Cysteine-scanning Mutagenesis around Transmembrane Segment III of Tn10-encoded Metal-Tetracycline/\(H^+\) Antiporter*

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Tomomi Kimura‡, Yasuko Shiina§, Tetsuo Sawai§, and Akihito Yamaguchi¶

From the Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka 565, Japan, and the Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Osaka University, Yamadaoka, Suita, Osaka 565, Japan

Each amino acid in the putative transmembrane helix III and its flanking regions (from Gly-62 to Tyr-98) of the Tn10-encoded metal-tetracycline/\(H^+\) antiporter (Tet(B)) was individually replaced with Cys. Out of these 37 cysteine-scanning mutants, the mutants from G62C to R70C and from S92C to Y98C showed high or intermediate reactivity with \(^{14}\)C-ethylmaleimide (NEM) except for the M64C mutant. On the other hand, the mutants from R71C to S91C showed almost no reactivity with NEM except for the P72C mutant. These results confirm that the transmembrane helix III is composed of 21 residues from Arg-71 to Ser-91. The majority of Cys replacement mutants retained high or moderate tetracycline transport activity. Cys replacements for Gly-62, Asp-66, Ser-77, Gly-80, and Asp-84 resulted in almost inactive Tet(B) (less than 3% of the wild-type activity). The Arg-70 \(\rightarrow\) Cys mutant retained very low activity due to a mercapptide between Co\(^{2+}\) and a SH group (Someya, Y., and Yamaguchi, A. (1996) Biochemistry 35, 9385–9391). Three of these six important residues (Ser-77, Gly-80, and Asp-84) are located in the transmembrane helix III and one (Arg-70) is located in the flanking region. These four functionally important residues are located on one side of the helical wheel. Only two of the residual 31 Cys mutants were inactivated by NEM (S65C and L97C). Ser-65 and Leu-97 are located on the cytoplasmic and periplasmic loops, respectively, in the topology of Tet(B). The degree of inactivation of these Cys mutants with SH reagents was dependent on the volume of substituents. In the presence of tetracycline, the reactivity of the S65C mutant with NEM was significantly increased, in contrast, the reactivity of L97C was greatly reduced, indicating that the cytoplasmic and periplasmic loop regions undergo substrate-induced conformational change in the mutually opposite direction.

The transposon Tn10-encoded Tet(B) protein is one of the family of bacterial tetracycline exporters of Gram-negative bacteria that includes Tet(A) to (H) (1). It is a polytopic cytoplasmic membrane protein that catalyzes stoichiometric metal-tetracycline/\(H^+\) antiport (2–4). It is composed of 401 amino acid residues (5, 6). Tet(B) belongs to a major facilitator superfamily (7) that includes uniporters and symporters in addition to bacterial multidrug exporters such as NorA (8) and Bmr (9). Most of the major facilitator family transporters are predicted to have a 12-membrane-spanning structure (7). With respect to tetracycline/\(H^+\) antiporters, Eckert and Beck (10) and Henderson and Maiden (11) proposed similar 12-membrane-spanning topologies of Tet(B) and pBR322-Tet(C) based on the results of hydropathy analysis. Allard and Bertrand (12) experimentally confirmed the topology of pBR322-Tet(C) by means of alkaline phosphatase (PhoA) gene fusions. Recently, we experimentally established the 12-membrane-spanning structure of Tet(B) by using the intact protein on the basis of the results of the competitive binding of membrane permeable and impermeable SH reagents to the cysteine residues introduced by site-directed mutagenesis (13).

The cysteine-scanning mutagenesis of lactose permease (14–22) and other membrane transporters (23) has been extensively studied and revealed to be useful for determination of the distribution of functionally essential residues and the arrangement of transmembrane helices (24, 25). We showed that the reactivity of the cysteine-scanning mutants of membrane proteins with NEM\(^1\) is useful to determine the range of the transmembrane segments and the exact boundaries between membrane-embedded regions and the loop regions exposed to the aqueous phase (26). We reported the exact range of the transmembrane helix IX and experimentally showed the presence of a short loop composed of three amino acid residues between helices IX and X exposed to the aqueous phase (26).

In this study, we established cysteine-scanning mutants around the putative helix III and its flanking regions. Tet(B) contains a conserved sequence motif, GXXXDXXGRR, in the putative cytoplasmic loop 2–3, which is common to the major facilitator superfamily transporters (7). Site-directed mutagenesis studies of Tet(B) suggest that the region containing this motif plays a role as an entrance gate for tetracycline translocation (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29). In addition, there is an Asp-84 in the middle of helix III, which is one of three essential transmembrane aspartic acid residues (30). The conserved quartet of residues is located around this motif (27). The importance of this motif was also confirmed in \(\alpha\)-ketoglutarate permease (28) and lactose permease (29).
EXPERIMENTAL PROCEDURES

Materials—N-[1-14C]ethylmaleimide ([14C]NEM) (1.5 GBq/mmol) and [7,3H]tetracycline were purchased from NEN Life Science Products. [α-32P]dCTP was purchased from Amersham Corp. 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 (32). For the mutagenesis, plasmid pCT1183 (33) was used as a template that carries the 2.45-kilobase Tn10 (tetAB) and tetR gene fragments. Mutations were detected based on 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 EcoRV-EcoRI fragment of the cysteine-scanning mutant tetA(B) genes with the corresponding fragment of the low copy number plasmid, pLGT2 (30), and used for determination of the reactivity with [14C]NEM.

Assay of the Reaction of [14C]NEM with Tet(B) Proteins—Sonicated membranes were prepared from Escherichia coli W3104 carrying pLGT2 or its derivatives as described previously (34) except that the cells were disrupted by brief sonication. The reaction of [14C]NEM with Tet(B) proteins in the sonicated membranes were performed as described previously (34). After solubilization, the [14C]NEM-bound Tet(B) proteins were immunoprecipitated with anti-Tet(B) C-terminal peptide antiserum (35) and Pansorbin Staphylococcus aureus cells (36). The resultant pellet was subjected to SDS-polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. The resultant membranes were solubilized in 1% Triton X-100 and 0.1% SDS, and then Tet(B) proteins were precipitated with C terminus-specific antiserum (35). After SDS-gel electrophoresis, Tet(B) proteins were detected by Coomassie Brilliant Blue staining (Fig. 2). There was no significant radioactive band detected in any lane. The result is a typical one out of at least three independent experiments. Since the density of the radioactive bands of the mutant proteins was increased in proportion to the incubation time with [14C]NEM, at least up to 10 min (data not shown), and wild-type Tet(B) did not show any radioactive band, the density reflects the reactivity of each Cys residue introduced in Tet(B). As shown in Fig. 2, Cys residues mutated into positions 62, 63, from 65 to 69, from 92 to 94, and 97 were highly reactive with NEM, and those introduced into positions 70, 72, 95, 96, and 98 showed moderate reactivity, indicating that the former residues are exposed to the aqueous phase and the latter ones are partially exposed. In contrast, Cys residues introduced into positions 64, 71, and from 73 to 91 showed almost no significant reactivity with NEM, indicating that these residues are embedded in the hydrophobic interior.

RESULTS

Construction of Cysteine-scanning Mutants—Each amino acid in the putative transmembrane helix III and its flanking regions (from Gly-62 to Tyr-98) (Fig. 1) was individually replaced with Cys by site-directed mutagenesis as described under “Experimental Procedures” using the mutagenic primers. Mutants from F72C to Y98C were constructed in this study, whereas those from G62C to R71C were constructed previously (27, 34, 38, 39). Membranes were prepared by brief sonication of E. coli W3104/pLGT2 after induction of tetA(B) gene expression. Resulting membranes were solubilized in 1% Triton X-100 and 0.1% SDS, and then Tet(B) proteins were precipitated with C terminus-specific antiserum (35). After SDS-gel electrophoresis, Tet(B) proteins were detected by Coomassie Brilliant Blue staining (Fig. 2A). There was no significant alteration observed in the level of expression of the Cys-scanning mutants.

Reactivity of the Cys-Scanning Mutants with [14C]NEM—The [14C]NEM binding to the Cys-scanning mutants is shown in Fig. 2. The result is a typical one out of at least three independent experiments. Since the density of the radioactive bands of the mutant proteins was increased in proportion to the incubation time with [14C]NEM, at least up to 10 min (data not shown), and wild-type Tet(B) did not show any radioactive band, the density reflects the reactivity of each Cys residue introduced in Tet(B). As shown in Fig. 2, Cys residues mutated into positions 62, 63, from 65 to 69, from 92 to 94, and 97 were highly reactive with NEM, and those introduced into positions 70, 72, 95, 96, and 98 showed moderate reactivity, indicating that the former residues are exposed to the aqueous phase and the latter ones are partially exposed. In contrast, Cys residues introduced into positions 64, 71, and from 73 to 91 showed almost no significant reactivity with NEM, indicating that these residues are embedded in the hydrophobic interior.

Helix III Cys Mutants of Tetracycline/H+ Antiporter

As judged by these observations, the transmembrane helix III is likely to range from Arg-71 to Ser-91. Out of nine residues of the putative cytoplasmic loop 2–3, only Met-64 is very slightly reactive with NEM as reported in our previous paper (27, 40), probably due to the fact that the side chain of this position is oriented to the protein interior. On the other hand, there was no moderately reactive position in the mid-transmembrane helix region.

Tetracycline Transport Activity of the Cys-Scanning Mutants—The initial rate (for a period of 30 s) of the tetracycline uptake by everted membrane vesicles containing each Cys-scanning mutant was measured (Fig. 3). The majority of the Cys-scanning mutants exhibited high or moderate tetracycline transport activity (>25% of the wild-type). Only three mutants (D66C, G80C, and D84C) exhibited complete loss of the activity. Two mutants (G62C and S77C) exhibited greatly reduced activity (less than 3% of the wild-type). Another one mutant (R70C) showed significant activity (about 8% of the wild-type); however, since other Arg-70 mutants, except for R70K and R70C, showed no activity (39), Arg-70 is also a functionally important residue. As reported previously, the residual activity of the R70C mutant is due to the mercaptide formed between Co2+ and the SH group acting as a functional positive charge (39). Thus, out of 37 residues of helix III and its flanking regions, only six residues are important for tetracycline transport. The importance and the possible roles of these six residues have been investigated in detail in our previous studies (27, 30, 31, 41).

Effect of NEM on Tetracycline Transport Activity—The initial rate of tetracycline uptake by each vesicle after 5 min of treatment with 2 mM NEM is indicated as a percentage of that
in the absence of NEM (Fig. 5). Fig. 5 shows the results for 31 Cys-scanning mutants that showed significant tetracycline transport activity in the absence of NEM. Out of these mutants, the transport activities of only two (S65C and L97C) were greatly inactivated by NEM. On the other hand, the activities of the other 29 Cys mutants were hardly affected by NEM. Although the K63C, R67C to G69C, and S92C to L94C mutants were highly reactive with NEM and the P72C, W95C, M96C, and Y98C mutants were moderately reactive as shown in Fig. 2, the tetracycline transport activities of all these mutants were not inhibited by NEM. This result indicates that positions 65 and 97 are unique hot spots for chemical modification.

Fig. 6 shows the dependence of inactivation of the transport activity of the S65C and L97C mutants on the concentration of SH reagents. As reported in our previous paper (37, 40, 42), the

**FIG. 2.** The binding of [14C]NEM to the cysteine-scanning mutants of Tn10-Tet(B). The sonicated membranes (0.5 mg of protein) of cysteine-scanning mutants were incubated with 0.5 mM [14C]NEM for 5 min at 30 °C, followed by solubilization and immunoprecipitation of Tet(B) proteins as described under “Experimental Procedures.” A, after SDS-PAGE, the protein bands and the radioactive bands were visualized by Coomassie Brilliant Blue staining (upper panels) and by use of a Bio-Imaging Analyzer BAS-1000 (lower panels), respectively. B, relative amount of bound [14C]NEM per Tet(B) protein calculated from band densities in panel A.

**FIG. 3.** Tetracycline transport activity of cysteine-scanning mutants of Tet(B). Initial rate of tetracycline uptake by everted membrane vesicles containing cysteine-scanning mutants was measured for 30 s in the presence of 10 μM [3H]tetracycline and 50 μM CoCl2 as described under “Experimental Procedures.”

**FIG. 4.** Helical wheel projection of residues in the N-terminal half of the putative helix III. Residues of Cys mutants that showed greatly reduced activity are highlighted by a dark background.
transport activity of the S65C mutant was completely inactivated by NEM at less than 1 mM, whereas the inactivation of the activity of the S65C mutant with methyl methanethiosulfonate (MMTS), which substitutes a small thiomethyl group, was saturated at the level of 40%. Since the activity of the MMTS-pretreated S65C mutant was no longer inactivated by NEM, it is clear that each molecule of the thiomethylated S65C mutant retains 60% activity (Fig. 6A). In the case of the L97C mutant, the maximum inactivation level by NEM was about 90%, which was reached at 2 mM MMTS for 5 min, and then the incubation was continued in the presence of indicated concentrations of NEM for 5 min. The initial rate of tetracycline transport was measured for 30 s as described under “Experimental Procedures.” Residual activity is presented as a percentage of the initial rate measured in the absence of NEM.

The Effect of Tetracycline on Reactivity of the Hot Spot Mutants with NEM—The substituent-volume dependence of the inactivation level of the activity of the S65C and L97C mutants with NEM suggests that these residues are located in the substrate translocation pathway. Thus, the effect of tetracycline on the reactivity of these mutants with [14C]NEM was investigated. Tetracycline significantly stimulates the reactivity of the S65C mutant with NEM as reported previously (43). In contrast, the reactivity of the L97C mutant with NEM was greatly inhibited by tetracycline (Fig. 7), indicating that the degree of exposure of the side chain at position 97 is reduced by the substrate-induced conformational change. When a D66A mutation was added to the L97C mutant, the substrate-induced change in the reactivity with NEM disappeared. On the other hand, the D66A/L97C double mutants retained the substrate-induced conformational change (Fig. 7). This results indicate that Asp-66 confers to the substrate-induced conformational change while Arg-70 may contribute to the step after the conformational change.

DISCUSSION

The results presented here confirm that the membrane-embedded regions and the aqueous phase-exposed regions can be distinguished on the basis of the reactivity of the cysteine-scanning mutants with NEM as reported previously (26). Out of 37 cysteine-scanning mutants established in this study, the 21 mutants barely reactive with NEM were clustered from Arg-71 to Ser-91, a region which corresponds to the putative transmembrane helix III. Several isolated barely reactive po-
sitions were presented in the aqueous phase-exposed regions, whereas there was no reactive positions in the transmembrane regions. The majority of the mutants retained transport activity. Only six mutants showed loss of or greatly reduced activity. Two NEM-sensitive hot spots were found in the water-exposed regions.

Cysteine-scanning mutants of lac permease were extensively studied by Kaback and his co-workers (14–22). The important results revealed from their studies are: 1) there are very few essential residues present in the permease, 2) NEM inactivates the transport activity of a few of these cysteine-scanning mutants, and 3) NEM-sensitive positions lie on one face of a putative transmembrane helix. Yan and Maloney (23) also reported that some cysteine mutants in the putative transmembrane segments of G6P/Pi antiporter (UhpT) are sensitive to SH reagents.

Two NEM-sensitive hot spots were found in the water-exposed regions. The majority of the mutants retained transport activity of a few of these cysteine-scanning mutants, and 3) NEM-sensitive positions lie on one face of a putative transmembrane helix. Yan and Maloney (23) also reported that some cysteine mutants in the putative transmembrane helix IX were barely reactive with NEM while the mutants of the flanking hydrophilic loop of the putative transmembrane helix IX were greatly stimulated in the presence of TDG due to the substrate-

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