Aspartic Acid-66 Is the Only Essential Negatively Charged Residue in the Putative Hydrophilic Loop Region of the Metal–Tetracycline/H^+ Antiporter Encoded by Transposon Tn10 of *Escherichia coli*†

Akihito Yamaguchi,* Mariko Nakatani, and Tetsuo Sawai

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan

Received March 10, 1992; Revised Manuscript Received June 2, 1992

ABSTRACT: Of the 16 acidic amino acid residues located in the hydrophilic region of the metal–tetracycline/H^+ antiporter of transposon Tn10, five glutamic acids and three aspartic acids are conserved among the tetracycline/H^+ antiporters of Gram-negative bacteria. When these conserved acidic residues were each replaced by a neutral polar residue, glutamine or asparagine, only the Asp 66 substitution mutants completely lost their transport activity. The substitution of Glu274, Asp120, Glu181, or Asp38 caused significant reduction of the transport activity, whereas the substitution of the other three residues had no detectable effect on the activity. These findings led to the conclusion that only Asp66 is essential for the transport function.

The metal–tetracycline/H^+ antiporter encoded by transposon Tn10 is responsible for high level bacterial resistance to tetracycline (Mendez et al., 1980). The antiporter excludes the monocationic metal–tetracycline chelation complex from the resistant cells coupled with an influx of protons, the stoichiometry being 1:1 (Yamaguchi et al., 1990a, 1991). The Tn10-antiporter (Tet B) belongs to a family of tetracycline resistance proteins of Gram-negative bacteria (Mendez et al., 1980). Three representative Tet proteins, Tet A encoded by transposon Tn1721 (Waters et al., 1983), Tet C encoded by plasmid pBR322 (Shutcliffe, 1978), and Tet B (Hillen & Schollmeier, 1983; Nguyen et al., 1983), have been sequenced and compared, which revealed about 50–70% homology (Waters et al., 1983) with essentially the same hydropathic pattern. Eckert and Beck (1989) presented a putative secondary structure model of Tn10–Tet B, including 12 transmembrane segments connected through interhelix loops, based on the hydropathy profile and the results of limited proteolysis. According to their model, the N- and C-terminals are both located on the cytoplasmic surface and the central loop is exceptionally large. The secondary structure is common to secondary solute transporters such as sugar/H^+ symporters (Henderson, 1990), indicating that the tetracycline/H^+ antiporter belongs to a super family of solute transporters. We confirmed and refined their model based on the results of site-directed antibody binding (Yamaguchi et al., 1990b). The main difference between their model and our model is that the charged residues located at the boundary between hydrophobic and hydrophilic segments are placed in the loop region of the membrane surface in our model (Yamaguchi et al., 1992). As a result, of the 19 acidic residues in the Tet B protein, only three aspartic acids are clearly located in the hydrophobic transmembrane segments in our model (Figure 1). These three transmembrane aspartic acids were replaced by glutamic acid, asparagine, or lysine in our previous work (Yamaguchi et al., 1992), which showed that these transmembrane negative charges are essential for the tetracycline transport function. These transmembrane negative charges probably form a transmembrane pathway for the monocationic metal–tetracycline complex.

Of the 16 negatively charged residues located in the putative hydrophilic loop regions, six (Glu32, Glu181, Glu236, Glu274, Asp66, and Asp120) are conserved among Tet proteins of classes, A, B, and C of Gram-negative bacteria, and the other two (Glu37 and Asp38) are conserved among two of these classes (Waters et al., 1983). Among them, two aspartic acids and two glutamic acids are postulated to be located on the cytoplasmic surface of the membrane (Figure 1), and the other one aspartic acid and three glutamic acids, on the periplasmic surface. These negatively charged residues on the membrane surface are expected to play some roles in the substrate–protein interaction at the start and finish of the transport process. In this study, we examined the roles of these surface negatively charged residues by means of the site-directed mutagenesis technique.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *Escherichia coli* W3104 (Yamamoto et al., 1981) was used for inverted vesicle preparation. *E. coli* TG1 (Taylor et al., 1985) was used for the transformation of mutated plasmids and the preparation of the plasmids. *E. coli* JM109 was used for the preparation of single-stranded DNA. *E. coli* CJ236 and BMH71-18 mutS (Kunkel, 1985) were used for mutagenesis with the Kunkel method.

pCT1182 (Yamaguchi et al., 1992) was made by subcloning the *tet* genes, *tet*A and *tet*R, of transposon Tn10 into pUC118 (Vieira & Messing, 1987) and used for mutagenesis. The low copy-number plasmid, pLGT2, was made by the subcloning of these *tet* genes into pLG339 (Stoker et al., 1982) and used for expression of the *tet* genes.

Site-Directed Mutagenesis. Mutagenesis was performed by the oligonucleotide-directed site-specific mutagenesis method of Kunkel (1985) or Taylor et al. (1985), using pCT1182. The mutagenic primers that are listed in Table I were synthesized with a Cyclone Plus DNA/RNA synthesizer (MilliGen Biosearch Co.). Each of the mutagenic primers contained an additional silent mismatch to make a new restriction site. The mutant plasmids were at first detected

† This work was supported in part by Grant-in-Aid for Scientific Research (C) and on Priority Areas by The Ministry of Education and a grant from the Naito Foundation, Japan.

0006-2960/92/0431-8344$03.00/0

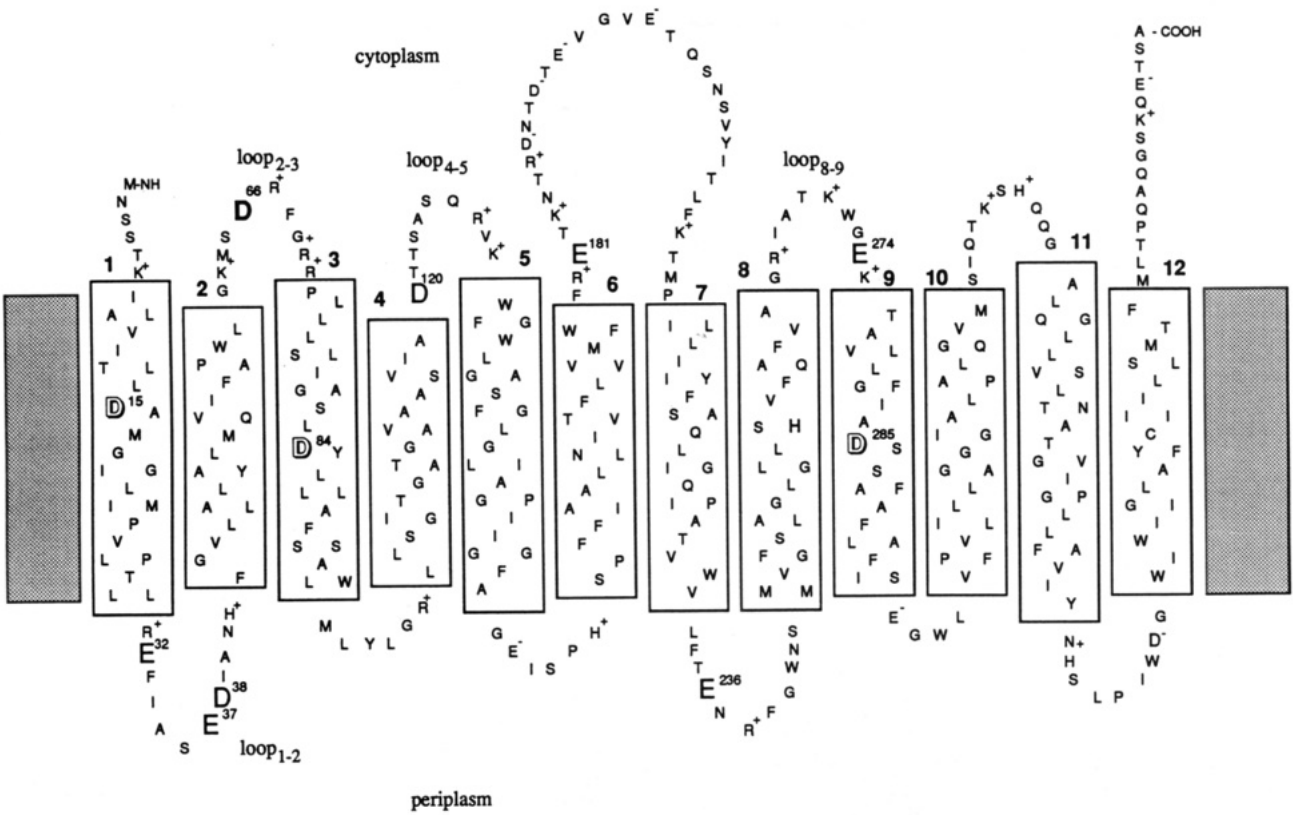

FIGURE 1: Putative secondary structure model of the Tn10–Tet protein. Hydrophobic α-herical segments are enclosed by boxes. The bold letters indicate the conserved negatively charged residues in the hydrophilic loop region, which were replaced in this study. Three essential Asp residues in the transmembrane segments are depicted by shadowed letters.

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

mutagenic primer	primer sequence	plasmid	amino acid substitution
E32Q	5′-AATAAATTGCCTTAAGAGCGTTGG-3′ (*Afl*II)	pLGE32Q	Glu32 → Gln
E37Q	5′-GATATCCTGCGAAGCAATGAATTC-3′ (*Eco*RI)	pLGE37Q	Glu37 → Gln
D38N	5′-GATGTTTTCCGAAGCAATGAATTC-3′ (*Eco*RI)	pLGD38N	Asp38 → Asn
D120N	5′-AGAAGCGCTGGTGGTGTTGGCAAT-3′ (*Eco*47III)	pLGD120N	Asp120 → Asn
E181Q	5′-ATCTCGAGTATTTTTGGTCTGACG-3′ (*Xho*I)	pLGE181Q	Glu181 → Gln
E236Q	5′-TCCGAAACGATTCTGGGTAAATAG-3′ (*Xmn*I)	pLGE236Q	Glu236 → Gln
E274Q	5′-TACTGCAGTTTTCTGGCCCCATTT-3′ (*Pst*I)	pLGE274Q	Glu274 → Gln

[a] The primers correspond to the complementary sequence of the coding strand. The mutagenic primers contained two kinds of mismatches: mismatches to cause amino acid replacements and silent mismatches to cause new restriction site(s). Asterisks indicate the mismatches.

as to the appearance of the new restriction site and then confirmed by DNA sequencing. The mutated regions of the *tet* genes were then restricted from pCT1182 and inserted into pLGT2 by means of fragment exchange.

Preparation of Inverted Membrane Vesicles. Cells were grown on the minimum medium supplemented with 0.2% glucose and 0.1% casamino acids. At the middle of the logarithmic phase, *tet*A gene expression was induced for 2 h by incubation with 0.25 mg/mL heat-inactivated chlorotetracycline. Inverted vesicles were prepared after the disruption of cells with a French press in 50 mM MOPS–KOH buffer (pH 6.6) containing 0.1 M KCl and 10 mM EDTA. Then the vesicles were washed once with 50 mM MOPS–KOH (pH 7.0) containing 0.1 M KCl (Yamaguchi et al., 1990c).

Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis of the inverted vesicles was followed by electroblotting of the proteins. The Tet protein was detected with an anti-carboxyl-terminal antibody, as described previously (Yamaguchi et al., 1990b).

Transport Assay. [7-^{3}H(N)]Tetracycline (Du Pont–New England Nuclear) uptake into the inverted membrane vesicles was measured as described previously (Yamaguchi et al.,

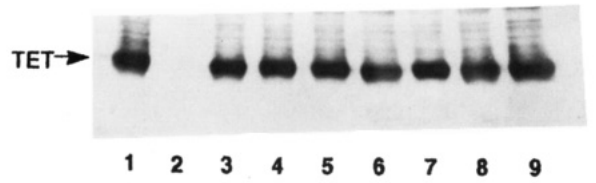

FIGURE 2: Immunoblot analysis of inverted membrane vesicles prepared from *E. coli* W3104 cells harboring the wild-type or a mutant plasmid. Each lane contained about 10 μg of total protein. Lane 1, pLGT2 (wild type); lane 2, no plasmid; lane 3, pLGE32Q; lane 4, pLGE37Q; lane 5, pLGD38N; lane 6, pLGD120N; lane 7, pLGE181Q; lane 8, pLGE236Q; lane 9, pLGE274Q.

1990a) in the presence of vesicles (0.7 mg of protein/mL), 50 μM $CoCl_2$, 0.1 M KCl, and 2.5 mM NADH in MOPS–KOH buffer, pH 7.0.

Measurement of Bacterial Resistance to Antibiotics. Bacterial resistance to tetracycline was measured by the agar dilution method using Heart Infusion Agar (Eiken Chemical Co., Tokyo) plate as described in our previous paper (Yamaguchi et al., 1990c).

RESULTS

Expression of Mutant tet Genes. Three aspartyl residues (Asp38, Asp66, and Asp120) and five glutamyl residues (Glu32, Glu37, Glu181, Glu236, and Glu274) were replaced one by one by asparagine or glutamine, respectively. The resulting plasmids encoding these mutant Tet proteins were named pLGD38N, pLGD66N, pLGD120N, pLGE32Q, pLGE37Q, pLGE181Q, pLGE236Q, and pLGE274Q, respectively. Among them, pLGD66N has been reported in our previous paper (Yamaguchi et al., 1990c).

The mutant protein production was detected by immunoblot analysis as described under Experimental Procedures. As shown in Figure 2, there was no significant difference in the amounts of the mutant and wild-type Tet proteins in the inverted membrane vesicles. The mutant protein encoded by pLGD66N was also normally produced (Yamaguchi et al., 1990c).

Effect of Replacement of Negatively Charged Residues Located on Cytoplasmic Surface of Membrane. Of the eight conserved acidic residues in the hydrophilic regions of the Tn10–Tet protein, four are postulated to be located on the cytoplasmic surface according to the putative secondary structure (Figure 1). In our previous study (Yamaguchi et al., 1990c), when Asp66 was replaced by asparagine, the transport activity was completely lost. Since the Glu66 mutant retained very low but significant tetracycline transport activity, it was concluded that the negative charge at position 66 is essential for the transport function (Yamaguchi et al., 1990c). When Asp120 and Glu274 were replaced by asparagine and glutamine, respectively, the initial rate of the tetracycline transport in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ was reduced to 85% and 25%, respectively, of the wild-type level (Figure 3). In contrast, when Glu181 was replaced by glutamine, there was no significant decrease in the initial rate of the transport in this condition (Figure 3).

These results clearly indicated that no conserved acidic residues on the cytoplasmic surface other than Asp66 are essential for the transport function.

Effect of Replacement of Negatively Charged Residues Located on Periplasmic Surface of Membrane. The postulated periplasmic loop regions contain seven negatively charged residues (Figure 1). Among them, Glu32, Glu37, Asp38, and Glu236 are conserved in at least two classes of the Tet proteins, A, B, and C (Waters et al., 1983). When Asp38 was

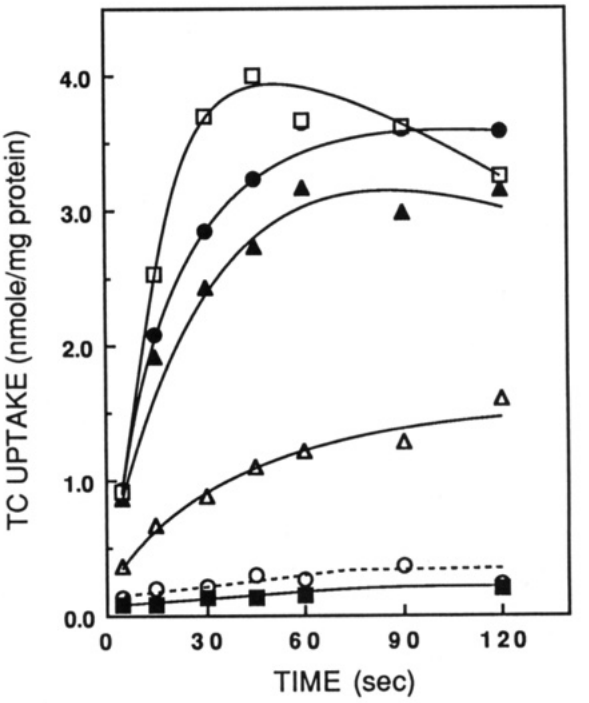

FIGURE 3: Tetracycline (TC) uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells containing wild type or a mutant Tet protein with substitution of a cytoplasmic surface-located negatively charged residue. The uptake was measured by addition of 2.5 mM NADH unless otherwise stated. Symbols: (●) wild type, (■) pLGD66N, (▲) pLGD120N, (□) pLGE181Q, (△) pLGE274Q, (- -○- -) wild type measured in the absence of NADH.

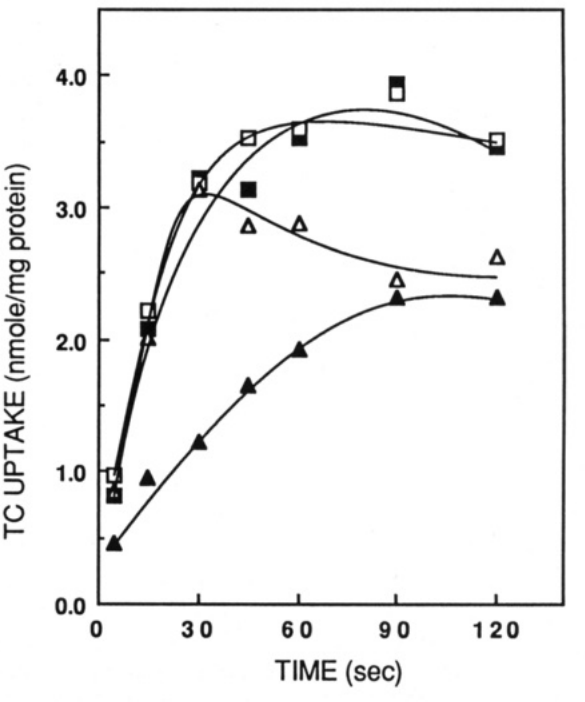

FIGURE 4: Tetracycline (TC) uptake by inverted membrane vesicles prepared from *E. coli* W3104 cells containing wild-type or a mutant Tet protein with substitution of a periplasmic surface-located negatively charged residue. The uptake was measured by addition of 2.5 mM NADH unless otherwise stated. Symbols: (■) pLGE32Q, (□) pLGE37Q, (▲) pLGD38N, (△) pLGE236Q.

replaced by asparagine, the initial transport activity was reduced to 40% of the wild-type level (Figure 4). In contrast, when Glu32 or Glu37 was replaced by glutamine, there was no significant effect on the transport activity (Figure 4). In the case of Glu236, the replacement with Gln caused no significant change in the initial rate of the tetracycline

Table II: Resistance Levels of *E. coli* W3104 Cells Harboring Plasmids Encoding Mutant *tet*A Genes[a]

plasmid	MIC, μg/mL	K_m, μM	V_{max}, nmol (mg of protein)$^{-1}$ min^{-1}
no plasmid	0.8		
pLGT2 (wild)	200	33.0	55.8
pLGE32Q	200	24.8	42.0
pLGE37Q	200	22.9	33.7
pLGD38N	200	25.0	12.4
PLGD66N	0.8		
pLGD120N	19	3.6	4.1
pLGE181Q	38	1.5	9.5
pLGE236Q	150	40.9	51.4
pLGE274Q	200	48.4	28.0

[a] Expressed as minimum inhibitory concentration (MIC) and the K_m – V_{max} values of the inverted vesicles calculated from the initial 30 s [^{3}H]tetracycline uptake in the presence of 1 mM $CoCl_2$ and 5–100 μM [^{3}H]tetracycline by Hanes–Woolf plot.

transport, while the accumulated tetracycline was gradually released from the mutant vesicles (Figure 4), probably due to the leakiness of the mutant vesicles. Therefore, none of the conserved negative charges on the periplasmic surface is essential for the transport function.

Tetracycline Resistance of Cells Carrying Mutant Plasmids. Table II shows the tetracycline resistance of the *E. coli* cells carrying the mutant plasmids. The strain carrying pLGD66N was the only strain that showed the same susceptibility to tetracycline as the host strain without plasmid, confirming that the only essential negatively charged residue in the hydrophilic region of the Tet protein is Asp66. On the other hand, the strains carrying pLGD120N or pLGE181Q showed the reduced resistance to tetracycline, whereas the other strains carrying pLGE32Q, pLGE37Q, pLGD38N, pLGE236Q, or pLGE274Q showed no significant alterlations in the resistance level. In comparison with the results of tetracycline transport, the resistance of the cells carrying pLGD120N and pLGE181Q was unexpectedly low, and in contrast, the resistance of ones carrying pLGE274Q and pLGD38N was unexpectedly high. These discrepancies between in vitro and in vivo results might be due to the assay conditions; that is, the divalent cation and tetracycline concentration in the nutrient agar plate were higher than those in the in vitro condition of Figures 3 and 4. Thus, the dependence of the transport activity of the mutant Tet proteins on tetracycline concentration was then measured in the presence of high divalent cation concentration as described below.

Kinetics of Tetracycline Transport by Mutant TET Proteins. The tetracycline concentration dependence of the transport activity of the inverted membrane vesicles under the high divalent cation concentration (1 mM $CoCl_2$) was measured and the K_m and V_{max} values were calculated from Hanes–Woolf plots. As shown in Table II, the K_m values of the D120N and E181Q mutants were significantly lower than those of the wild type so that the tetracycline transport of these mutants were saturated at low tetracycline concentration, resulting in the far lower V_{max} values than those expected from the initial rate as in Figure 3. In contrast, since the K_m values of the E274Q and D38N mutants were higher or similar to the wild-type K_m value, V_{max} values were larger than those of the D120N and E181Q mutants. The in vivo resistance level corresponded to the V_{max} value, although the resistance level was saturated when V_{max} value was more than 12 nmol (mg of protein)$^{-1}$ min^{-1}. The slightly low resistance level of the cells carrying pLGE236Q might be related to the leakage of tetracycline through the membrane as shown in Figure 4.

DISCUSSION

Our results clearly indicate that Asp66 is the only essential negatively charged residue in the hydrophilic loop regions of the Tet protein. The mutation at Glu274 caused the reduction in the affinity of the substrate binding. On the other hand, the mutations at Asp120, Glu181, and Asp38 caused a significant decrease in the maximum velocity of the substrate translocation. In contrast, the mutations at the other three conserved negatively charged residues showed no significant change in both in vivo and in vitro function of the Tet protein.

Asp66 is located in the loop between helices 2 and 3. This loop contains a highly conserved sequence motif, GXXXXRX-GRR, and is postulated to act as an entrance gate for a substrate (Yamaguchi et al., 1990c). On the other hand, Glu274 is located in the loop between helices 8 and 9. This loop contains another conserved motif, GXXXXK(/R)-XGEK(/R), and according to the tandem duplication hypothesis (Rubin et al., 1990), both loops might play complemental roles. The reduction in the substrate affinity by the Glu274 substitution might indicate some contribution of this residue to the putative gating function.

The dramatic decrease in the K_m value by substitution of Asp120 and Glu181 is likely due to the decrease in the turnover rate of the tetracycline translocation. Taking the positions of these residues into consideration, the effect of the substitution of these residues might not be due to the contribution of these residues to the specific rate-limiting step of the substrate translocation process, but rather it might be caused by the partial distortion of the Tet protein conformation probably due to breakdown of the salt bridge composed of these residues. Asp120 is located in $loop_{4-5}$, which is next to $loop_{2-3}$ on the cytoplasmic surface. It is possible that this Asp residue may interact with the positive charge in polycationic $loop_{2-3}$ and may contribute to the protein stability. Glu181 is located in the central large loop, of which the sequence homology between Tet proteins is very low, that is, only two residues, Glu181 and Ser199, are conserved out of 30 residues of this putative loop. Glu181 is near the neck of this loop and may have some structural role.

It is surprising that none of the periplasmic negative charge is essential for the membrane protein function, considering that most transmembrane proteins are greatly negatively charged on the periplasmic surface and greatly positively charged on their cytoplasmic surface. Such an asymmetrical distribution of the charged residues is believed to be necessary for the orientation of the protein in the membrane. Our results suggest that such a role of the negative charges does not depend on a specific residue but may be necessary for retention of the electric dipole moment.

Among the negatively charged residues on the periplasmic surface, only the substitution of Asp38 affected the transport activity. Regarding our previous results (Yamaguchi et al., 1992), helices 2 and 3 probably form a substrate transport channel, because essential residues in these helices are arranged on the same vertical stripes of the hydrophilic side of the amphipathic cylinder of the helices. Asp38 is located on the neck of $loop_{1-2}$ connected with helix 2. Therefore, Asp38 may play some role in the exit of the transport channel.

REFERENCES

Eckert, B., & Beck, C. F. (1989) *J. Biol. Chem. 264*, 11663–11670.

Henderson, P. J. F. (1990) *J. Bioenerg. Biomembr. 22*, 525–569.

Hillen, W., & Schollmeier, K. (1983) *Nucleic Acids Res. 11*, 525–539.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Mendez, B., Tachibana, C., & Levy, S. B. (1980) *Plasmid 3*, 99–108.
Nguyen, T. T., Postle, K., & Bertrand, K. P. (1983) *Gene 25*, 83–92.
Rubin, R. A., Levy, S. B., Heinrikson, R. L., & Kezdy, F. J. (1990) *Gene 87*, 7–13.
Stoker, N. G., Fairweather, N. F., & Spratt, B. G. (1982) *Gene 18*, 335–341.
Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764–8785.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3–11.
Waters, S. H., Rogowsky, P., Grinsted, J., Altenbuchner, J., & Schmitt, R. (1983) *Nucleic Acids Res. 11*, 6089–6105.
Yamaguchi, A., Udagawa, T., & Sawai, T. (1990a) *J. Biol. Chem. 265*, 4809–4813.
Yamaguchi, A., Adachi, K., & Sawai, T. (1990b) *FEBS Lett. 265*, 17–19.
Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T., & Sawai, T. (1990c) *J. Biol. Chem. 265*, 15525–15530.
Yamaguchi, A., Iwasaki-Ohba, Y., Ono, N., Kaneko-Ohdera, M., & Sawai, T. (1991) *FEBS Lett. 282*, 415–418.
Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., & Sawai, T. (1992) *J. Biol. Chem. 267*, 7490–7498.
Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y., & Yamagishi, S. (1981) *J. Bacteriol. 145*, 808–813.

Registry No. Asp, 56-84-8; Glu, 56-86-0; H^+, 12408-02-5; TC, 60-54-8.