Roles of Conserved Arginine Residues in the Metal−Tetracycline/H^+ Antiporter of *Escherichia coli*†

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Received December 31, 1997; Revised Manuscript Received February 18, 1998

ABSTRACT: Seven arginine residues are conserved in all the tetracycline/H^+ antiporters of Gram-negative bacteria. Four (Arg67, -70, -71, and -127) of them are located in the putative cytoplasmic loop regions and three (Arg31, -101, and -238) in the putative periplasmic loop regions [Eckert, B., and Beck, C. F. (1989) *J. Biol. Chem. 264*, 11663−11670]. These arginine residues were replaced by alanine, lysine, or cysteine one by one through site-directed mutagenesis. None of the mutants showed significant alteration of the protein expression level. The mutants resulting in the replacement of Arg31, Arg67, Arg71, and Arg238 with either Ala, Cys, or Lys retained tetracycline resistance levels comparable to that of the wild type. Among them, only the Arg238 → Ala mutant showed very low transport activity in everted membrane vesicles, probably due to the instability of the mutant protein. The replacement of Arg70 and Arg127 with Ala or Cys resulted in a drastic decrease in the drug resistance and almost complete loss of the transport activity, while the Lys replacement mutants retained significant resistance and transport activity, indicating that the positively charged side chains at these positions conferred the transport function. On the other hand, neither the Ala, Cys, nor Lys replacement mutant of Arg101 exhibited any drug resistance or transport activity. As for the reactivity of the Cys replacement mutants, only two (Arg71 → Cys and Arg101 → Cys) were not reactive with NEM, the other five mutants being highly or moderately reactive. The reactivity of the cysteine-scanning mutants around Arg101 with NEM revealed that Arg101 is located in transmembrane helix IV. It is not likely that Arg101 confers the protein folding through a salt bridge with a transmembrane acidic residue because no double mutants involving Arg101 → Ala and the replacement of one of three transmembrane acidic residues (Asp15, Asp84, and Asp285) showed the recovery of any tetracycline resistance or transport activity. The effect of tetracycline on the [^{14}C]NEM binding to the combined mutants S65C/R101A and L97C/R101A suggests that Arg101 may cause a substrate-induced conformational change of the putative exit gate of TetA(B).

The transposon Tn10-encoded metal−tetracycline/H^+ antiporter [TetA(B)][1] (*1*−*3*) is a polytopic cytoplasmic membrane protein (*4*, *5*), which confers a high level of tetracycline resistance (*6*). A 12 membrane-spanning structure of TetA(B) was proposed (*7*) and experimentally confirmed (*8*). It belongs to the major facilitator superfamily that contains a conserved sequence motif, DRXGRR (*9*), in the hydrophilic loop region between putative helices 2 and 3. The possible roles of this motif have been studied by site-directed mutagenesis; the importance of the first Asp residue (Asp66) and the fifth Arg residue (Arg70) were revealed, while the second and sixth Arg residues do not confer the function (*10*). The importance of the first Asp residue of this motif was commonly observed in α-ketoglutarate permease (*11*) and lac permease (*12*). However, the sixth Arg residue is important in α-ketoglutarate permease but not in lac permease. The typical tetracycline efflux proteins of Gram-negative bacteria classified into TetA(A) to TetA(C) have 11 conserved acidic residues (*13*). Of these conserved acidic residues, three transmembrane Asp residues are all essential for the function (*14*). On the other hand, the only essential acidic residue in the hydrophilic loop region is Asp66 (*15*). In lac permease (*16*−*21*), some acidic residues in the putative transmembrane region are stabilized through the formation of ion pairs with basic residues.

TetA(B) contains seven conserved arginine residues (Figure 1), which are all located in the hydrophilic loop region in the putative topology (*7*, *8*). Of these seven arginine residues, three are located in the conserved motif (Arg67, -70, and -71 in loop 2−3). One additional conserved arginine residue is also located on the cytoplasmic side (Arg127 in loop 4−5). The other three conserved arginines are located on the periplasmic side (Arg31 in loop 1−2, Arg101 in loop 3−4, and Arg238 in loop 7−8). In this

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan and by a Grant-in-Aid for The Joint Research between the Institute for Protein Research and the Institute of Scientific and Industrial Research, Osaka University, "Protein Functions and Creation of New Materials" from The Ministry of Education, Science, Sport and Culture.

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[1] Abbreviations: MIC, minimum inhibitory concentration; MOPS, 3-(morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

S0006-2960(97)03188-7 CCC: $15.00
Published on Web 03/28/1998

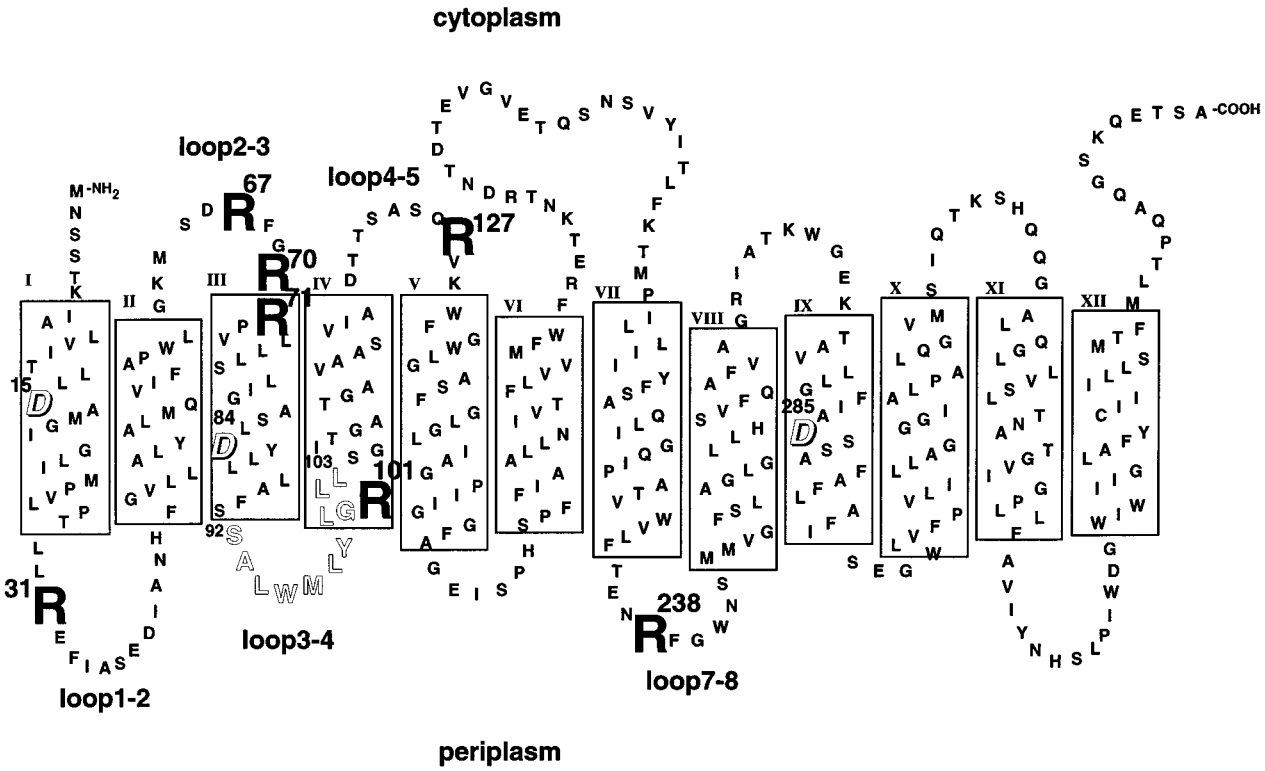

FIGURE 1: Putative secondary structure of TetA(B). The model is slightly modified from that proposed by Kimura et al. (*8*) on the basis of the results of site-directed chemical labeling (*23, 29*). Bold letters represent the arginine residues conserved in tetracycline efflux proteins of Gram-negative bacteria (*13*). Outlined italic letters represent the important transmembrane acidic residues. Outlined letters represent the residues replaced by Cys in the Cys-scanning mutants.

study, these conserved arginines were replaced with Ala, Cys, or Lys, one by one, and then their roles in the transport function were investigated.

EXPERIMENTAL PROCEDURES

Materials. *N*-[1-^{14}C]Ethylmaleimide and [7-^{3}H]tetracycline were purchased from DuPont-New England Nuclear. [α-^{32}P]dCTP was purchased from Amersham. All other materials were of reagent grade and were obtained from commercial sources.

Site-Directed Mutagenesis. Site-directed mutagenesis were performed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (*22*) using the mutagenic primers listed in Table 1. For the mutagenesis, plasmid pCT1182 (*14*), which carries the 2.45 kb Tn10-*tetA-(B)* and *tetR* gene fragments, was used as a template. Mutations were detected as the appearance of a newly introduced restriction site and then verified by DNA sequencing. The R31C (*23*), R67C (*10*), R70C (*24*), R71C (*10*), and R238C (*8*) mutants were constructed previously.

Low-copy number mutant plasmids were constructed through exchange of the *Bgl*II−*Eco*RV (Arg31), *Eco*RV−*Eco*RI (Arg67, -70, -71, -101, and -127), or *Eco*RI−*Bam*HI (Arg238) fragment of the mutant *tetA(B)* genes with the corresponding fragment of the low-copy number plasmid, pLGT2 (*14*).

Determination of Tetracycline Resistance. Tetracycline resistance was determined by an agar dilution method as described previously (*25*) and expressed as the minimum inhibitory concentration.

Preparation of Everted Membrane Vesicles. *Escherichia coli* W3104 (*26*) cells carrying pLGT2 or a mutant plasmid were grown on a minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. After a 2 h induction of *tet* gene expression with 0.25 μg/mL heat-inactivated chlorotetracycline, everted vesicles were prepared by cell disruption with a French press at 5000 psi, as described in a

Table 1: Mutagenic Primers Used for Site-Directed Mutagenesis[a]

Mutation	Primer sequence	codon change
R31A	5′-gcaatGaattcaGCtaataa-3′ *Eco*RI	CGT→GCT
R67A	5′-cgaccaaaCGCgtcagacat-3′ *Mlu*I	CGA→GCG
R71A	5′-agcactggAGCTcgaccaaa-3′ *Sac*I	CGC→GCT
R101A	5′-tgaaagcaGaGCTcctaaat-3′ *Sac*I	CGT→GCT
R127A	5′-ttcacgGCttgagaagcGCTggtg-3′ *Eco*47III	CGC→GCC
R238A	5′-catccaaaCGCGttttcggt-3′ *Mlu*I	CGT→GCG
R31K	5′-aagcaatGaattcTTTtaataacg-3′ *Eco*RI	CGT→AAA
R101K	5′-ccctgaaagAaGCTTgcctaaatac-3′ *Hind*III	CGT→AAG
R127K	5′-cacttcacTTTttgagaagcGCTggtggta-3′ *Eco*47III	CGC→AAA
R238K	5′-tccatccGaaTTTattCtcggtaaa-3′ *Xmn*I	CGT→AAA
L99C	5′-tgaaagcaGTcgAccGCaatacagc-3′ *Hinc*II	TTA→TGC
G100C	5′-aaagcaGTcgAcAtaaata-3′ *Hinc*II	GGC→TGT
R101C	5′-gcaaacACccGaGatacagc-3′ *Ava*I	CGT→TGT
L102C	5′-gatcccCgaGagGCaacggc-3′ *Ava*I	TTG→TGC
L103C	5′-tgatcccCgaGCAcaaacgg-3′ *Ava*I	CTT→TGC
R127C	5′-tcacgcAttgGCTagctgag-3′ *Nhe*I	CGC→TGC

[a] The mutagenic primers contained two kinds of mismatches, i.e. mismatches for causing amino acid replacements and silent ones for causing new restriction site(s). Capital letters indicate the mismatches. Underlined letters indicate the new restriction sites.

previous paper (*14*). The vesicles were suspended in 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and stored at −80 °C.

Transport Assays. [^{3}H]Tetracycline uptake by everted membrane vesicles was assayed as described previously (*14*) in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ in 50 mM MOPS-KOH buffer (pH 7.0).

Assay of the Reaction of N-[^{14}C]Ethylmaleimide with TetA-(B) Proteins. The [^{14}C]NEM binding experiment was performed as described previously (*27*). *E. coli* W3104 cells carrying pLGT2 or one of its derivatives were grown in medium A supplemented with 0.1% casamino acids and 0.2% glucose to the logarithmic phase. After the induction of *tetA*(B) gene expression with 0.25 μg/mL heat-inactivated chlorotetracycline for 2 h, the cells were harvested by centrifugation and washed with a preparation buffer [50 mM MOPS-KOH (pH 6.6), 100 mM KCl, and 10 mM EDTA]. Then the cells were disrupted by brief sonication in the same buffer. After removal of unbroken cells, the membrane fraction was collected by ultracentrifugation. The resultant pellet was washed once with an assay buffer [50 mM MOPS-KOH (pH 7.0) and 100 mM KCl] and then suspended in the same buffer to a final concentration of 5 mg of protein/mL. A 100 μL aliquot of the membrane suspension was incubated with 0.5 mM [^{14}C]NEM for 5 min at 30 °C. The reaction was stopped by dilution with the same buffer containing 5 mM unlabeled NEM, and then the membranes were collected by ultracentrifugation. The pellet was solubilized in 1% Triton X-100 and 0.1% SDS in phosphate-buffered saline containing 5 mM unlabeled NEM. Then TetA(B) proteins were immunoprecipitated with anti-TetA(B) C-terminal peptide antiserum (*28*) and Pansorbin *Staphylococcus aureus* cells (Sigma Chemical Co.). The resultant pellet was subjected to SDS−polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining. The resultant gel was soaked in Amplify prior to drying. The dried gel was exposed to an imaging plate for visualization using a BAS-1000 Bio-Imaging Analyzer (Fuji Film Co., Tokyo).

RESULTS AND DISCUSSION

Effects of the Replacement of Arg Residues. Everted membrane vesicles were prepared as described in Experimental Procedures, and the amounts of the mutant TetA(B) proteins were determined by Western blotting using anti-TetA(B)-C-terminal antiserum (*28*). The amounts of the mutant TetA(B) proteins were approximately equal to that of the wild-type TetA(B) (data not shown).

Table 2 shows the tetracycline resistance levels of *E. coli* W3104 cells harboring pLGT2 or one of its derivatives encoding a mutant TetA(B) protein. With respect to four arginine residues (Arg31, -67, -71, and -238), replacement with Ala, Cys, or Lys did not greatly affect the tetracycline resistance level except in the R71C and R238C mutants. These two cysteine mutants showed moderate resistance levels (50 μg of tetracycline/mL). However, since the Ala to Arg mutants showed resistance comparable to that of the wild type, the positive charges at these positions are not important for the function. The replacement of Arg127 with a neutral amino acid resulted in a great decrease in the resistance level, whereas the replacement with Lys did not have a significant effect, indicating the importance of a positive charge at this position. The replacement of Arg70 and Arg101 caused drastic decreases in the resistance level. The charge-conserved Arg mutants tended to show slightly higher levels than those of the neutral mutants.

Table 2: Tetracycline Resistance Levels of *E. coli* W3104 Cells Harboring Plasmids Encoding the Wild-Type or Mutant TetA(B) Proteins[a]

plasmid	MIC of tetracycline (μg/mL)
no plasmid	1.6
pLGT2 (wild type)	200

	MIC of tetracycline (μg/mL)		
plasmid	X = A	X = K	X = C
pLGR31X	200	200	200
pLGR67X	100		200
pLGR70X	6.3	12.5	12.5
pLGR71X	100		50
pLGR101X	3.1	6.3	0.8
pLGR127X	12.5	100	6.3
pLGR238X	200	200	50

[a] The resistance levels are expressed as the minimum inhibitory concentrations (MICs) determined by the agar dilution method.

Tetracycline Transport Activity of Arg Mutants. The tetracycline transport activities of the mutants in which Arg residues were replaced with Ala or Lys are shown in Figure 2. When Arg67 and -71 were replaced by Ala, the resultant tetracycline uptake activities were rather increased (Figure 2). When Arg31 was replaced by Ala or Lys, the activity was the same as that of the wild type. Therefore, these three arginine residues do not confer tetracycline transport, consistent with the results with minimum inhibitory concentrations (Table 2). On the other hand, the tetracycline transport activities of the Arg238 mutants greatly differed from the resistance levels of intact cells. The R238K mutants showed only 40% of the wild-type activity and the Ala mutant almost no activity at all (Figure 2), whereas both the R238A and R238K mutants retained full resistance in intact cells. When everted membrane vesicles of the R238A mutant were prepared in the presence of 10% glycerol, the resultant vesicles showed about 30% of the wild-type activity (Figure 3), indicating that the transport activity of the R238A mutant was lost during preparation of the vesicles in the absence of glycerol. Since no degradation product was detected on Western blotting in the R238A or R238K mutant vesicles (data not shown), the loss of the transport activity was not due to the degradation of the mutant proteins. Although it still remains unknown why the transport activity does not quantitatively reflect the full level resistance of the R238A and R238K mutants, it is clear that Arg238 is not essential for the TetA(B) function.

With respect to Arg70 and Arg127, only the Lys mutants retained significant activity, while the Ala mutants completely lost their activity. This tendency is consistent with the resistance levels in intact cells, whereas the tetracycline transport activity of the R127K mutant was lower than that expected from the high-level resistance (100 μg/mL). Neither the R70A nor the R127A mutant showed recovery of the transport activity when its vesicles were prepared in the presence of glycerol (Figure 3).

On the other hand, no mutants of Arg101 showed any transport activity (Figure 2), although the resistance level of the R101K mutant (6.3 μg/mL) was slightly higher than

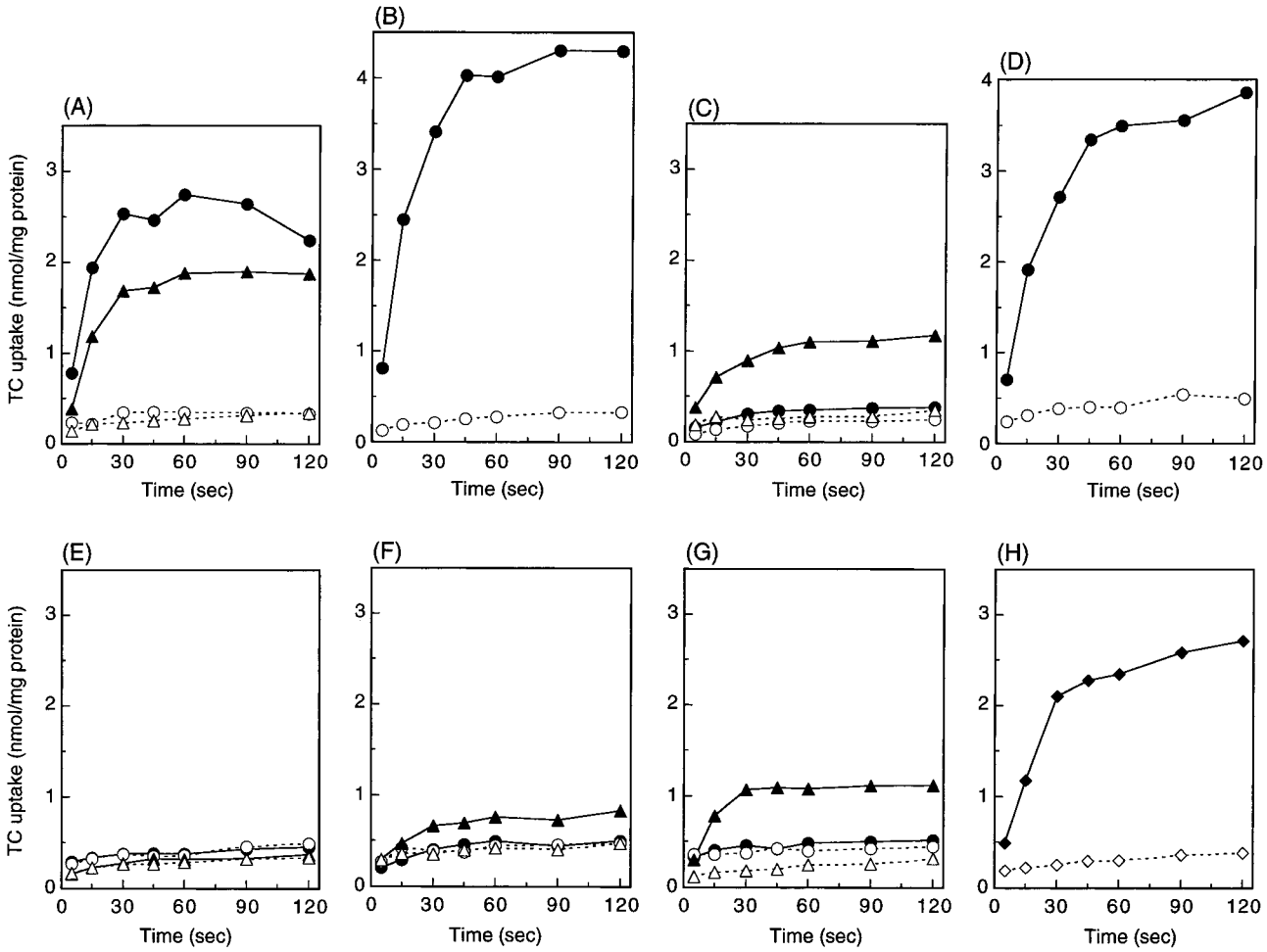

FIGURE 2: Tetracycline uptake by everted membrane vesicles prepared from *E. coli* W3104 cells harboring a mutant plasmid: (A) Arg31 mutants, (B) Arg67 mutants, (C) Arg70 mutants, (D) Arg71 mutants, (E) Arg101 mutants, (F) Arg127 mutants, (G) Arg238 mutants, and (H) wild type. Circles represent mutants with replacement with Ala. Triangles represent mutants with replacement with Lys. Diamonds represent the wild type. Closed symbols represent vesicles energized with NADH. Open symbols represent vesicles not energized.

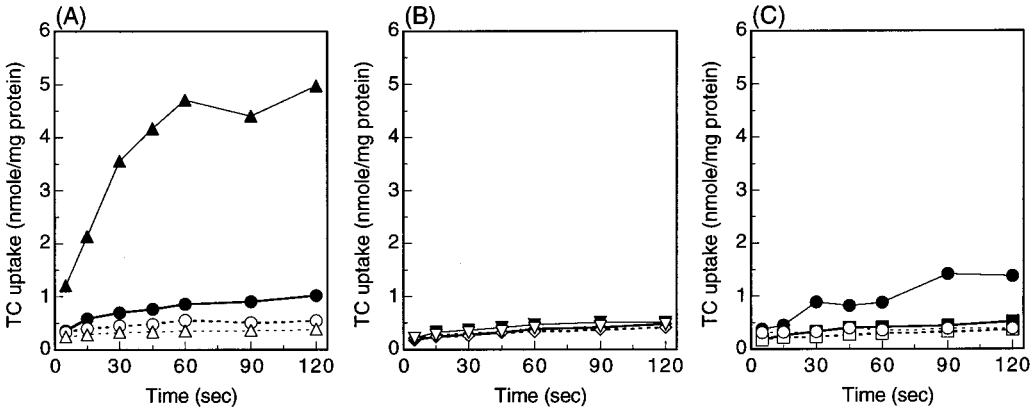

FIGURE 3: Tetracycline uptake by everted membrane vesicles prepared in the presence of 10% glycerol. (A) Uptake by the wild type and the R70A mutant: triangles, wild type; and circles, R70A. (B) Uptake by the R101A and R101K mutants: diamonds, R101A; and inverted triangles, R101K. (C) Uptake by the R127A and R238A mutants: squares, R127A; and circles, R238A. Closed and open symbols indicate that the vesicles were energized with NADH and not energized, respectively.

that of the R101A mutant (3.1 μg/mL). The R101A mutant also showed no activity when its vesicles were prepared in the presence of glycerol (Figure 3).

[^{14}C]NEM Binding to Arg → Cys Mutants. To determine whether these arginine residues are exposed to the aqueous phase or buried in the membrane, the reactivity of Arg → Cys mutants was measured in everted membrane vesicles as described in Experimental Procedures. Of these seven Cys mutants, five (R31C, R67C, R70C, R127C, and R238C) showed high-level binding of [^{14}C]NEM (Figure 4A). Two mutants (R71C and R101C) unexpectedly showed no binding of [^{14}C]NEM. In a recent study, we found that Arg71 is located at the boundary between helix III and loop 2−3 (*29*). With respect to Arg101, the topology proposed by Eckert and Beck predicted that it is located at the boundary between periplasmic loop 3−4 and helix IV (*7*). To confirm this

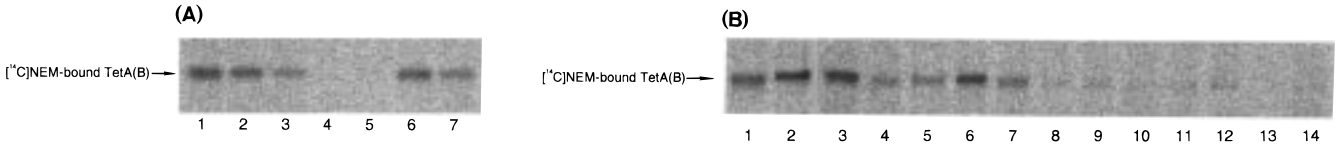

FIGURE 4: Binding of [^{14}C]NEM to the Cys mutants. Everted membrane vesicles (0.5 mg of protein) containing mutant TetA(B) proteins were incubated with 0.5 mM [^{14}C]NEM for 5 min at 30 °C, followed by solubilization and immunoprecipitation of TetA(B) proteins as described in Experimental Procedures. After SDS−PAGE, the gel was dried and the radioactive bands were visualized with a BAS-1000 Bio-Imaging Analyzer. (A) The binding to the conserved Arg → Cys mutants: lane 1, R31C; lane 2, R67C; lane 3, R70C; lane 4, R71C; lane 5, R101C; lane 6, R127C; and lane 7, R238C. (B) The binding to the cysteine-scanning mutants around the putative periplasmic loop 3−4: lane 1, S92C; lane 2, A93C; lane 3, L94C; lane 4, W95C; lane 5, M96C; lane 6, L97C; lane 7, Y98C; lane 8, L99C; lane 9, G100C; lane 10, R101C; lane 11, L102C; lane 12, L103C; lane 13, host cells without the *tetA(B)* gene; and lane 14, wild type.

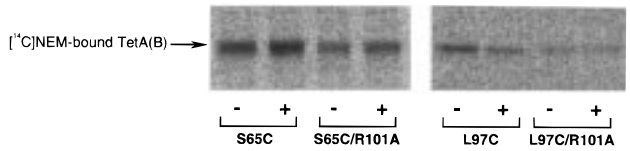

FIGURE 5: Effect of tetracycline on the [^{14}C]NEM binding to the S65C and L97C single mutants and the double mutants with each of these mutations combined with the R101A mutation. The binding assay was performed under the same conditions as in Figure 4 except for the presence or absence of 1 mM tetracycline and 5 mM $MgSO_4$. − and + indicate the absence and presence of Mg−tetracycline, respectively.

prediction, we constructed five additional cysteine-scanning mutants of Leu99−Leu103. Figure 4B shows the [^{14}C]NEM binding to these five cysteine-scanning mutants in addition to seven cysteine-scanning mutants of loop 3−4 (*29*). As shown in this figure, helix IV starts from Leu99 and thus Arg101 is the third residue from the N-terminal end of helix IV.

Effects of Combinations of Mutations at Arg101 and Three Transmembrane Aspartic Acid Residues. The results described above indicate that Arg101 is a transmembrane basic residue. Some basic residues of secondary transporters are stabilized through ion pairs with transmembrane acidic residues (*16*−*21*). The ion pairs are used to be presumed on the basis of the effect of the combination of mutations of a basic and an acidic residue on the protein function; that is, when each of these charged residues was replaced by a neutral residue, the resulting mutant lost the activity, while the double mutant retained significant transport activity. In some cases, on the mutational exchange of these charged residues with each other, the activity was often retained. To check the possibility of ion pair formation of Arg101 with any other transmembrane acidic residues, charge-neutralized or charge-exchanged double mutants were constructed. In the charge-neutralized ones, the R101A mutation was combined with the D15N, D84N, or D285N mutation. In the charge-exchanged ones, the R101D mutation was combined with the D15K, D84K, or D285K mutation. None of these double mutants showed any distinct recovery of the drug resistance or tetracycline transport activity, indicating that Arg101 acts alone as a positive charge (data not shown).

Effects of Tetracycline on the [^{14}C]NEM Binding to the Combined Mutants, S65C/R101A and L97C/R101A. The reactivity of [^{14}C]NEM with the S65C and L97C mutants was stimulated and repressed, respectively, on the addition of tetracycline (*29*, *30*). When the R101A mutation was combined with the S65C mutation, the response of the reactivity of the resulting double mutant, S65C/R101A, with [^{14}C]NEM on tetracycline was greatly reduced (Figure 5). On the other hand, the reactivity of the L97C/R101A double mutant with [^{14}C]NEM was no longer affected by tetracycline (Figure 5). That is, the R101A mutation prevented the substrate-induced conformational change, especially around the exit gate, suggesting the role of Arg101 in the gating function.

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BI973188G