His257 Is a Uniquely Important Histidine Residue for Tetracycline/H^+ Antiport Function but Not Mandatory for Full Activity of the Transposon Tn10-Encoded Metal−Tetracycline/H^+ Antiporter†

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Received September 6, 1995; Revised Manuscript Received January 18, 1996⊗

ABSTRACT: His257 is the only histidine residue located in the putative transmembrane region of the Tn10-encoded metal−tetracycline/H^+ antiporter (TetA) and contributes to the substrate/H^+ coupling [Yamaguchi, A., Adachi, K., Akasaka, T., Ono, N., & Sawai, T. (1991) *J. Biol. Chem. 266,* 6045−6051]. Tn10-TetA contains five histidine residues, including His257. When these histidine residues were replaced by alanine one by one, only the His257Ala mutant showed almost complete loss of the tetracycline transport activity, whereas the other four His → Ala mutants, H42A, H158A, H329A, and H359A, retained transport activity comparable to that of the wild type. The mutant which contains only one histidine, His257, retained about 80% of the wild-type activity, whereas the histidine-less mutant, in which all five histidine residues were replaced by Ala, exhibited little activity. These results clearly indicated that His257 is a unique histidine residue in TetA responsible for the transport activity. The His257Tyr mutant, irrespective of the presence or absence of the other four histidine residues, retained about 30% of the wild-type tetracycline transport activity and showed corresponding tetracycline-coupled H^+ translocation, indicating that an imidazole group is not necessary at position 257 for the substrate/H^+ coupling. A histidine-specific reagent, diethyl pyrocarbonate (DEPC), equally inactivated the wild-type and one-histidine mutant TetA, whereas the H257Y mutant was hardly inactivated by DEPC irrespective of the presence or absence of the other four histidine residues, indicating that the inactivation by DEPC is due to the modification of His257.

The metal−tetracycline/H^+ antiporter of *Escherichia coli* [TetA(B)] (Yamaguchi et al., 1990a) belongs to a superfamily of H^+-coupled secondary transporters (Henderson, 1990), which are commonly composed of 12 putative transmembrane segments. One of the fundamental problems concerning the molecular mechanism of the secondary transporters is elucidation of the H^+ translocation pathway. Acidic residues located in the transmembrane segments of such polytopic membrane proteins play a central role in H^+ translocation in H^+ pumps (Mogi et al., 1988; Marinetti et al., 1989; Otto et al., 1989; Krebs & Khorana, 1993), H^+ channels (Zhang & Fillingame, 1994), and H^+-coupled symporters (Carrasco et al., 1989; Franco & Brooker, 1994; Poolman et al., 1995). In lactose permease, Glu325, which is located in transmembrane helix X, is deduced to play an essential role in H^+ translocation (Carrasco et al., 1989; Lee et al., 1989; Franco & Brooker, 1994). In TetA(B), Asp285, which is located in putative transmembrane helix IX, plays a corresponding essential role in the tetracycline/H^+ coupling (Yamaguchi et al., 1992).

Does a proton pass to the essential acidic residue directly through water molecules or through other dissociable residue(s)? His322 of LacY is a unique histidine residue important for the LacY function (Puttner et al., 1989), which is located in helix X on the same side as Glu325. His322 was proposed to participate in proton translocation in combination with Glu325 and Arg302 via a charge relay system (Carrasco et al., 1986; Menick et al., 1987; Lee et al., 1989). His322 certainly plays a role in the substrate/H^+ coupling because some mutants of His322 showed an uncoupled phenotype without substrate-coupled proton translocation. However, the imidazole group of His322 may not be essential for the proton translocation because the H322Y and H322F mutants retained the substrate-coupled H^+ translocation ability, although these mutants did not actively accumulate lactose (King & Wilson, 1989, 1990). The role of His322 in the substrate recognition has also been pointed out (Franco & Brooker, 1991).

In order to elucidate the general role of the transmembrane histidine residue, it is important to determine the roles of corresponding histidine residues in other secondary transporters. TetA(B) contains a unique histidine residue, His257, in putative transmembrane helix VIII, which is conserved throughout the tetracycline/H^+ antiporters of Gram-negative bacteria (Waters et al., 1983). We previously reported that His257 plays a role in H^+ tetracycline coupling (Yamaguchi et al., 1991). The importance of the transmembrane histidine residue in the substrate recognition and H^+ substrate coupling has also been reported for LacS of *Streptococcus thermophilus* (Poolman et al., 1992).

The TetA(B) protein contains five histidine residues, His42, His158, His257, His329, and His359. The former three histidine residues are conserved in the tetracycline/H^+ antiporters of Gram-negative bacteria (Waters et al., 1983). A putative secondary structure of TetA(B) was proposed by Eckert and Beck (1989). In the putative topology, His42, His158, and His359 are located on the periplasmic surface, and His329 is located on the cytoplasmic surface. Only

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

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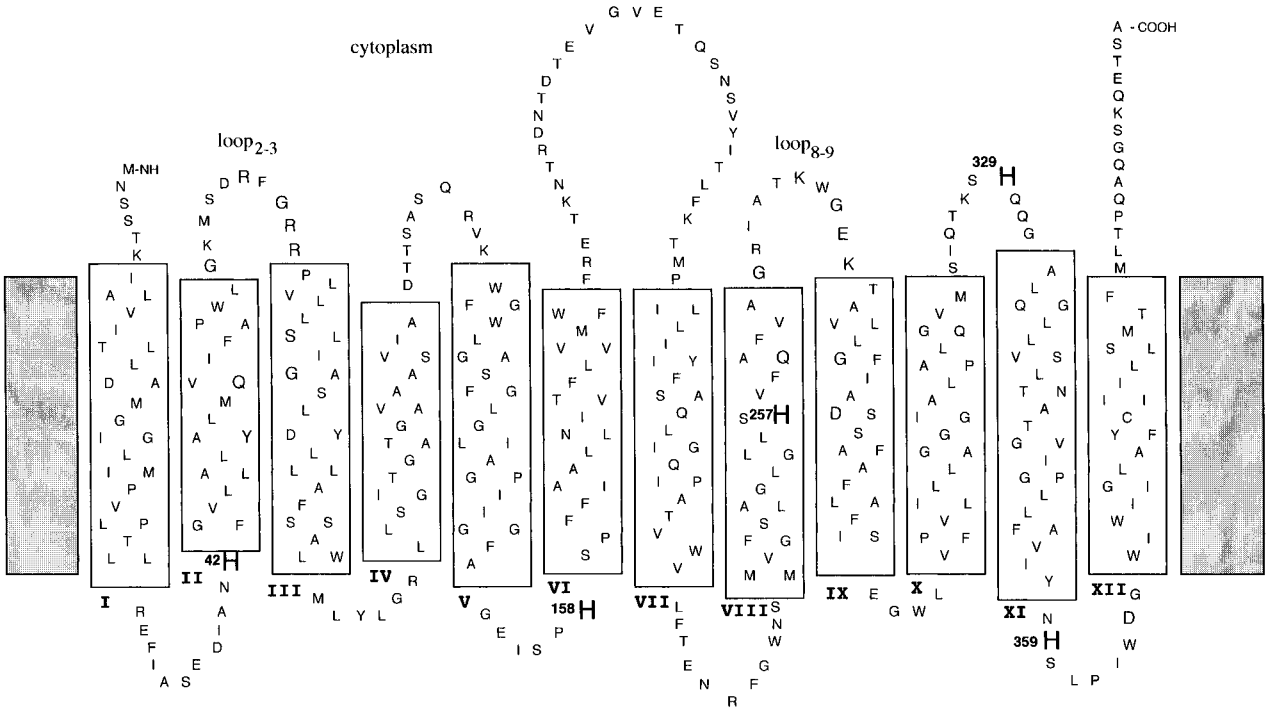

FIGURE 1: Putative secondary structure of TetA(B) according to Eckert and Beck (1989). Histidine residues are depicted as bold letters with numbers representing their positions.

His257 is located in the transmembrane region (Figure 1). In this study, each of these five histidine residues was replaced by another amino acid residue. As a result, only His257 was found to be important for the TetA(B) function, and His257 alone is enough as a functional histidine residue to confer the wild-type transport activity to TetA(B). However, when His257 was replaced by tyrosine, decreased but significant activity remained. Therefore, an imidazole side chain of His257 is required for neither tetracycline transport nor tetracycline-coupled H^+ translocation.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Tetracycline and 5′-[α-^{32}P]dCTP were purchased from Du Pont-New England Nuclear and Amersham, respectively. Diethyl pyrocarbonate was purchased from Sigma. Restriction enzymes and other DNA-modifying enzymes were obtained from Takara (Kyoto, Japan), Toyobo (Osaka, Japan), and Nippon Gene (Toyama, Japan). All other reagents used were of reagent grade and were obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* TG1 (Taylor et al., 1985) and W3104 (Yamamoto et al., 1981) were used for transformation after mutagenesis and inverted vesicle preparation, respectively. *E. coli* JM109 (Yanish-Perron et al., 1985), CJ236 (Kunkel, 1985), and BMH71-18 *mut*S (Kunkel, 1985) were used for oligonucleotide-directed mutagenesis by the Kunkel method (Kunkel, 1985). pCT1183 (Someya et al., 1995) and pLGT2 (Yamaguchi et al., 1992) are plasmids carrying 2.45 kilobase Tn10-*tet*A(B) and *tet*R gene fragments cloned into pUC118 (Vieira & Messing, 1987) and pLG339 (Stoker et al., 1982), respectively.

Site-Directed Mutagenesis. Mutagenesis was performed by the Kunkel method (Kunkel, 1985) using synthetic mutagenic primers and pCT1183 as a template. Mutagenic primers were synthesized with a Beckman Oligo 1000 DNA synthesizer. The mutagenic primers used in this experiment contained a silent mismatch generating a new restriction site in addition to the mismatch for the codon change. Mutant plasmids were at first selected as to the appearance of the restriction site, and then the sequences were confirmed by DNA sequencing. The mutant *tet*A(B) gene was then subcloned into the low-copy plasmid, pLGT2, by corresponding restriction fragment exchange.

Preparation of Inverted Membrane Vesicles. Inverted membrane vesicles were prepared according to a previous paper (Yamaguchi et al., 1990a). Briefly, cells harboring a low-copy plasmid carrying mutant and wild-type *tet*A(B) genes were grown in 1 L of minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. After 2 h of induction of *tet*A(B) gene expression of mid-log phase cells with 0.25 μg/mL heat-inactivated chlortetracycline, the cells were harvested, washed, and disrupted with a French press in 50 mM MOPS/KOH buffer (pH 6.6) containing 0.1 M KCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 100 μg/mL DNase II. Then the vesicles were washed once with 50 mM MOPS/KOH (pH 7.0) containing 0.1 M KCl and stored at −80 °C.

Transport Assay. [^{3}H]Tetracycline uptake by inverted membrane vesicles was assayed as described previously (Yamaguchi et al., 1990a). In brief, a mixture of 10 μL of the vesicle suspension (3.5 mg of protein/mL) and 0.5 μL of 250 mM NADH was preincubated at 30 °C for 1 min. The tetracycline uptake was initiated by the addition of 40 μL of MOPS/KOH buffer (pH 7.0) containing 0.1 M KCl, $CoCl_2$ (final concentration, 50 μM), and [^{3}H]tetracycline (final concentration, 10 μM), unless otherwise stated. After incubation at 30 °C for the indicated periods, the uptake was stopped by the addition of 2 mL of 5 mM MOPS/KOH buffer (pH 7.0) containing 0.15 M LiCl. The mixture was immediately filtered through a Millipore filter (pore size, 0.45 μm) and then washed once with the same buffer. Then the radioactivity on the filter was measured.

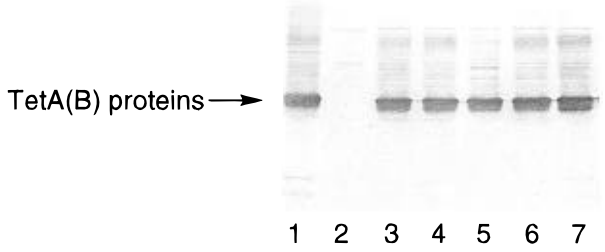

FIGURE 2: Immunoblot analysis of inverted membrane vesicles prepared from *E. coli* W3104 cells harboring the wild-type or a mutant plasmid. Each lane contained 10 μg of total protein: lane 1, pLGT2 (wild type); lane 2, no plasmid; lane 3, pLGH42A; lane 4, pLGH158A; lane 5, pLGH257A; lane 6, pLGH329A; lane 7, pLGH359A.

Immunoblot Analysis. TetA(B) proteins were detected by immunoblot analysis using anti-C-terminal peptide antiserum (Yamaguchi et al., 1990b) as described previously (Yamaguchi et al., 1990c).

RESULTS

Effects of Single Histidine to Alanine Mutations on Tetracycline Transport Activity. The TetA(B) protein possesses five histidine residues, His42, His158, His257, His329, and His359 (Figure 1). Each histidine residue was replaced by alanine through site-directed mutagenesis using the following mutagenic primers: 5′-ATCGCTAACGCGTTTG-GCGT-3′ for H42A, 5′-GATTTCGCCGGCTAGTCCCT-3′ for H158A, 5′-GGGTCTAGGCCTTTTAGCCTCAGT-3′ for H257A, 5′-CAAAGAGCGCTCAGCAA-3′ for H328A, and 5′-ATTTATAACGCGTCACTACC-3′ for H359A. The underlined letters indicate the bases mismatched with the wild-type sequence. The mutations were verified by DNA sequencing, and the mutant *tet*A(B) genes were cloned into a low-copy number plasmid, pLGT2, by fragment exchange. Inverted membrane vesicles were prepared from the cells after induction of the *tet*A(B) gene, and the TetA(B) proteins were detected by Western blotting using anti-C-terminal peptide antibodies (Yamaguchi et al., 1990b). The amounts of the mutant TetA(B) proteins were approximately equal to that of the wild-type TetA(B) (Figure 2).

The tetracycline resistance levels of *E. coli* W3104 cells harboring these mutant plasmids were about the same as that of the cells harboring the wild-type plasmid, pLGT2, except for the cells having pLGH257A, the resistance level of which was drastically lower than that of the other cells having the mutant and wild-type plasmids (data not shown).

Active uptake of [^{3}H]tetracycline by inverted membrane vesicles prepared from *E. coli* W3104 cells carrying these mutant plasmids was measured (Figure 3). The rates of tetracycline uptake by the vesicles containing the H42A, H329A, and H359A mutants were approximately the same as that of the vesicles containing the wild-type TetA(B) within experimental error. On the other hand, the rate of uptake by the H158A mutant vesicles was significantly lower than that of the wild-type vesicles by a factor of about 2. Among these five single histidine mutant vesicles, only the H257A mutant vesicles almost completely lost the ability of active uptake of tetracycline. These results clearly indicated that His257 is a unique histidine residue important for the TetA(B) function.

Properties of Double Histidine Mutants. While constructing a histidine-less mutant of the TetA(B) protein, we

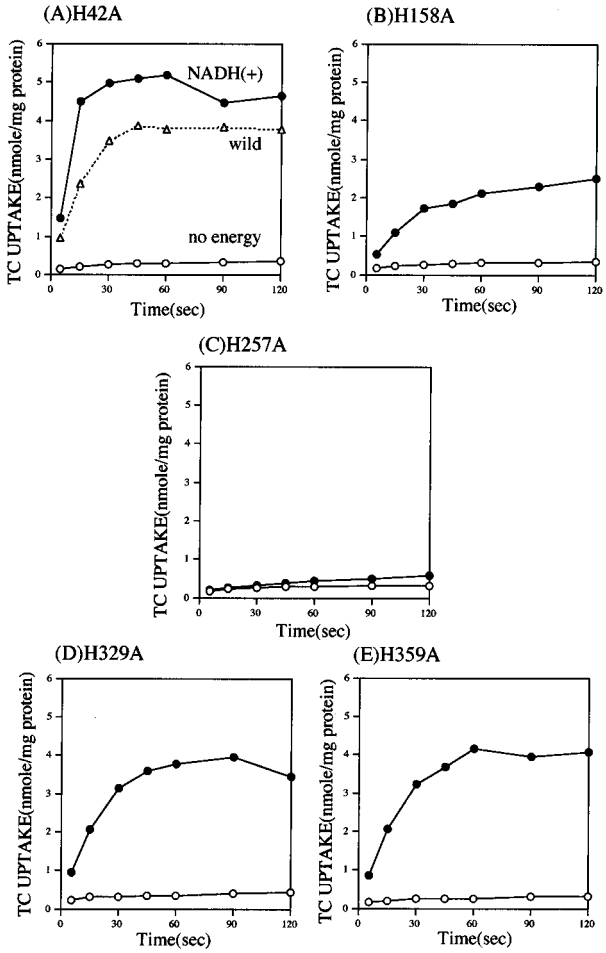

FIGURE 3: Tetracycline uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells harboring low-copy number plasmids encoding single histidine mutant *tet*A(B) genes: (A) pLGH42A, (B) pLGH158A, (C) pLGH257A, (D) pLGH329A, (E) pLGH359A. Closed and open circles indicate the uptake by the mutant vesicles in the presence and absence of NADH as an energy source, respectively. The dashed line with open triangles in panel A indicates the uptake by the energized wild-type vesicles.

obtained two kinds of double histidine mutants, H42A/H158A and H329A/H359A. The H42A/H158A double mutant was constructed by site-directed mutagenesis using a mutagenic primer of H158A and a template prepared from pCTH42A. The H329A/H359A double mutant was constructed in a similar fashion using a H359A primer and pCTH329A as a template.

Interestingly, the rates of tetracycline uptake by the inverted vesicles containing these double histidine mutants were the same as that of the wild-type vesicle (Figure 4). It is not obvious why the transport rate of the H42A/H158A double mutant vesicles was so clearly higher than that of the H158A single mutant vesicles; however, the result confirms the previous conclusion that His158 is not important for the TetA(B) function.

Properties of H257 One-Histidine TetA(B) and Histidine-less TetA(B). We constructed mutants in which all histidine residues except for His257 were replaced by alanine as follows. The *Eco*RI-*Bam*HI fragment of pCTH42A/H158A was replaced by the corresponding fragment of pCTH329A/H359A (Figure 5). A histidine-less mutant was constructed by replacement of the *Eco*RI-*Bgl*I fragment of the pCTH42A/H158A/H329A/H359A quadruple mutant plasmid with the corresponding fragment of pCTH257A or pCTH257Y (Fig-

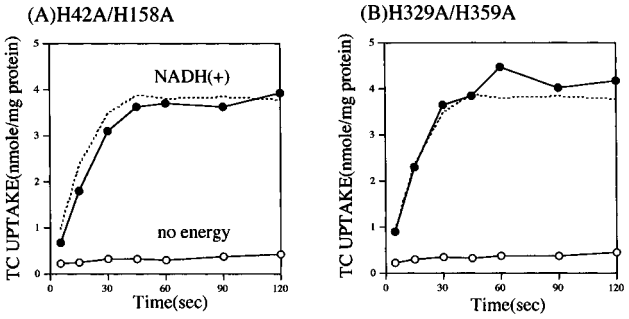

FIGURE 4: Tetracycline uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells carrying double histidine mutant *tet*A(B) genes: (A) pLGH42A/H158A, (B) pLGH329A/H359A. Closed and open circles indicate the uptake by the mutant vesicles in the presence and absence of NADH as an energy source, respectively. The dashed lines indicate the uptake by the energized wild-type vesicles.

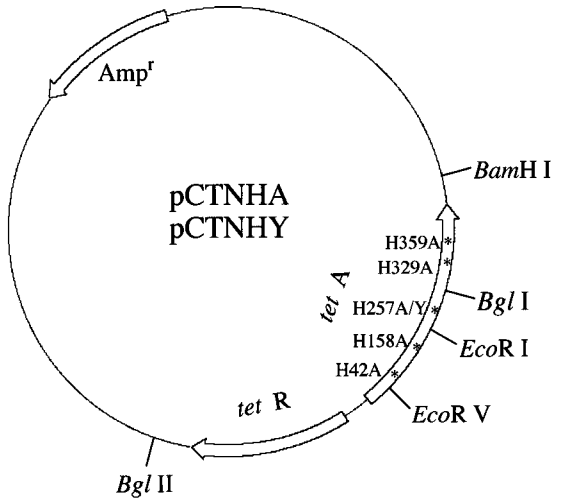

FIGURE 5: High-copy number plasmid carrying His-less *tet*A(B) genes derived from pUC118. pCTNHA and pCTNHY represent the plasmids carrying His-less *tet*A(B) genes containing H257A and H257Y mutations, respectively. Asterisks indicate the positions of histidine codons in the *tet*A(B) gene. The restriction sites used for construction of these multimutant genes are also indicated.

ure 5). pCTH257Y was constructed by site-directed mutagenesis using a mutagenic primer, 5′-TTAAGCGGGTCT-AGGCC TTTTATATTCAGTA-3′, and a template, pCT1183. The resulting mutant *tet*A(B) genes were subcloned into pLGT2 by fragment exchange.

H257 one-histidine TetA(B) (NHH mutant) showed a rate of tetracycline uptake of about 80% of that of wild-type TetA(B) (Figure 6A), confirming that the four histidine residues other than His257 are not important for the function. In contrast, histidine-less TetA(B) (NHA mutant), in which the last His257 was replaced by alanine in addition to the other four histidines, showed less than 5% of the transport rate of the wild type, which was similar to that of the single H257A mutant (Figure 6B). So, it is clear that only His257 is critical for the TetA(B) function.

When His257 was replaced by tyrosine in addition to the mutations of the other four histidine residues to alanine, the resulting NHY mutant retained about 30% of the tetracycline transport rate of wild-type TetA(B) (Figure 6C). The K_m value of the tetracycline transport by the NHY mutant was 23 μM, which is similar to that of the wild type. On the other hand, the V_{max} value of the NHY mutant [10.9 nmole (mg of protein)$^{-1}$ min^{-1}] was about one-third of that of the wild type [32.6 nmol (mg of protein)$^{-1}$ min^{-1}]. The significant transport activity of the NHY mutant indicates that an imidazole group is not obligatory at position 257.

The characteristics of the single H257Y mutant were very similar to those of the NHY mutant. The initial rate of tetracycline transport of the H257Y mutant was 33% of the wild-type rate under the same conditions. The results confirm that the histidine residues except for His257 hardly contribute to the transport function.

Effects of Histidine Mutations on the Inhibiton of Tetracycline Transport by Diethyl Pyrocarbonate. Diethyl pyrocarbonate (DEPC) is a histidine-specific modifier of proteins (Padan et al., 1979). We previously showed that DEPC specifically inhibits the tetracycline transport mediated by TetA(B) (Yamaguchi et al., 1991). The concentration dependency of the inhibitory effect of DEPC on the histidine mutants was measured (Figure 7). In the case of the wild-type vesicles, 1 mM DEPC was enough for complete inhibition. The single His257 (NHH) mutant showed a profile similar to that of the wild type, and in the presence of 1 mM DEPC, the residual activity of the mutant was less than 5%. In contrast, the degree of inhibition of the histidine-less (NHY) mutant was far lower than that of the one-histidine or wild-type TetA(B). The NHY mutant retained about 75% of the transport activity after treatment with 1 mM DEPC. The majority of the inhibition of the NHY mutant reflected inhibition of the energized state of the vesicles by DEPC. In the case of the single H257Y mutant, the inhibition profile was similar to but somewhat significantly different from that of the NHY mutant. In the presence of 1 mM DEPC, the H257Y mutant retained about 60% activity. The difference in the degree of inhibition between the single H257Y mutant and the NHY mutant may reflect the effect of modification of histidine residues other than His257 by DEPC. These results clearly indicated that the inhibition of tetracycline transport activity of TetA(B) by DEPC is mainly due to the modification of His257.

DISCUSSION

The present work revealed that (1) His257 is the only histidine residue responsible for the TetA(B) function, (2) abolition of tetracycline transport activity by DEPC is due to the chemical modification of His257, and (3) an imidazole group at position 257 is necessary for neither tetracycline transport nor tetracycline/H^+ coupling. These observations are interesting to compare with those for His322 in LacY (Puttner et al., 1989; King & Wilson, 1989). His257 in TetA(B) may be a residue playing a role similar to that of His322 in LacY in the substrate/H^+ coupling and/or substrate recognition; however, the role of His257 is not mandatory for the TetA(B) function.

A structural role of His257 through the charge-neutralizing pairing to Asp285, which is an essential transmembrane acidic residue probably present sterically close to His257, is not likely for the following reasons. (1) Some site-directed mutants of His257 with neutral amino acids retain significant transport activity (Yamaguchi et al., 1991). (2) A mutation of His257 was not found in the second site suppressor mutants from the Asp285Asn mutant (Yamaguchi et al., 1993). (3) The double mutant in which His257 and Asp285 were replaced by neutral residues showed no recovery of the transport activity (data not shown). Replacement with

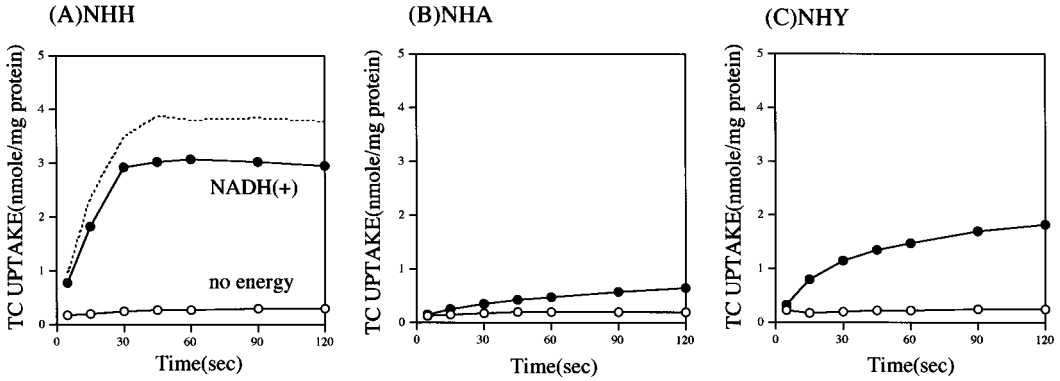

FIGURE 6: Tetracycline uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells carrying low-copy number plasmids encoding His-less *tet*A(B) genes with a His257, Ala257, or Tyr257 codon: (A) pLGNHH, which encodes His-less *tet*A(B) with a His257 codon; (B) pLGNHA, which encodes His-less *tet*A(B) with an Ala257 codon; (C) pLGNHY, which encodes His-less *tet*A(B) with a Tyr257 codon. All histidine residues in TetA(B) except for His257 were replaced with Ala in these mutants. Closed and open circles indicate the uptake by the mutant vesicles in the presence and absence of NADH as an energy source, respectively. The dashed line in panel A indicates the uptake by the energized wild-type vesicles.

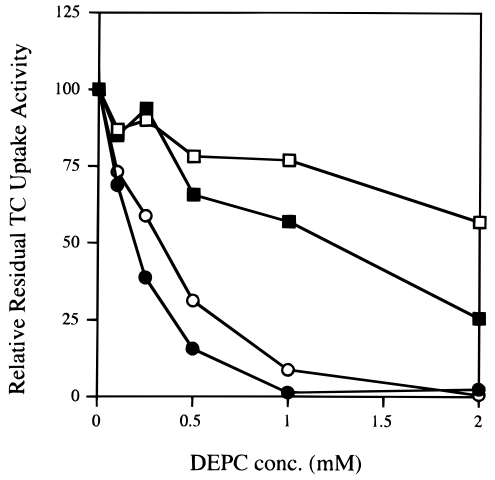

FIGURE 7: Abolition of the tetracycline transport activity of inverted membrane vesicles by diethyl pyrocarbonate (DEPC). Vesicles were treated with the indicated concentrations of DEPC in 50 mM MOPS/KOH buffer (pH 6.5) containing 0.1 M KCl at room temperature for 30 min. Then unreacted DEPC was removed by washing the vesicles by ultracentrifugation using a Beckman TLA100.2 instrument, followed by resuspension in the same buffer. The initial rate of tetracycline uptake was measured for 30 s in a reaction mixture containing 500 μM $CoCl_2$ and 50 μM [^{3}H]tetracycline. The vesicles were energized with 20 mM ascorbate and 100 μM phenazine methosulfate (PMS) in place of NADH. The relative residual rates of the uptake by the DEPC-treated vesicles are plotted as percentages against the rate of the uptake by each type of vesicle treated in the absence of DEPC: closed circles, pLGT2 [wild-type TetA(B)]; open circles, pLGNHH [His-less TetA(B) with His257]; closed squares, pLGH257Y [H257Y single mutant TetA(B)]; open squares, pLGNHY [His-less TetA(B) with Tyr257].

alanine is in general a replacement causing little disturbance of the protein structure except for the loss of a charge. In fact, the replacement of histidines except for His257 had almost no effect on the tetracycline transport activity of TetA(B). Therefore, the drastic decrease in the activity caused by the replacement of His257 with Ala is not likely due to structural disturbance by the introduction of an alanine side chain. His257 must be important for confering the transport activity.

A transmembrane histidine residue not only is distributed in LacY and TetA but also is suggested to be possibly located in LacS (Poolman et al., 1992) and some other substrate/H^+ symporters (Poolman et al., 1995), whereas there is no corresponding transmembrane histidine residue in MelB (Yazhu et al., 1984) or some other drug exporters homologous to TetA (Yoshida et al., 1990; Neyfakh, 1992). On the basis of the results indicating that the transmembrane histidine residue is certainly important for the function but an imidazole group is not necessary, in the latter group of transporters, some other amino acid residues such as tyrosine may play a role in place of histidine.

It is clear that some kinds of mutants of this transmembrane histidine residue show uncoupling between the substrate transport and the proton translocation (Puttner et al., 1989; Yamaguchi et al., 1991), indicating the role of these histidine residues in a substrate/H^+ coupling. However, the role of the transmembrane histidine residue in proton translocation may be an auxiliary one because some mutants such as H257Y retain not only substrate transport activity but also tetracycline/H^+ coupling activity.

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BI952116R