C | 200 | |
| V195C | 400 | G224C | 400 | F248C | 400 | A314C | 200 | I379C | 400 | |
| E196C | 400 | Q225C | 400 | S249C | 400 | L315C | 200 | I380C | 200 | |
| T197C | 400 | I226C | 400 | L250C | 400 | P316C | 200 | L381C | 200 | |
| Q198C | 400 | P227C | 200 | A251C | 200 | A317C | 50 | L382C | 50 | |
| S199C | 400 | A228C | 400 | G252C | 400 | L318C | 400 | S383C | 200 | |
| N200C | 400 | T229C | 400 | L255C | 400 | Q319C | 12.5 | M384C | 200 | |
| S201C | 25 | V230C | 200 | G254C | 6.3 | G320C | 200 | T385C | 25 | |
| V202C | 400 | W231C | 400 | L255C | 400 | V321C | 200 | F386C | 200 | |
| Y203C | 400 | V232C | 200 | L256C | 200 | M322C | 400 | M387C | 400 | |
| I204C | 400 | L233C | 100 | H257C | 12.5 | S323C | 100 | L388C | 200 | |
| T205C | 400 | F234C | 50 | S258C | 25 | I324C | 200 | T389C | 200 | |
| L206C | 400 | T235C | 200 | V259C | 400 | Q325C | 400 | P390C | 200 | |
| F207C | 200 | E236C | 100 | F260C | 400 | T326C | 400 | Q391C | 400 | |
| K208C | 400 | Q261C | 400 | K327C | 400 | A392C | 200 | |
| T209C | 400 | A262C | 200 | Q393C | 400 | |
| M210C | 50 | F263C | 400 | G394C | 200 | |
| P211C | 100 | V264C | 400 | S395C | 200 | |
| I212C | 400 | A265C | 400 | K396C | 200 | |
LEGENDS TO FIGURES

Fig. 1. **Putative membrane topology of the Tn10-encoded metal-tetracycline/H\(^+\) antiporter (TetA(B))**. This structure was constructed on the basis of the results of hydropathy analysis (4) and our previous results as to site-directed chemical modification of Cys mutants of TetA(B) (5, 6, 7, 8, 21, 22). Putative transmembrane segments are enclosed in squares. Gray-colored squares indicate the transmembrane segments of which the precise boundaries were determined by site-directed chemical modification of cysteine-scanning mutants (6, 7, 8, 21, 22). The residues of the cysteine-scanning mutants constructed in this study are depicted as encircled letters. *The closed circle with a white letter* indicates the residue mutated in Cys-free TetA(B).

Fig. 2. **Reactivity of cysteine-scanning mutants with [\(^{14}\)C]N-ethylmaleimide**. Sonicated membranes (0.5 mg protein) including cysteine-scanning mutants were incubated with 0.5 mM [\(^{14}\)C]NEM for 5 min at 30°C, followed by solubilization and immunoprecipitation of the TetA(B) proteins with anti-TetA(B)-C-terminal peptide antiserum, and then the protein bands were visualized after SDS-PAGE as described under Experimental Procedures. (A) The radioactive bands visualized with a Bioimaging Analyzer BAS-1000. As to the C-terminal region, the NEM binding of Q397C and E398C could not be measured because these mutant proteins were not immunoprecipitated with anti-C-terminal peptide antibody probably due to these residues being the common part of the epitopes of this polyclonal antibody. (B) The amounts of [\(^{14}\)C]NEM binding per TetA(B) protein (mole/mole) were calculated from the radioactive band densities in panel (A) and the Coomassie-brilliant blue-stained protein bands (data not shown) as described in the previous paper (6).
Fig. 3. **Helical wheel projection of the residues in TM7, TM8 and TM10 viewed from periplasmic side.** The residues of which cysteine mutants gave NEM-reactivity higher than 0.1 mole NEM per mole TetA(B) in Fig. 2B are depicted as *outlined letters on a black background*. As to TM7 and TM10, only the N-terminal amphiphilic halves were shown.

Fig. 4. **Effect of AMS on $[^{14}\text{C}]$NEM binding to the cysteine-scanning mutant TetA(B) proteins in intact cells and sonicated membranes.** Each upper panel: Intact cells expressing the cysteine-scanning mutants were preincubated in the presence (+) or absence (-) of 5 mM AMS for 30 min at 30˚C, followed by incubation with 0.5 mM $[^{14}\text{C}]$NEM for 5 min. After stopping the labeling by dilution with excess non-labeled NEM, the cells were disrupted by brief sonication. The membrane fractions were solubilized and then the TetA(B) proteins were immunoprecipitated. The radioactive bands on SDS-PAGE gels were visualized with a BAS1000 Bio-imaging Analyzer. Each lower panel: Sonicated membranes were preincubated in the presence (+) or absence (-) of AMS, followed by labeling with $[^{14}\text{C}]$NEM, and then the radioactive bands were visualized as described in Fig. 2. (A) TM7, (B) TM8, and (C) TM10.

Fig. 5. **The detailed topology of the C-terminal half of TetA(B) determined in this study.** Transmembrane regions are shown in *boxes*. The residues of which Cys mutants exhibited the NEM binding more than 0.1 mole/mole TetA(B) are indicated by *blue bold letters*, and those of which Cys mutants were almost no reactive with NEM (less than 0.1 mole/mole TetA(B)) are indicated by *red bold letters*. *Pink and light blue regions* indicate the portions embedded in the hydrophobic environment and ones facing the water-filled channel, respectively. Yellow circles indicate the residues to which AMS can gain access in intact cells, and *open circles* indicate those to which AMS can not gain access in intact cells. The *open square* indicates the residue to which AMS can not gain access in either intact cells nor sonicated.
membranes. The green line indicates the permeability barrier for AMS.

Fig. 6. Effect of tetracycline on [14C]NEM binding to the cysteine-scanning mutants as to TM8 and its flanking regions of TetA(B). Sonicated membranes containing Cys-scanning mutant TetA(B) were labeled with [14C]NEM in the presence (+) or absence (-) of 1 mM tetracycline (TC) as described in the previous paper (22). The radioactive bands were visualized as in Fig. 4. (A) The effect of tetracycline on NEM binding to the NEM-reactive Cys mutants as to TM8 and its flanking regions. (B) The effect of tetracycline on NEM binding to the Cys mutants which were newly found out in the current study that their NEM binding are sensitive to tetracycline.

Fig. 7. The entire topology of TetA(B) determined by the site-directed chemical modification method. Transmembrane regions are shown in boxes. The residues of which Cys mutants exhibited high or moderate NEM reactivity are indicated by blue bold letters, and those of which Cys mutants were almost not reactive with NEM are indicated by red bold letters. Pink and light blue regions indicate the portions embedded in the hydrophobic environment and ones facing the water-filled channel, respectively. The green line indicates the permeability barrier for AMS. Yellow Circles and white squares indicate the functionally or structurally important residues of which cysteine mutants exhibited drastically-reduced tetracycline resistance (less than 10 µg/ml) and moderately-reduced resistance (less than 50 µg/ml), respectively. The green upward and downward arrows indicate the residues of which NEM binding to Cys mutants were stimulated and inhibited by tetracycline, respectively. Outlined letters in brown ovals indicate the numbers of residues in the boundaries in each transmembrane segment.

Fig. 8. Model of the helix arrangement of TetA(B). All α-helices project from the periplasmic side. White letters in a blue background indicate NEM-reactive positions.
Helices in pink, light blue and light green backgrounds indicate the entirely hydrophobic, “amphiphilic” and “partially amphiphilic” helices described in the text. (A) Helix arrangement on the cytoplasmic side. (B) Helix arrangement on the periplasmic side. In each wheel, residues in the cytoplasmic or periplasmic half are depicted.
Central loop and TM7 region

\[ ^{14}C \text{NEM-bound TetA(B)} \]

Fig. 2A-1

![Image of a gel electrophoresis showing bands labeled with amino acid positions and labels indicating central loops and TM7 regions.]
[14C]NEM-bound TetA(B) loop7-8

TM8 region

Fig. 2A-2

[14C]NEM-bound TetA(B) loop8-9

TM8

N237C R238C F239C G240C W241C N242C S243C M244C M245C V246C G247C F248C S249C L250C A251C G252C L253C G254C L255C L256C H257C S258C V259C F260C Q261C A262C F263C V264C A265C

TM8
**TM10 region**

[\[^{14}C\]NEM-bound TetA(B) → ]

---

**TM10 region**

[\[^{14}C\]NEM-bound TetA(B) → ]

---

**Fig. 2A-3**

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**TM10**

- P303C
- V304C
- L305C
- I306C
- L307C
- L308C
- A309C
- G310C
- G311C
- G312C
- I313C
- A314C
- L315C
- P316C
- A317C
- L318C
- Q319C
- G320C

**loop10-11**

- V321C
- M322C
- S323C
- I324C
- Q325C
- T326C
- K327C

**loop10-11**
Fig. 2A-4

**TM12 region**

[14C]NEM-bound TetA(B) →

| TM12 | W367C | I 368C | W369C | I 370C | I 371C | G372C | L373C | A374C | F375C | Y376C | C377C | I 378C | I 379C | I 380C | L381C | L382C | S383C | M384C |
|------|-------|--------|-------|--------|--------|-------|-------|-------|-------|-------|-------|-------|--------|--------|--------|-------|-------|-------|-------|

**[14C]NEM-bound TetA(B) →**

| C-terminal end | T385C | F386C | M387C | L388C | T389C | P390C | Q391C | A392C | Q393C | G394C | S395C | K396C | T399C | S400C | A401C |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|-------|-------|-------|-------|
Fig. 3

Helix 7

Helix 8

Water-filled Channel

Helix 10
(A) TM7

**intact cell**

\[
[^{14}C]\text{NEM-bound TetA(B)}
\]

| AMS | G224C | A228C | V232C | L233C |
|-----|-------|-------|-------|-------|
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |

**membrane**

\[
[^{14}C]\text{NEM-bound TetA(B)}
\]

| AMS | G224C | A228C | V232C | L233C |
|-----|-------|-------|-------|-------|
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |
|     | +     | -     | +     | -     |
|     | -     | +     | -     | +     |

Fig. 4-1
(B) TM8

intact cell

[\textsuperscript{14}C]NEM-bound TetA(B)

\begin{tabular}{ccccccccccccc}
AMS & + & + & + & + & + & + & + & + & + & + & + & + \\
S243C & - & - & - & - & - & - & - & - & - & - & - & - \\
M244C & - & - & - & - & - & - & - & - & - & - & - & - \\
M245C & - & - & - & - & - & - & - & - & - & - & - & - \\
V247C & - & - & - & - & - & - & - & - & - & - & - & - \\
L250C & - & - & - & - & - & - & - & - & - & - & - & - \\
L253C & - & - & - & - & - & - & - & - & - & - & - & - \\
G254C & - & - & - & - & - & - & - & - & - & - & - & - \\
H257C & - & - & - & - & - & - & - & - & - & - & - & - \\
S258C & - & - & - & - & - & - & - & - & - & - & - & - \\
V259C & - & - & - & - & - & - & - & - & - & - & - & - \\
\end{tabular}

membrane

[\textsuperscript{14}C]NEM-bound TetA(B)

\begin{tabular}{cccccccccccc}
AMS & + & + & + & + & + & + & + & + & + & + & + \\
S243C & - & - & - & - & - & - & - & - & - & - & - \\
M244C & - & - & - & - & - & - & - & - & - & - & - \\
M245C & - & - & - & - & - & - & - & - & - & - & - \\
V247C & - & - & - & - & - & - & - & - & - & - & - \\
L250C & - & - & - & - & - & - & - & - & - & - & - \\
L253C & - & - & - & - & - & - & - & - & - & - & - \\
G254C & - & - & - & - & - & - & - & - & - & - & - \\
H257C & - & - & - & - & - & - & - & - & - & - & - \\
S258C & - & - & - & - & - & - & - & - & - & - & - \\
V259C & - & - & - & - & - & - & - & - & - & - & - \\
\end{tabular}
(C) TM10

intact cell

[\textsuperscript{14}C]NEM-bound TetA(B)

\begin{tabular}{c|c|c|c|c|c}
AMS & + & - & + & - & + & - \\
G311C & - & - & - & - & - & - \\
L315C & + & + & + & + & + & + \\
P316C & - & - & - & - & - & - \\
Q319C & + & + & + & + & + & + \\
G320C & - & - & - & - & - & - \\
\end{tabular}

membrane

[\textsuperscript{14}C]NEM-bound TetA(B)

\begin{tabular}{c|c|c|c|c|c}
AMS & + & - & + & - & + & - \\
G311C & - & - & - & - & - & - \\
L315C & + & + & + & + & + & + \\
P316C & - & - & - & - & - & - \\
Q319C & + & + & + & + & + & + \\
G320C & - & - & - & - & - & - \\
\end{tabular}
Fig. 5
Fig. 6

(A) TetA(B) 

[\(^{14}\text{C}\)NEM-bound TetA(B)

TC  
S243C  M244C  M245C  G247C  L250C  L253C  G254C  H257C  S258C  V259C

(B) TetA(B)

[\(^{14}\text{C}\)NEM-bound TetA(B)

TC  
G20C  M23C  L47C  A51C  L351C  A354C
(A) Cytoplasmic side

(B) Periplasmic side

Fig. 8
Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H+ antiporter
Norihisa Tamura, Satoko Konishi, Shinobu Iwaki, Tomomi Kimura-Someya, Shigeyuki Nada and Akihito Yamaguchi

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