Determination of a Transmembrane Segment Using Cysteine-Scanning Mutants of Transposon Tn10-Encoded Metal−Tetracycline/H^+ Antiporter†

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Received June 28, 1996; Revised Manuscript Received October 1, 1996⊗

ABSTRACT: The metal−tetracycline/H^+ antiporter is a bacterial plasma membrane protein belonging to the 12-transmembrane transporter family. The boundaries of membrane-embedded domains can be clearly determined by means of a method involving cysteine-scanning mutants and their reactivity to *N*-ethylmaleimide. The results for 37 cysteine-scanning mutants around putative transmembrane helix 9 indicate that the residues from 266 to 275 and from 296 to 298 form water extruding loops, while residues from 276 to 295 and from 299 to 302 are embedded in the membrane. This method is generally useful for determination of the transmembrane regions of a polytopic membrane protein.

The bacterial metal−tetracycline/H^+ antiporter (TetA) belongs to the major facilitator family having putative 12 α-helical transmembrane segments (Eckert & Beck, 1989; Allard & Bertrand, 1992). The secondary structure of TetA was first determined by hydropathy analysis, and from the results of limited proteolysis (Eckert & Beck, 1989), immunological examinations (Yamaguchi et al., 1990a), and analyses of PhoA-fused proteins (Allard & Bertrand, 1992). However, none of these approaches can reveal the boundaries between membrane-embedded regions and water-extruding loops. In this paper, we report the determination of such boundaries by means of a new method based on the reactivity to *N*-ethylmaleimide (NEM) of site-directed cysteine-scanning mutants derived from a cysteine-less TetA mutant. The theoretical background of this method is that the reactivity to NEM of a sulfhydryl group embedded in the hydrophobic interior should be lower than that of one exposed to the medium because the molecular species of a sulfhydryl group reactive with NEM is a deprotonated form.

EXPERIMENTAL PROCEDURES

Materials. *N*-([1-^{14}C]Ethyl)maleimide (1.5 GBq/mmol) and [α-^{32}P]dCTP were purchased from DuPont-New England Nuclear and Amersham, respectively. 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 (1985). For the mutagenesis, plasmid pCTC377A was used as a template, in which a Cys377→Ala mutation was introduced into pCT1183 (Someya et al., 1995), which carries the 2.45 kb Tn10 *tetA* and *tetR* gene fragment. 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 *Eco*RI−*Bam*HI fragment of the cysteine-scanning mutant *tetA* genes with the corresponding fragment of the low-copy-number plasmid, pLGT2 (Yamaguchi et al., 1992a) and used for determination of tetracycline resistance.

Preparation of Everted Membrane Vesicles. Everted membrane vesicles were prepared from *Escherichia coli* TG1 (Taylor et al., 1985) cells carrying high-copy-number plasmids, pCTC377A, or its derivatives encoding cysteine-scanning mutants, as described previously (Yamaguchi et al., 1992b).

Assaying of the Reaction of N-([1-^{14}C]Ethyl)maleimide with TetA Proteins. The [^{14}C]NEM-binding experiment was performed as described previously (Yamaguchi et al., 1993, 1994). Briefly, everted membrane vesicles (0.5 mg of protein) were incubated in 100 μL of 50 mM MOPS-KOH (pH 7.0) buffer containing 0.1 M KCl and 0.5 mM *N*-([1-^{14}C]ethyl)maleimide for 5 min at 30 °C. Then the reaction mixture was diluted with 0.9 mL of 50 mM MOPS-KOH (pH 7.0) buffer containing 0.1 M KCl and 10 mM unlabeled NEM, followed by ultracentrifugation. The resultant precipitate was solubilized with 1% Triton X-100 and 0.1% SDS in phosphate-buffered saline containing unlabeled NEM. TetA proteins were precipitated from the soluble materials by successive treatment with anti-TetA carboxyl-terminal peptide antiserum (Yamaguchi et al., 1990a) and Pansorbin (Philipson et al., 1978). The immunoprecipitates were subjected to SDS−polyacrylamide gel electrophoresis. Then gels were soaked in Amplify (as an enhancer; Amersham) prior to being dried. The dried gels were exposed to X-ray film for 40 days at −80 °C.

RESULTS AND DISCUSSION

TetA(B) has only one cysteine residue, at position 377; however, Cys377 does not confer the TetA function because

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan and a Grant-in-Aid for the Promotion of Science of Ciba-Geigy Foundation (Japan).

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

S0006-2960(96)01568-1 CCC: $12.00

Table 1: Tetracycline Resistance Levels of *E. coli* W3104 (Yamamoto et al., 1981) Cells Harboring the Low-Copy-Number Mutant Plasmids

mutants	MIC (μg/mL)[a]	mutants	MIC (μg/mL)[a]	mutants	MIC (μg/mL)[a]
wild-type	200	T276C	200	A290C	200
Cys-less TetA	200	A277C	100	F291C	200
(no plasmid)	0.8	V278C	200	L292C	200
G266C	100	L279C	200	A293C	100
R267C	100	L280C	200	F294C	100
I268C	200	G281C	50	I295C	100
A269C	100	F282C	100	S296C	200
T270C	200	I283C	100	E297C	100
K271C	200	A284C	200	G298C	100
W272C	200	D285C	0.2	W299C	200
G273C	200	S286C	200	L300C	200
E274C	100	S287C	200	V301C	200
K275C	200	A288C	200	F302C	200
		F289C	200		

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

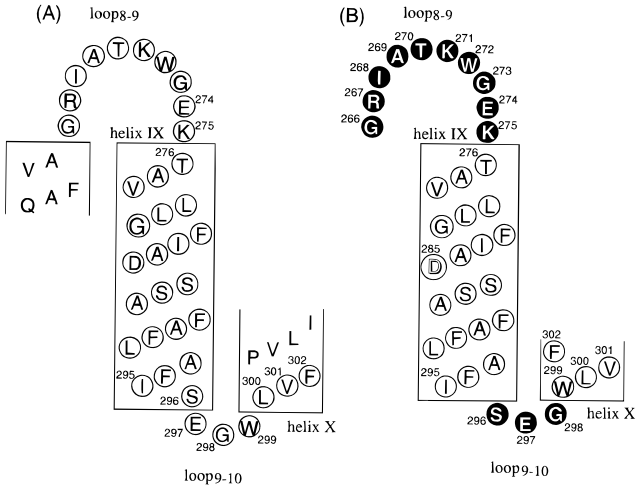

FIGURE 1: The topology of the putative transmembrane helix 9 and its flanking loops of TetA(B). (A) Predicted by hydropathy analysis (Eckert & Beck, 1989). Residues substituted with cysteine in this study are enclosed in circles. (B) Revised topology based on the results of this study. The positions at which the cysteine mutants showed high reactivity to NEM are indicated by closed circles with white letters. The only functionally-essential residue in this region, Asp285, is depicted by an outlined letter.

the Cys377→Ala mutant of TetA (Cys-less TetA) exhibited wild-type resistance against tetracycline (Table 1). We constructed 37 cysteine-scanning mutants, from Gly266 to Phe302, from Cys-less TetA. These residues are located around putative transmembrane segment 9 and its flanking loops (Figure 1A) (Eckert & Beck, 1989), which contain a functionally-essential residue, Asp285 (Yamaguchi et al., 1992a).

The tetracycline resistance levels of transformants with low-copy-number plasmids encoding the cysteine-scanning mutants of Cys-less TetA were measured (Table 1). Among the 37 cysteine-scanning mutants, only the D285C one completely lost the tetracycline resistance, the others retaining the wild-type or significant resistance, indicating that Asp285 is the only essential residue in this region and that the replacement of the residues in this region caused no significant conformational change.

Everted membrane vesicles (Yamaguchi et al., 1992b) were prepared from *Escherichia coli* cells harboring high-copy-number plasmids encoding the cysteine-scanning mutants after induction of *tetA* gene expression. Each lot of mutant vesicles was incubated with *N*-([^{14}C]ethyl)maleimide, followed by solubilization and immunoprecipitation of TetA with an anti-C-terminal peptide antibody. The precipitated materials were subjected to SDS−polyacrylamide gel electrophoresis (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue, dried, and then exposed to X-ray film for 40 days. As shown in Figure 2, NEM highly bound to mutants E274C to K275C, and S296C to G298C. In contrast, it hardly bound to mutants T276C to I295C, and W299C to F302C. The high reactivity to [^{14}C]NEM of mutants G266C to G273C has already been reported previously (Yamaguchi et al., 1993).

The amounts of [^{14}C]NEM bound to the cysteine-scanning TetA mutants were determined with a BAS-2000II Bio-Imaging Analyzer (Fuji Film Co., Tokyo, Japan). Under these conditions, the reactivity to [^{14}C]NEM of each mutant TetA protein was neither saturated nor complete. So, the amount of binding reflects the reactivity to NEM of the sulfhydryl groups of the mutants. Figure 3 shows the number of [^{14}C]NEM molecules bound to a molecule of each mutant TetA. The numbers of bound NEM molecules per molecule of the mutant TetA (bNEM/TetA) from G266C to E274C, under these conditions, approximately ranged between 0.4 and 0.8. The maximum difference in this region was within a factor of 2. Then, the bNEM/TetA values discontinuously changed between K275C and T276C. The former and latter values were about 0.38 and 0.08, respectively. So, it was indicated that the reactivity was almost 5-fold decreased at this point. The low reactivity continued from T276C to I295C. In this region, the bNEM/TetA values were around or less than 0.1. There was a second discontinuity in the reactivity between I295C and S296C. The bNEM/TetA values of I295C and S296C were 0.09 and 0.46, respectively, showing an about 5-fold increase. Three mutants, S296C to G298C, showed relatively high reactivity, and then the reactivity again discontinuously decreased to 0.03 for the W299C mutant. In summary, there were three discontinuous points between G266C and F302C, representing the boundaries between the membrane-embedded regions and the water-extruding loops. These results clearly indicated that the regions of Gly266 to Lys275, and Ser296 to Gly298, comprise the water-extruding loops, and the region of Thr276 to Ile295 and the residues downstream from Trp299 comprise the membrane-embedded segments.

Among the mutants in the membrane-embedded regions, the reactivity of the F282C mutant and that of the mutants around Asp285 (D285C, S286C, S287C, and A288C) were relatively higher than that of the other mutants. Asp285 is the only essential residue in this region, and Phe282 is the residue located on the same side of Asp285 at position one α-helical turn upward in this helix. Asp285 plays an important role in the substrate binding (Kimura & Yamaguchi, 1996). Therefore, the moderate reactivity of the mutants around these residues may indicate the presence of the water-filled cavity comprising the substrate-binding site. Some periodic appearance of the moderately reactive mutants between T276C and D285C seems to be present, probably suggesting the presence of water-filled channel along the helix up to the substrate-binding site.

It should be noted that [^{14}C]NEM molecules have to penetrate into the interior of everted vesicles through the

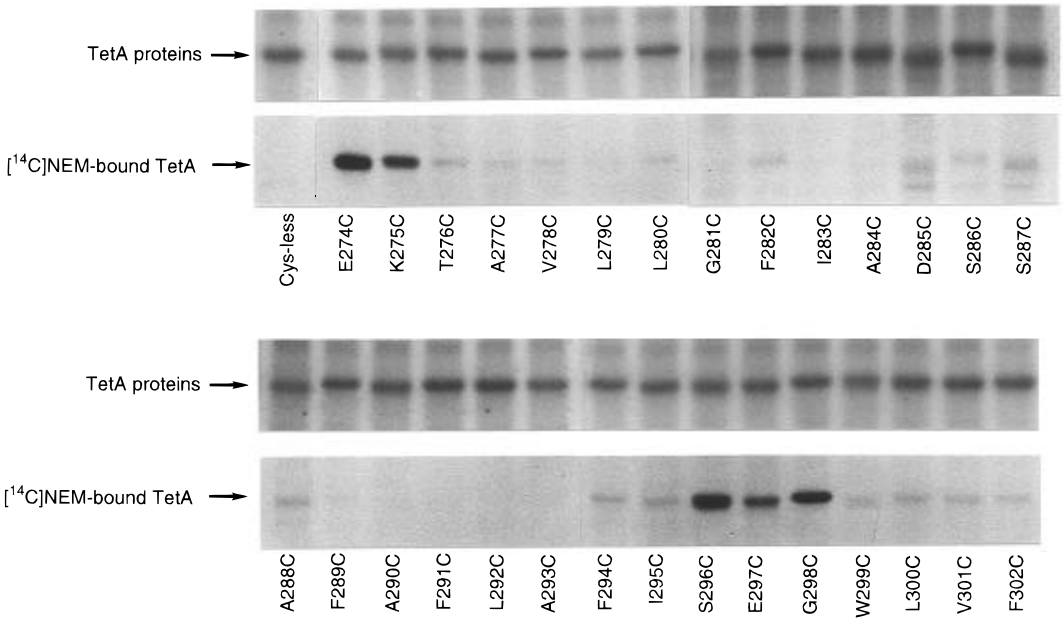

FIGURE 2: The binding of *N*-([14C]ethyl)maleimide to the cysteine-scanning mutants. Everted membrane vesicles (0.5 mg of protein) containing cysteine-scanning mutants of TetA proteins were incubated with 0.5 mM [14C]NEM for 5 min at 30 °C, followed by solubilization and immunoprecipitation of TetA proteins as described under the Experimental Procedures. After SDS−PAGE, gels were dried and exposed to X-ray film for 40 days at −80 °C. Upper panels for each mutant are TetA proteins stained with Coomassie Brilliant Blue. Lower panels are autoradiographs of [14C]NEM-bound TetA proteins.

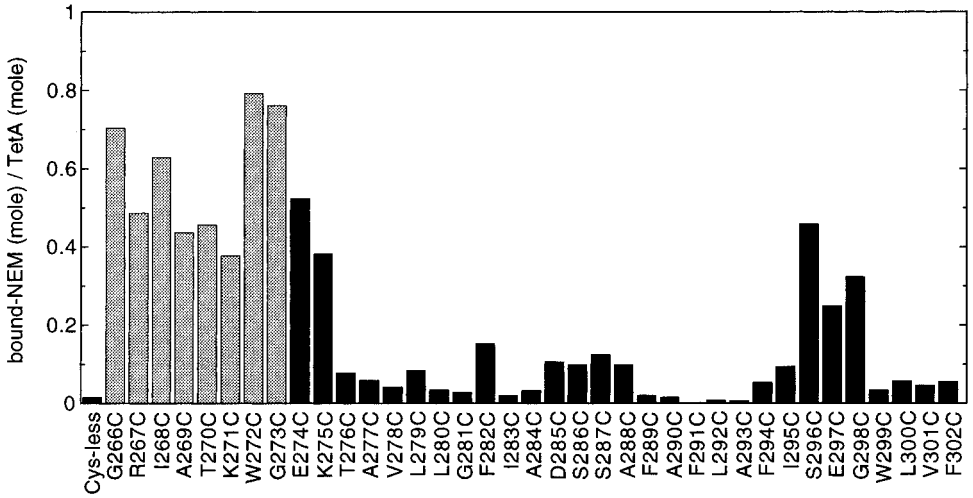

FIGURE 3: Relative amounts of *N*-([14C]ethyl)maleimide bound to TetA. The amount of bound [14C]NEM was calculated from the radioactivity of the band quantified with a BAS-2000II Bio-Imaging Analyzer (Fuji Film Co., Tokyo, Japan). The amount of TetA in the band was calculated from the intensity of the Coomassie Brilliant Blue-stained band measured with a dual-wavelength TLC scanner (Shimadzu, Kyoto, Japan). The levels of NEM binding to mutants G266C to G273C were cited from the previous paper (Yamaguchi et al., 1993), which were depicted as faintly-shadowed bars. Two series of experiments gave similar results.

cytoplasmic membrane prior to reaction with the residues located on the periplasmic side. The relatively lower reactivity of the second loop region mutants Ser296 to Gly298, than that of the first loop region mutants, may be due to the sidedness of these loop regions across the membrane.

Compared with the original topology (Figure 1A), the water-extruding loop between Gly266 to Lys275 is exactly consistent with $loop_{8-9}$, and the membrane-embedded region from Thr276 to Ile295 corresponds to transmembrane segment 9, which appears to be only one residue shorter than the original one (Figure 1B). $Loop_{9-10}$ is between Ser296 and Gly298 instead of between Glu297 and Trp299 in the original one. These results suggest that the reactivity to NEM of cysteine-scanning mutants of Cys-less polytopic membrane proteins is clearly useful for determining the exact boundaries between the membrane-embedded regions and the water-extruding loops. It is striking that the result of secondary structure prediction based on that of hydropathy analysis is highly consistent with the result of NEM-binding experiments, which appears to confirm the fundamental validity of the 12-transmembrane structure of TetA(B).

We have found that the TetA(B) has two essential acidic residues, Asp66 and Asp285 (Yamaguchi et al., 1990b, 1992a). The results in this paper also confirm that Asp285 is actually in the middle of the membrane bilayer, whereas Asp66 is in the hydrophilic loop region (Yamaguchi et al., manuscript in preparation). In general, *phoA*-fusion approach is often used for topology determination of membrane proteins. Tetracycline/H^+ antiporter was also analyzed using

TetA-PhoA fusions of TetA(C) (tetracycline resistance protein encoded by the plasmid pBR322), supporting a TetA topology model consisting of 12 transmembrane segments (Allard & Bertrand, 1992). But some fusion proteins in which the fusion junctions were near the middle of transmembrane segments gave the same expected PhoA activity as the other fusion proteins having fusion junctions in the hydrophilic regions. Hence, our approach based on the reactivity to NEM of Cys mutants has an advantage in that it can determine the position of each amino acid residue in detail and in that it can use any membrane vesicles other than *E. coli* membranes, over the approaches of fusion protein analysis.

The topology of the human MDR protein was also examined with a cysteine-less mutant by reintroducing cysteine residues into predicted extracellular or cytoplasmic loops, and using membrane-permeant and -impermeant thiol-specific reagents (Loo & Clarke, 1995). However, determination of the topology of a protein based on the reactivity of a cysteine-scanning mutant with NEM is reported for the first time in this paper. Wu et al. (1995a) reported that a few lactose permease mutants with a single Cys residue at the periplasmic end of putative helix I were readily labeled with [^{14}C]NEM, while the other transmembrane Cys mutants reacted less effectively. In our case, there were certainly some differences in the reactivity with [^{14}C]NEM between Cys residues in the putative membrane-embedded region; however, the reactivity of the putative transmembrane Cys residues was clearly less than the reactivity of those located on the putative loop region.

In bacteriorhodopsin and rhodopsin, Altenbach et al. (1990) and Farahbakhsh et al. (1995) tried to determine the boundaries of membrane-embedded domains by means of a method based on the combination of site-specific mutagenesis and nitroxide spin labeling. Their method involves purification and reconstitution of the membrane proteins. The method we described in this paper can be performed using intact membranes, because the [^{14}C]NEM-bound carrier can be specifically precipitated with an antibody after the binding reaction. This method is expected to be applicable to other polytopic membrane proteins as a general method for determining their exact topologies. A recent study on the lactose permease showed that site-directed chemical cleavage could determine the helix−helix packing *in situ* (Wu et al., 1995b). Combination of the approaches illustrated above, such as the site-directed chemical cleavage (Wu et al., 1995b), site-directed labeling by membrane-permeant and -impermeant thiol reagents (Loo & Clarke, 1995), and comparing the reactivity of NEM to the cysteine-scanning mutants (this study), will be able to determine the three-dimensional structure of intrinsic membrane proteins without purification, crystallization, and X-ray crystallography.

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BI961568G