Site-directed Chemical Modification of Cysteine-scanning Mutants as to Transmembrane Segment II and Its Flanking Regions of the Tn10-encoded Metal-Tetracycline/H⁺ Antiporter Reveals a Transmembrane Water-filled Channel*

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Cysteine-scanning mutants, E32C to G62C, of the metal-tetracycline/H⁺ antiporter were constructed in order to precisely determine the membrane topology around putative transmembrane segment II. None of the mutants lost the ability to confer tetracycline resistance, indicating that the cysteine mutation in each mutant did not alter the protein conformation. [14C]-N-Ethylmaleimide (NEM) binding to these cysteine mutants in isolated membranes was then investigated. The peptide chain of this region passes through the membrane at least once because residues 36 and 65 are exposed on the outside and inside surfaces of the membrane, respectively (Kimura, T., Ohnuma, M., Sawai, T., and Yamaguchi, A. (1997) J. Biol. Chem. 272, 580–585). However, there was no continuous segment in which all of the introduced cysteine residues showed almost no reactivity with [14C]-NEM. The proportion of the unbound positions in the second half downstream from position 45 was 55% (10/18), which was clearly higher than that in the first half (21%; 3/14), suggesting that the second half is a transmembrane segment. Positions reactive to NEM appear periodically in the second half. They are located on one side of the helical wheel, suggesting that this side of the transmembrane helix faces a water-filled channel. The cysteine mutants as to the reactive positions in the second half were severely inactivated by NEM except for the P59C mutant, whereas the A40C mutant was the only one inactivated by NEM in the first half. These results suggest that the water-filled channel along this helical region may be a substrate translocation pathway.

The tetracycline/H⁺ antiporter (Tet(B)) is a tetracycline exporter existing in the cytoplasmic membrane of Gram-negative bacteria (1–3). This transporter is a member of the major facilitator superfamily (4, 5) including transporters for sugars, amino acids, antibiotics, and neurotransmitters. These transporters were predicted to have 12 transmembrane segments in common (4). The secondary structures of most intrinsic membrane proteins have been determined by the gene fusion method (6, 7). We established a new method for determining the membrane topology on the basis of the reactivity of SH reagents to cysteine-scanning mutants of the tetracycline/H⁺ antiporter (Fig. 1) (8). Cysteine-scanning mutagenesis of intrinsic membrane proteins, especially lactose permease, has been widely used for site-directed chemical modification analysis (9–21). We found that Tet(B) has some segments composed of about 20 consecutive residues at which cysteine-scanning mutants were hardly reactive with NEM1 (22, 23). These segments are embedded in the hydrophobic interior of the membrane. On the basis of this finding, we succeeded in determining the precise boundaries of transmembrane segment (TM) III (23) and TM IX (22). Similar results were obtained for the second transmembrane segment of EmrE.2 However, these observations were inconsistent with the results for lactose permease (10–16) and UhpT (9), of which some cysteine mutants as to the putative transmembrane regions were inactivated by NEM or p-chloromercuribenzoate, indicating that these transmembrane cysteine residues are reactive with SH reagents. Therefore, in order to determine whether or not the NEM-reactive positions are also present in the transmembrane region of the Tet(B) protein, we constructed further cysteine-scanning mutants as to putative TM II.

The first cytoplasmic loop region, loop2–3, contains a widely conserved motif, GXXDXRXGR (4). The first and eighth Gly, fifth Asp, and ninth Arg were revealed to be important for the tetracycline transport function or maintenance of the protein conformation (24). We isolated second-site suppressor mutants of the G62L, D66C, and R70A mutants (25–27). The second-site mutations of the former two mutants (G62L and D66C) occurred in the first periplasmic loop region, loop1–2 (25, 26). The mutation at Gly-62 had a remote-conformational effect on Tyr-50 and Gln-54 at mid-transmembrane positions of TM II, which are conserved in the tetracycline/H⁺ antiporters of Gram-negative bacteria, play some role in the substrate recognition (28). Thus, TM II may play a role in the substrate transport function. In this study, cysteine-scanning mutants of TM II and its flanking regions were constructed and analyzed.

EXPERIMENTAL PROCEDURES

Materials—N-ethyl-1-14C]Maleimide (1.5 Gbq/mmol) and [7-3H]tetracycline (3.7 Gbq/mmol) were purchased from NEN Life Science Products. All other materials were of reagent grade and obtained from commercial sources.

Site-directed Mutagenesis—Cysteine-scanning mutants except for

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1 The abbreviations used are: NEM, N-ethylmaleimide; TM, transmembrane.

* S. Schuldiner, personal communication.
Fig. 1. Putative membrane topology of the metal-tetracycline/H\textsuperscript{+} antiporter. This structure was constructed on the basis of previous results of site-directed chemical modification of Cys mutants of Tet(B) (8, 22, 23, 25). The residues subjected to cysteine-scanning mutagenesis in this study are depicted as bold letters.

the L46C one were constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (29) using synthetic oligonucleotides. For the mutagenesis, plasmid pCT1183 (30) was used as a template that carries the 2.45-kilobase pair Tn10 tetA and tetR gene fragments. The L46C mutant was constructed by the oligonucleotide-directed dual amber method (31) using Mutan\textsuperscript{®}-Super Express gene fragments. The L46C mutant was constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (29) using synthetic oligonucleotides. For the mutagenesis, plasmid pKFB3, which includes the Km (Takara-shuzo Co., Japan) according to the manufacturer’s instructions, was used for the oligonucleotide-directed dual amber method. 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 the BglII-EcoRV fragment for E23C to E27C, the BglII-EcoRI fragment for D38C to A40C, or the EcoRI-EcoRV fragment for N41C to L61C of the cysteine-scanning mutant tetA(B) with the corresponding fragment of the low copy number plasmid, pLGCC377A, which encodes a cysteine-less mutant Tet(B) (22), and then used for tetA(B) gene expression.

Determination of Tetracycline Resistance—Tetracycline resistance was determined by the two-fold agar dilution method as described previously (32), and expressed as the minimum inhibitory concentration.

Assaying of the Reaction of [\textsuperscript{14}C]N-Ethylmaleimide with Tet(B) Proteins—The [\textsuperscript{14}C]NEM-binding experiment was performed as described previously (33).

Assaying of Tetracycline Transport by Everted Membrane Vesicles—Everted membrane vesicles were prepared from Escherichia coli W3104 cells carrying pLGCC377A or one of its derivatives, and tetracycline transport assay was performed as described previously (34).

RESULTS

Activities of Cysteine-scanning Mutants—Cysteine-scanning mutants as to the putative TM II segment and its flanking regions were constructed in order to identify functional residues and to precisely determine the topology around TM II of Tet(B). TM II was determined to be present between Ser-36 and Gly-62 because these residues are located on the outside and inside surfaces of the membrane, respectively (8, 25). We introduced cysteine mutations at positions 29, 30, 31, and 36, and from 62 to 103 in our previous study (8, 23–25). The remaining positions around TM II and its flanking hydrophilic regions, i.e. positions from 32 to 35 and from 37 to 61, were mutagenized in this study. The C377A mutant was used as a template, which is a Cys-less mutant retaining full activity comparable to that of the wild-type (22). All the mutations were confirmed by DNA sequencing and then transferred to the low copy number plasmid, pLGCC377A, as described under “Experimental Procedures.” The expression levels of the mutant tetA(B) genes were determined by Western blotting using anti-Tet(B) C-terminal peptide antiserum (35). The amount of each mutant Tet(B) protein was comparable to that of the wild-type in the membrane fraction of E. coli W3104 cells (data not shown).

The tetracycline resistance levels of E. coli cells including these mutants were measured by the agar dilution method (Table I). None of them lost the resistance, indicating that these amino acid residues of this region are not directly involved in the mechanism of tetracycline transport. These results also indicate that the replacement of the residues in this region did not cause a drastic conformational change. Only the G44C mutant showed a reduction to a moderate resistance level. The tetracycline transport activity of the G44C mutant in everted membrane vesicles was greatly reduced (Fig. 2A). Gly-44 may be important for maintenance of the local conformation as in the cases reported previously (24, 37).

As to Y50C and Q54C, the tetracycline transport activities were quite low as shown in Fig. 2 (B and C), whereas the drug resistance were not largely decreased. We reported that Tyr-50 and Gln-54 were involved in the tetracycline transport function because all substitution mutants of these residues showed largely reduced activity (28). Out of the mutants reported in the previous paper (28), the data as to Y50C mutant, which showed no transport activity, were incorrect. As shown in Fig. 2A, Y50C showed very low but significant tetracycline transport activity, which was highly reproducible. Although the reason for the discrepancy between the drug resistance and the transport activity is not clear, the drug resistance seems to be saturated at relatively low tetracycline efflux activity if any other characteristics of the transporter are not altered. Thus, it seems likely that Tyr-50 and Gln-54 play some roles in tetracycline transport function. Similar to Y50C and Q54C, the P59C mutant also showed the greatly reduced transport activity without apparent change in the drug resistance (Fig. 2D). We consider that Pro-59 is also involved in the transport func-
tion through maintaining the local protein conformation because the reduced transport activity of P59C mutant was recovered by modification with NEM (Fig. 5).

[^14C]N-Ethylmaleimide Binding to the Cys-scanning Mutants—The binding of [14C]NEM to Cys-scanning mutants R31C to G62C was examined (Fig. 3). The reactivity of the I34C, F37C, I39C, V45C, A48C, L52C, M53C, V55C, I56C, F57C, W60C, and L61C mutants with NEM was very low. The other Cys mutants showed high reactivity with NEM (Fig. 3). There was no segment in which there were more than four consecutive low reactive positions. When this region was separated into two segments at the boundary after position 44, the frequencies of low reactive positions in the first and second segments were 21% (3 of 14) and 55% (10 of 18), respectively. This indicates that the second segment is located in a more hydrophobic environment than the first one. The peptide chain of this region must pass through the membrane, because positions 36 and 62 are located on the outside and inside surfaces of the membrane, respectively (8, 25). These results indicate that the second half is a transmembrane segment.

Positions reactive with [14C]NEM appeared periodically in the putative TM II. When the residues of TM II were projected as a helical wheel, these positions were found to be located on one side of the wheel (Fig. 4), suggesting that this side of TM II faces a hydrophilic environment. The chemical reaction of a maleimide derivative with a sulfhydryl group is a nucleophilic attack in which the active molecular species of a sulfhydryl group is a deprotonated form. Since deprotonation of an SH group required a water molecule as a proton acceptor, these results strongly suggest the existence of a water-filled transmembrane channel. Tyr-50 and Gln-54, which are predicted to be involved in substrate recognition (28), are located on the hydrophilic side of the amphiphilic α-helix and cysteine mutants of them are highly reactive to NEM (Fig. 3). This observation also supports the idea that TM II faces the substrate translocation pathway.

### Table I

| Mutation | MIC (µg/ml) | Mutation | MIC (µg/ml) |
|----------|-------------|----------|-------------|
| Wild type          | 200         | L46C     | 100         |
| (No plasmid)       | 0.8         | L47C     | 100         |
| E32C            | 200         | A48C     | 100         |
| F33C            | 200         | L49C     | 50          |
| I34C            | 200         | Y50C     | 100         |
| A35C            | 200         | A51C     | 100         |
| S36C            | 200         | L52C     | 100         |
| E37C            | 200         | M53C     | 100         |
| I38C            | 50          | Q54C     | 200         |
| E39C            | 200         | V55C     | 400         |
| A40C            | 100         | I56C     | 400         |
| N41C            | 100         | F57C     | 100         |
| H42C            | 100         | A58C     | 400         |
| F43C            | 100         | P59C     | 200         |
| G44C            | 12.5        | W60C     | 200         |
| V45C            | 200         | L61C     | 200         |

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**FIG. 2.** Tetracycline uptake by everted membrane vesicles prepared from *E. coli* W3104 cells harboring plasmids encoding each cysteine-scanning mutant Tet(B) proteins. Membrane vesicles were energized with NADH. Closed circles with solid lines and open circles with broken lines indicate the uptake in the presence and absence of NADH, respectively. A, G44C; B, Y50C; C, Q54C; D, P59C; E, wild-type.
Effect of NEM on the Tetracycline Transport Activity of Cys-scanning Mutants—For the Cys mutants reactive to NEM, the effect of NEM on the tetracycline transport activity was measured in everted membrane vesicles. In our previous study, it was revealed that an excess (5 mM) of NEM does not affect the transport activity of Cys mutants, which exhibit very low reactivity with NEM (23). Thus, the effect of NEM on the low-reactive Cys mutants was not examined in this study. The results for Cys mutants R31C to M64C are shown in Fig. 5. The G44C mutant was not examined because it retained only very little transport activity (Fig. 2A). In the putative transmembrane region (TM II; Val-45 to Leu-61), the Y50C, Q54C, and A58C mutants were completely inactivated by NEM. The L46C and A51C mutants were greatly inactivated. The L47C mutant was inhibited about 50% under these conditions (Fig. 4). As to the L46C, L47C, and A51C mutants, the inhibition levels were measured with a higher concentration (5 mM) of NEM. As a result, the inhibition levels were increased (90, 60, and 100% inhibition, respectively). In contrast, only the P59C mutant was activated by NEM more than 2-fold. In summary, all of the NEM-reactive mutants as to TM II were inactivated except for the P59C mutant. On the other hand, in the putative periplasmic segment between Arg-31 and Phe-43, the A40C mutant was a unique one, which was greatly inactivated by NEM (80% inhibition). Such unique hot spots for NEM inhibition were also found in other hydrophilic loop regions, loop2–3 (24), loop3–4 (23), and loop8–9 (37). The inhibition pattern of the putative TM II was clearly different from those of the loop regions. Positions 46, 47, 50, 51, 54, and 58 face to the putative water-filled transmembrane channel (Fig. 4). It seems likely that the tetracycline transport was sterically inhibited by NEM bound to the Cys residues on this side of TM II, suggesting that the channel is a substrate translocation pathway.

Pro-59 is located on the hydrophobic side of TM II (Fig. 4). The tetracycline transport activity of the P59C mutant was reduced to 25% of that of the wild type (Fig. 2B). Thus the activity of the NEM-modified P59C mutant was nearer that of the wild-type than that of the unmodified one, probably reflecting the fact that the maleimidyl side chain fitted the local conformation more than the free SH group. The kink in the peptide backbone at Pro-59 may play a role in the maintenance of the conformation of TM II.

**DISCUSSION**

Previously, we showed that cysteine-scanning mutants as to transmembrane segments III (23) and IX (22) were not reactive with NEM. We established the precise boundaries of these segments and confirmed the whole topology of Tet(B) by means of the reactivity of the mutants with sulfhydryl reagents (22, 23). In this study, we investigated the reactivity of Cys-scanning mutants of Tet(B), R31C to G62C. Although the peptide chain of this region must pass across the membrane, no continuous segment was found in which all residues exhibited no reactivity with NEM, such as TM III and TM IX. Since the hydrophobicity of the latter half of the region between positions 31 and 62 is higher than that of the former region, TM II seems to be located between Val-45 and Leu-61. In this putative TM II, highly reactive positions appeared periodically. Moreover, the highly reactive positions were located on one side of the
brane vesicles were treated with 1 mM NEM for 5 min at 30 °C prior to the tetracycline transport assay. The initial rate of [3H]tetracycline transport was measured as described under “Experimental Procedures.” Residual activities are presented as percentages of the initial rate measured in the absence of NEM. The residual transport activity of the mutants that were not examined because of their very low reactivity with NEM is predicted to be 100% and is shown as 100% by the stippled bars.

Several cysteine mutants as to the putative transmembrane segment of lactose permease (LacY) or glucose 6-phosphate/phosphate exchanger (UhpT) were shown to be inactivated by NEM. This observation is consistent with the substrate or proton in the course of turnover.

The current data suggest the presence of the transmembrane water-filled channel, the channel should not be continuous throughout the membrane because, if it is continuous, the proton permeability will be quite high and the protein acts as an uncoupler. Yan and Maloney (9) reported that, in UhpT, there are three kinds of transmembrane positions to which chloromercuribenzenesulfonate is accessible from the inside, the outside, and both sides, respectively. Since NEM is a membrane-permeable SH reagent, it can bind from both sides of the membrane vesicle. The interception of the water-filled channel is likely to be less than 10 Å, which may not be detected by this method. An alternative possibility is that an immobilized water is present on the putative substrate translocation pathway and plays as a proton acceptor without increasing the proton conductance.

TM III and TM IX contain functional residues, Asp-54 and Asp-285, at the mid-transmembrane positions, respectively. These Asp residues were predicted to play a role for substrate and/or H+ binding (36, 38). It was shown that TM III and TM IX are very hydrophobic, as judged by the NEM reactivity of the Cys-scanning mutants, so that these helices are not likely to compose the water-filled channel. Rather, it is likely that these two aspartic residues face toward the hydrophobic interior of the membrane in the resting state and that they may interact with the substrate or proton in the course of turnover.

If the water-filled channel is completely parallel to TM II, NEM-reactive positions should appear at every 3.6 residues. The highly reactive positions of TM II were 46, 50, 54, and 58, which is every 4 residues. This may suggest that TM II is tilted as to the water-filled channel (Fig. 6). The substrate may be translocated along the tilted helix from the cytoplasm to the periplasm over the residues at positions 58, 54, 50, and 46.

REFERENCES
1. Levy, S. B. (1992) Antimicrob. Agents Chemother. 36, 695–703
2. McMurry, L., Petrucci, R. E., Jr., and Levy, S. B. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3974–3977
3. Kaneko, M., Yamaguchi, A., and Sawai, T. (1985) FEBS Lett. 193, 194–198
4. Henderson, P. J. F. (1990) J. Bioenerg. Biomembr. 22, 525–569
5. Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1996) Microb. Rev. 60, 575–608
6. Mansil, C., and Beckwith, J. (1986) Science 233, 1043–1048
7. Allard, J. D., and Bertrand, K. P. (1992) J. Biol. Chem. 267, 17809–17819
8. Kimura, T., Ohtuma, M., Sawai, T., and Yamaguchi, A. (1997) J. Biol. Chem. 272, 580–586

FIG. 5. Effect of N-ethylmaleimide on the tetracycline uptake by everted membrane vesicles including cysteine-scanning mutants. Everted membrane vesicles were treated with 1 mM NEM at 30 °C prior to the tetracycline uptake by everted membrane vesicles including cysteine-scanning mutants. The residual transport activity of the mutants that were not examined because of their very low reactivity with NEM is predicted to be 100% and is shown as 100% by the stippled bars.

FIG. 6. A model of the amino acid arrangement of transmembrane segment 2 predicted from the results of this study. The arrow indicates the putative pathway for tetracycline translocation.
9. Yan, R.-T., and Maloney, P. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5973–5976
10. Sahin-Toth, M., and Kaback, H. R. (1993) *Protein Sci.* **2**, 1024–1033
11. Dunten, R. L., Sahin-Toth, M., and Kaback, H. R. (1995) *Biochemistry* **34**, 12644–12650
12. Frillingos, S., Sahin-Toth, M., Persson, B., and Kaback, H. R. (1994) *Biochemistry* **33**, 8074–8081
13. Weitzman, C., and Kaback, H. R. (1995) *Biochemistry* **34**, 9374–9379
14. He, M. M., Sun, J., and Kaback, H. R. (1996) *Biochemistry* **35**, 9374–9379
15. Frillingos, S., Sun, J., Gonzalez, A., and Kaback, H. R. (1997) *Biochemistry* **36**, 269–273
16. Frillingos, S., Ujwal, M. L., Sun, J., and Kaback, H. R. (1997) *Protein Sci.* **6**, 431–437
17. Sahin-Toth, M., Persson, B., Schwieger, J., Cohan, P., and Kaback, H. R. (1994) *Protein Sci.* **3**, 240–247
18. Sahin-Toth, M., Frillingos, S., Bibi, E., Gonzalez, A., and Kaback, H. R. (1994) *Protein Sci.* **3**, 2302–2310
19. Frillingos, S., and Kaback, H. R. (1996) *Biochemistry* **35**, 5333–5338
20. Frillingos, S., Gonzalez, A., and Kaback, H. R. (1997) *Biochemistry* **36**, 14284–14290
21. Frillingos, S., Wu, J., Venkatesan, P., and Kaback, H. R. (1997) *Biochemistry* **36**, 6408–6414
22. Kimura, T., Suzuki, M., Sawai, T., and Yamaguchi, A. (1996) *Biochemistry* **35**, 15896–15901
23. Kimura, T., Shina, Y., Sawai, T., and Yamaguchi, A. (1998) *J. Biol. Chem.* **273**, 5243–5247
24. Yamaguchi, A., Someya, Y., and Sawai, T. (1992) *J. Biol. Chem.* **267**, 19155–19162
25. Kimura, T., Sawai, T., and Yamaguchi, A. (1997) *Biochemistry* **36**, 6941–6946
26. Yamaguchi, A., Inagaki, Y., and Sawai, T. (1995) *Biochemistry* **34**, 11800–11806
27. Someya, Y., and Yamaguchi, A. (1997) *Biochim. Biophys. Acta* **1322**, 230–236
28. Yamaguchi, A., Akasaka, T., Kimura, T., Sakai, T., Adachi, Y., and Sawai, T. (1993) *Biochemistry* **32**, 5688–5704
29. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492
30. Someya, Y., Niwa, A., Sawai, T., and Yamaguchi, A. (1995) *Biochemistry* **34**, 7–12
31. Hashimoto-Gotoh, T., Mizuno T., Ogasahara Y., and Nakagawa M. (1995) *Gene (Amst.)* **152**, 271–275
32. Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T., and Sawai, T. (1990) *J. Biol. Chem.* **265**, 15525–15530
33. Kimura, T., Nakatani, M., Kawabe, T., and Yamaguchi, A. (1998) *Biochemistry* **37**, 5475–5480
34. Yamaguchi, A., Samejima, T., Kimura, T., and Sawai, T. (1996) *Biochemistry* **35**, 4359–4364
35. Yamaguchi, A., Adachi, K., and Sawai, T. (1990) *FEBS Lett.* **265**, 17–19
36. Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., and Sawai, T. (1992) *J. Biol. Chem.* **267**, 7490–7498
37. Yamaguchi, A., Kimura, T., Someya, Y., and Sawai, T. (1993) *J. Biol. Chem.* **268**, 6496–6504
38. Kimura, T., and Yamaguchi, A. (1996) *FEBS Lett.* **388**, 50–52
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