Cysteine-scanning mutagenesis around transmembrane segment VI of Tn10-encoded metal-tetracycline/H^+ antiporter

Satoko Konishi[a,b], Shinobu Iwaki[a,b], Tomomi Kimura-Someya[a,b], Akihito Yamaguchi[a,b,c,*]

[a] *Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan*
[b] *Faculty of Pharmaceutical Science, Osaka University, Suita, Osaka 565-0871, Japan*
[c] *CREST, Japan Science and Technology Corporation, Osaka, Japan*

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Abstract Each amino acid in putative transmembrane helix VI and its flanking regions, from Ser-156 to Thr-185, of a Cys-free mutant of the Tn10-encoded metal-tetracycline/H^+ antiporter (TetA(B)) was individually replaced by Cys. All of the cysteine-scanning mutants showed a normal level of tetracycline resistance except for the S156C mutant, which showed moderate resistance, indicating that there is no essential residue located in this region. All 20 mutants from S159C to W178C showed no reactivity with *N*-ethylmaleimide (NEM), whereas the mutants of the flanking regions from S156C to H158C and F179C to T185C were highly or moderately reactive with NEM. These results indicate that like transmembrane helices III and IX, the transmembrane helix VI comprising residues Ser-159–Trp-178 is totally embedded in the hydrophobic environment.

Key words: Cysteine-scanning; Transmembrane helix; Tetracycline

1. Introduction

The transposon Tn10-encoded metal-tetracycline/H^+ antiporter (TetA(B)) is a typical tetracycline exporter of Gram-negative bacteria [1,2], of which the transport mechanism and molecular structure have been studied as a paradigm of bacterial drug efflux transporters [3]. The 12 transmembrane structure of TetA(B) has been established by site-directed competitive chemical modification of cysteine-introduced mutants on the basis of a cysteine-free TetA(B) [4]. We also showed that the reactivity of cysteine-scanning mutants of the cysteine-free TetA(B) with a sulfhydryl reagent, *N*-ethylmaleimide (NEM), is useful for determining the precise boundaries between the membrane-embedded segments and the water-exposed loop regions [5,6]. Some transmembrane segments such as TM III and TM IX are totally embedded in the hydrophobic interior of the membrane. In these segments, about 20 cysteine-scanning mutants are continuously non-reactive with NEM, whereas mutants of the flanking regions are generally reactive with NEM. The presence of such totally embedded segments has recently been found for other polytopic membrane proteins such as erythrocyte anion exchanger [7] and the bacterial multidrug exporter (EmrE) [8], also on the basis of chemical modification of cysteine-scanning mutants. Recently, we found that some transmembrane segments such as TM II had no continuous non-reactive region [9]. Instead of a continuous non-reactive region, TM II showed the periodic appearance of non-reactive positions. The cycle of reactive and non-reactive positions was consistent with the α-helical turn of a polypeptide chain. The transmembrane NEM-reactive positions could be distinguished from the membrane surface positions by the fact that the former Cys mutants were generally inactivated by NEM, while the activities of the latter mutants were not affected by NEM binding except at a few hot spots [10]. The periodic reactivity of TM II with NEM indicates that one side of the helix faces a water-filled transmembrane channel. Although this water-filled channel must be interrupted in the middle in order to avoid uncoupling, the interruption site seems to be too short to be detected by the chemical reactivity. Anyway, it is of interest to determine which transmembrane helices face the channel.

In this study, we chose transmembrane helix VI for cysteine-scanning mutagenesis. The presence of a close conformational linkage between this transmembrane helix and the putative cytoplasmic gating region, loop 2-3, was predicted because the second-site mutation at Thr-171, which is located in the middle of TM VI, suppresses the first charge-neutralized mutation of Arg-70, which is a functionally important residue located in the putative gating region, loop 2-3 [11].

2. Materials and methods

2.1. Materials

[^{14}C]NEM (1.5 GBq/mmol) was purchased from NEN Life Science Products. All other materials were of reagent grade and obtained from commercial sources.

2.2. Site-directed mutagenesis

Cysteine-scanning mutants were constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel [12] using synthetic oligonucleotides. For the mutagenesis, plasmid pCT1183 [13], 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 the *Eco*RV-*Eco*RI fragment of the cysteine-scanning mutant *tetA*(B) genes with the corresponding fragment of the low-copy number plasmid, pLGC377A, which encodes a cysteine-less mutant TetA(B) [5], and then used for *tetA*(B) gene expression.

2.3. Determination of tetracycline resistance

Tetracycline resistance was determined by a 2-fold agar dilution method as described previously [14] and expressed as the minimum inhibitory concentration.

2.4. Assay of the reaction of [^{14}C]NEM 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 from *Escherichia coli* W3104 cells carrying pLGC377A or

*Corresponding author. Fax: (81) (6) 6879-8549.
E-mail: akihito@sanken.osaka-u.ac.jp

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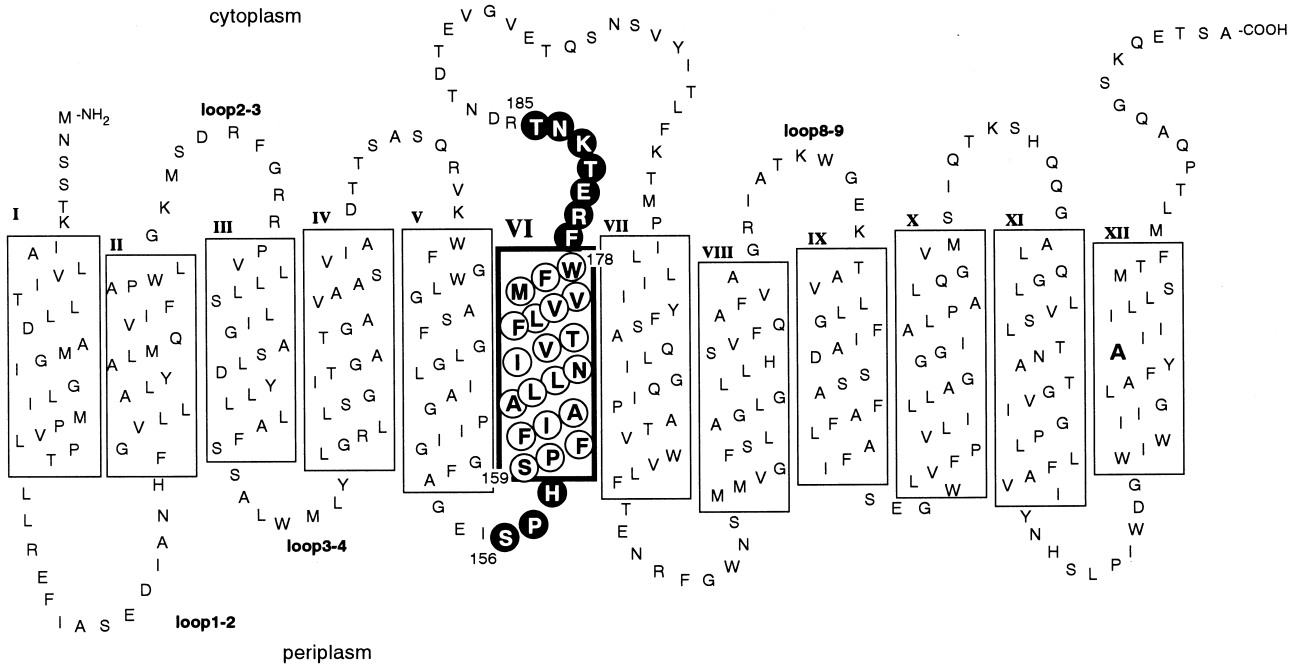

Fig. 1. A secondary structure model of Tn10-TetA(B) and the reactivity of the cysteine-scanning mutants with [^{14}C]NEM. Each of the encircled bold-letter residues was replaced with Cys in the Cys-free TetA(B) and the reactivity with [^{14}C]NEM was examined. Black circles, residues reactive with NEM; open circles, residues not reactive with NEM.

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% sodium dodecyl sulfate (SDS) in phosphate-buffered saline containing 5 mM unlabelled NEM and then, TetA(B) proteins were immunoprecipitated with anti-TetA(B) C-terminal peptide antiserum [15] and Pansorbin *Staphylococcus aureus* cells [16]. The TetA(B) protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE), 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, Tokyo, Japan).

3. Results

3.1. Tetracycline resistance of cysteine-scanning mutants

In our previous study [4], it was revealed that Ser-156 and Ser-199 are located on the outside and inside surfaces of the cytoplasmic membrane, respectively. Therefore, the polypeptide chain of TetA(B) must cross the membrane at least once between positions 156 and 199 (Fig. 1). In order to determine the transmembrane region, we constructed 30 cysteine-scanning mutants from Ser-156 to Thr-185. When the expression of the cysteine mutants was detected by Western blotting using anti-C-terminal peptide antiserum, they were found to be normally expressed compared with the wild-type (data not shown). The tetracycline resistance levels of *E. coli* cells containing these mutants were measured by the agar dilution method (Table 1). All mutants showed the same resistance as the wild-type TetA(B) (200 μg/ml) except for the S156C mutant, which showed moderate resistance (25 μg/ml), indicating that there are no functionally essential residues in this region. This observation is consistent with the previous conclusion that the suppression of the mutation of Arg-70 by the second-site mutation of Thr-171 was not due to a functional substitution but due to a remote-conformational distortion [11].

Table 1
Tetracycline resistance levels of *E. coli* cells harboring plasmids encoding wild-type or cysteine-scanning mutant TetA(B) proteins

Mutants	MIC (μg/ml)[a]
No plasmid	0.8
Wild	200
S156C	25
P157C	200
H158C	200
S159C	200
P160C	200
F161C	200
F162C	200
I163C	200
A164C	200
A165C	200
L166C	200
L167C	200
N168C	200
I169C	200
V170C	200
T171C	200
F172C	200
L173C	200
V174C	200
V175C	200
M176C	200
F177C	200
W178C	200
F179C	200
R180C	200
E181C	200
T182C	200
K183C	200
N184C	200
T185C	200

[a]MIC: Minimum inhibitory concentration for cell growth.

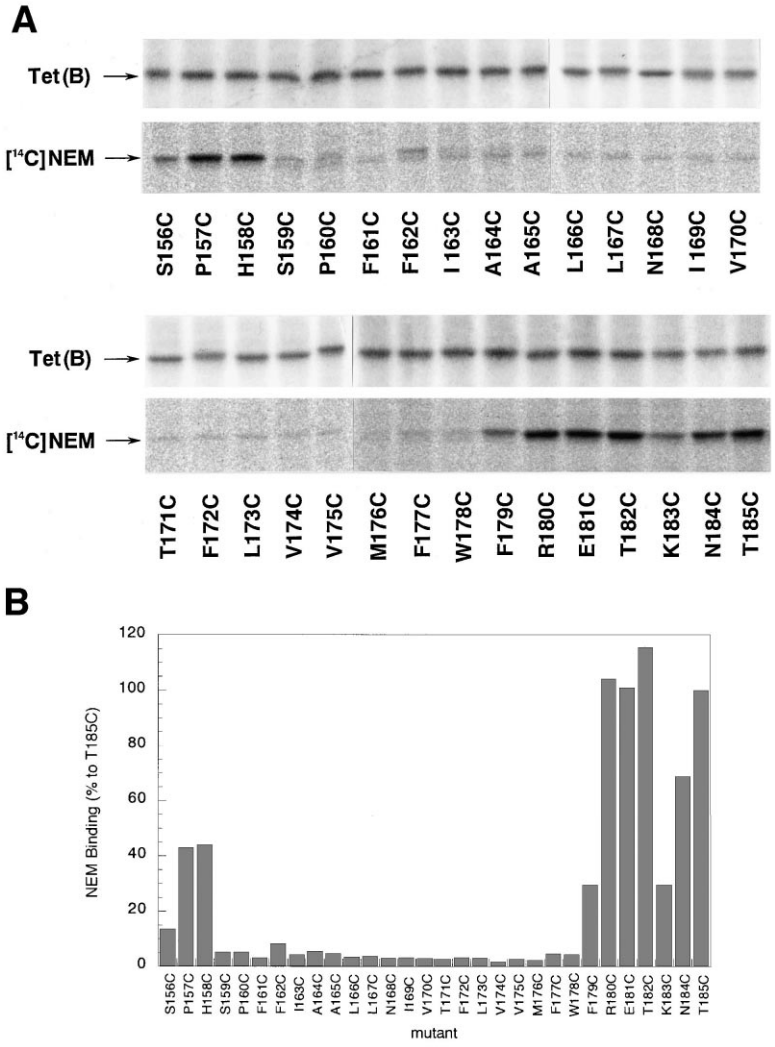

Fig. 2. The binding of [^{14}C]NEM to the cysteine-scanning mutants of Tn10-TetA(B). Sonicated membranes (0.5 mg protein) from bacteria harboring the cysteine-scanning mutants were incubated with 0.5 mM [^{14}C]NEM for 5 min at 30°C, followed by solubilization and immunoprecipitation of TetA(B) proteins as described under Section 2. (A) After SDS-PAGE, the protein bands were visualized by Coomassie brilliant blue staining (upper panels) and the radioactivity by use of a Bio-Imaging Analyzer BAS-1000 (lower panels), respectively. (B) Relative amounts of bound [^{14}C]NEM calculated from band densities in A.

3.2. [^{14}C]NAM binding to cysteine-scanning mutants

The binding of [^{14}C]NEM to the cysteine-scanning mutants S156C to T185C was examined (Fig. 2). The reactivity of the P157C and H158C mutants was very high and that of the S156C mutant was moderate. Since Ser-156 was confirmed to be located on the periplasmic surface in our previous study, the region including these three residues is a part of the periplasmically exposed loop region. The seven mutants from F179C to T185C also showed high reactivity with NEM, except for F179C and K183C, which showed moderate reactivity. In contrast, the 20 cysteine-scanning mutants from S159C to W178C showed almost no reactivity with NEM, indicating that this region is totally embedded in the hydrophobic environment. The 20 amino acid length of this region is consistent with the length required for crossing the hydrophobic core of the lipid bilayer membrane once in the form of an α-helix. Thr-171 is located just in the middle of this transmembrane region, as predicted by hydropathy analysis.

4. Discussion

In this study, we showed that transmembrane segment VI of TetA(B) is a helix totally embedded in the hydrophobic interior of the membrane, as are TM III [6] and TM IX [5]. This indicates that the total embedding of some transmembrane segments in the hydrophobic environment is not an exception for a few transmembrane segments of TetA(B), but rather one of the general rules for transmembrane segment construction. Although TM III and TM IX contain functionally essential aspartic acid residues, Asp-84 and Asp-285 [17], TM VI does not contain any essential residues. In addition, the TM VI region contains neither glycine nor proline residues in spite of the general abundance of these residues in the other transmembrane regions of TetA(B), suggesting that TM VI forms a straight helical structure without a kink or bend.

The reactivity of maleimide derivatives with SH groups is useful for determining whether or not the SH groups are ex-

posed to an aqueous phase, because deprotonation of a SH group is necessary for its nucleophilic attack against a nitrogen atom of a maleimide ring. On the basis of the reactivity of maleimide derivatives, transmembrane segments that are totally embedded in the hydrophobic milieu of the membrane were also found in other transporters, such as the erythrocyte anion exchanger [7] and the bacterial small multidrug exporter, EmrE [8]. In the case of EmrE, none of the residues in putative transmembrane segments reacted with NEM. On the other hand, in both lactose permease [18] and UhpT [19], NEM-reactive residues are certainly present in the vicinity of the binding site for hydrophilic substrates. In our TetA(B) protein, there are both types of transmembrane segments. TM III, VI and IX are totally embedded in the hydrophobic milieu [5,6] and TM II faces a water-filled cavity [9]. These results are also consistent with a model of the arrangement of transmembrane segments within the major facilitator superfamily transporters on the basis of hydropathy analysis [20].

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