Volume 307, number 2, 229–232 FEBS 11336 July 1992

Serine residues responsible for tetracycline transport are on a vertical stripe including Asp-84 on one side of transmembrane helix 3 in transposon Tn10-encoded tetracycline/H^+ antiporter of *Escherichia coli*

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Received 3 June 1992

Putative transmembrane helix 3 of the tetracycline/H^+ antiporter encoded by a transposon, Tn10, contains four serine residues, Ser-77, Ser-82, Ser-91 and Ser-92. Each of these serine residues was replaced by site-directed mutagenesis. Of these four serine residues, Ser-77 was important for the transport function, and a bulky side chain at position 91 hindered substrate translocation, whereas Ser-82 and Ser-92 did not play any role. Ser-77 and Ser-91 are on the same vertical stripe, that includes the essential Asp-84, on the hydrophilic side of putative helix 3. These observations suggest that helix 3 is part of the tetracycline translocation channel across the membrane.

Tetracycline; Antiporter; Serine; Tetracycline/H^+ antiporter; Site-directed mutagenesis; Antibiotic resistance

1. INTRODUCTION

The transposon Tn10-encoded tetracycline resistance protein (Tn10-TET) is a metal-tetracycline/H^+ antiporter [1]. Tn10-TET is a membrane protein [2] composed of 401 amino acid residues [3,4]. On the basis of its hydropathy profile, and the results of protease digestion and antibody binding, a topological model containing 12 transmembrane helices and 11 interhelix loops was proposed (Fig. 1) [5,6]. Although Tn10-TET contains 43 charged residues, only four are located in the transmembrane helix region. Of these transmembrane charged residues, Asp-84 is one of the essential residues for tetracycline translocation [7]. Asp-84 seems to play a role in substrate recognition in combination with Asp-15 and Asp-285 through their negative charges [7]. Asp-84 is located in putative helix 3 (Fig. 1). We also found that Asp-66, located in cytoplasmic $loop_{2-3}$ (Fig. 1), is also essential for tetracycline translocation, probably through a gating role [8]. Cytoplasmic $loop_{2-3}$ (Fig. 1) is connected with helix 3. Thus, helix 3 must be important as a route for substrate migration from a putative gate, $loop_{2-3}$, to a proposed substrate recognition site, Asp-84. It is reasonable to expect that some of the polar residues in this route are involved in substrate translocation.

Helix 3 contains four serine residues, of which Ser-77 and Ser-82 are located between $loop_{2-3}$ and Asp-84. Only Ser-77 is conserved in all TET proteins of Gram-negative bacteria so far sequenced belonging to classes A, B and C [9]. The other three serine residues are not conserved, however, site-directed replacement of these unconserved residues can provide a clue as to whether or not they are located in the substrate translocation route. In this study, each serine residue in helix 3 was replaced with a cysteine, alanine, or threonine by site-directed mutagenesis.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]Tetracycline and 5-α-[^{35}S]dCTP were purchased from Du Pont-New England Nuclear and Amersham, respectively. All other materials were of reagent grade and obtained from commercial sources.

2.2. *Site-directed mutagenesis*

Mutagenesis was performed by the method of Taylor et al. [10] with an oligonucleotide-directed in vitro mutagenesis system (Version 2; Amersham) using pER as a template as described in our previous paper [7]. The mutagenic primers (Table I) were synthesized with an Applied Biosystems DNA synthesizer Model 380B or CYCLONE Plus DNA/RNA Synthesizer (MilliGen Biosearch Co.). Mutant genes were transferred to pLGT [8] by fragment exchange.

2.3. *Transport assays*

The inverted membrane vesicles were prepared from *E. coli* W3104 [11] cells containing the wild-type or a mutant plasmid, as described previously [12]. [^{3}H]Tetracycline uptake by inverted membrane vesicles was assayed as described previously [8] in the presence of 50 μM $CoCl_2$, 10 μM [^{3}H]tetracycline and 2.5 mM NADH in MOPS-KOH buffer (pH 7.0) unless otherwise stated.

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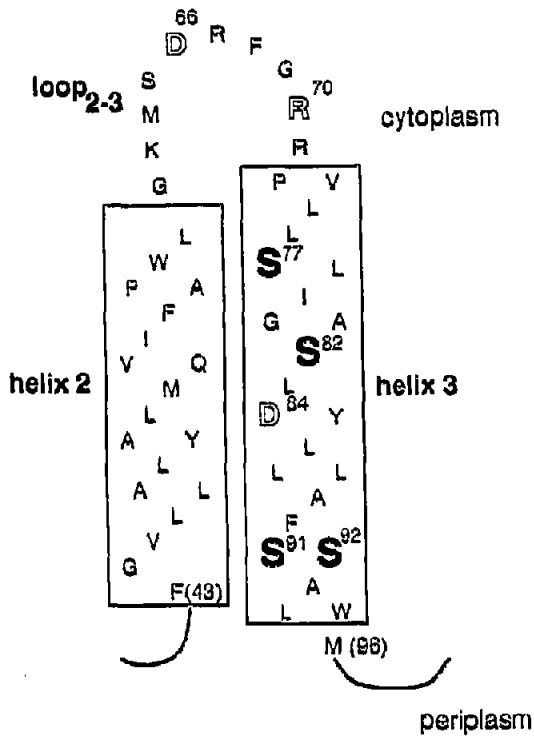

Fig. 1. Secondary structure model of the helix 2-loop$_{2-3}$-helix 3 region of the TnlO-TET protein. Amino acid residues are depicted by one letter symbols. Hydrophobic domains are shown in *boxes* as transmembrane α-helices. Serine residues mutated in the present work are depicted by *bold letters* with the numbers of their positions in the primary sequence. Essential residues for tetracycline transport, which have been found in our previous papers [7,8,12], are indicated by *outlined letters*.

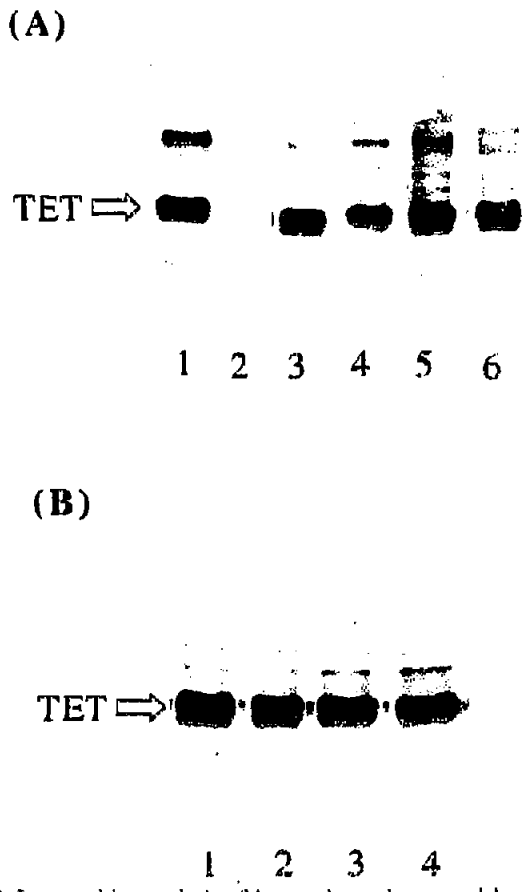

Fig. 2. Immunoblot analysis of inverted membrane vesicles from *E. coli* W3104 cells harboring the wild-type or a mutant plasmid. (A) Wild-type and Cys mutants: lane 1, wild-type; lane 2, no plasmid; lane 3, S77C; lane 4, S82C; lane 5, S91C; lane 6, S92C. (B) Ala or Thr mutants: lane 1, S77A; lane 2, S77T; lane 3, S91A; lane 4, S91T.

3. RESULTS AND DISCUSSION

3.1. *The effect of replacement of serine residues in helix 3 with cysteine*

Four serine residues in helix 3 were replaced by a cysteine residue by oligonucleotide-directed site-specific mutagenesis. Inverted membrane vesicles prepared from *E. coli* W3104 carrying the mutant plasmids contained the normal amount of TET proteins detected by immunoblot analysis (Fig. 2A).

As shown in Fig. 3, among the four Cys mutants, only the Cys-77 mutant vesicles had completely lost tetracycline transport activity. This finding is consistent with the fact that Ser-77 is the only conserved serine residue in helix 3 [9]. Of the three other Cys mutants, Cys-91 showed a significant decrease in tetracycline transport (Fig. 3), which was about 20% the wild-type activity (initial rate for 30 s). In contrast, the Cys-82 and Cys-92 mutants showed similar activity to the wild-type (Fig. 3). Therefore, it is concluded that Ser-82 and Ser-92 do not play a role in the TET function, whereas Ser-91 may have some relationship to the TET function.

3.2. *Replacement of Ser-77 or Ser-91 with alanine or threonine*

The volume of the side chain of a cysteine residue is about 1.5-times larger than that of serine [13], and is hydrophobic and shows weak hydrogen bonding. In order to reveal whether the defect in the activity of the Cys mutants is due to the steric hindrance of the massive side chain or to the lack of hydrogen bonding, Ser-77 or Ser-91 were replaced by Thr or Ala. These mutations did not affect the expression of the TET protein (Fig. 2B).

The replacement of Ser-77 by Thr caused complete loss of activity (Fig. 4A) as did the replacement by Cys. However, the Ala-77 mutant showed unexpectedly high activity of about 45% the wild-type. Therefore, it is concluded that an hydroxyl group at position 77 is not obligatory for the tetracycline transport function. K_m values of the initial tetracycline uptake for 30 s were calculated from Lineweaver–Burk, Hanes–Woolf and Eadie plots. In any plot, the K_m value of the wild-type TET was 46 μM, which was higher than the previously reported K_m value [8], probably due to the difference in

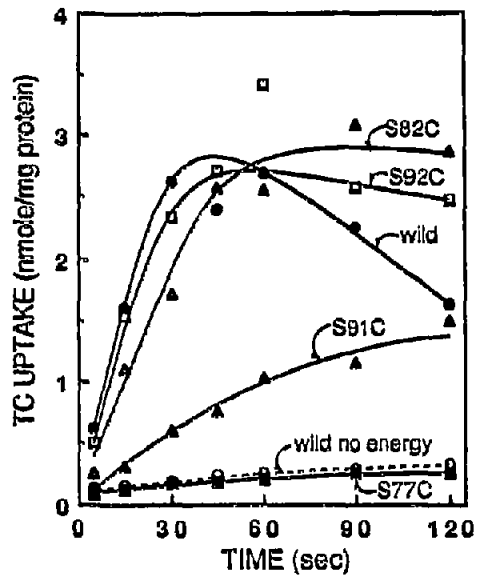

Fig. 3. Tetracycline (TC) uptake by inverted membrane vesicles from *E. coli* W3104 cells harboring the wild-type or Cys mutant plasmid. [^{3}H]Tetracycline uptake was examined in the presence of 50 μM $CoCl_2$. ●, wild-type; ■, S77C; △, S82C; ▲, S91C; □, S92C. Open circles with broken line indicate the background uptake by the wild-type vesicles in the absence of NADH.

the assay conditions. The K_m value of the Ala-77 mutant was about 100 μM. Thus, the affinity of the Ala-77 mutant to tetracycline was clearly lower than that of the wild-type, indicating that an hydroxyl group at position 77 contributes to the affinity for substrate binding. The complete loss of the activity in the Thr-77 mutant indicated that the strict arrangement of the hydroxyl group at position 77 is absolutely needed for its contribution to the activity. Since the Ala-77 mutant showed significant residual activity, the loss of activity in the Thr and Cys mutants seems to be due to the steric hindrance of the massive side chain.

On the other hand, the Thr-91 mutant showed a decreased but significant activity similar to the Cys-91 mutant, whereas the Ala-91 mutant showed normal activity, i.e. similar to the wild-type, indicating that the hydroxyl group at position 91 did not contribute to the transport activity (Fig. 4B). Since the Thr-91 and Cys-91 mutants showed clearly lower activity than the wild-type and Ala mutant, the massive side chain at position 91 is also likely to inhibit the tetracycline transport, however, the degree of strictness of the side chain arrangement at position 91 required for activity seems to be far less than that at position 77.

Table I

Mutagenic primers used for site-directed mutagenesis, and codons changed in the mutant plasmids

Plasmid	Primer sequence	Substituting residue		
		Position	Codon	Amino acid
pS77C	5′-GTGCTGTTGTTGTGCCTAATAGGCGCATCG-3′ (*BglI*)	77	TCA→TGC	Ser→Cys
pS82C	5′-TTAATAGGCGCATGCCTGGATTACTTATTG-3′ (*SphI*)	82	TCG→TGC	Ser→Cys
pS91C	5′-TTATTGCTGGCTTCTGCAGTGCGCTTTGG-3′ (*PstI*)	91	TTT→TTC	Ser→Cys
pS92C	5′-CTGGCTTTTTCATGCGCGCTTTGGATGCTG-3′ (*BssHII*)	92	AGT→TGC	Ser→Cys
pS77A	5′-GTGCTGTTGCTAGCGCTAATAGGC-3′ (*Eco47III*)	77	TCA→GCG	Ser→Ala
pS77T	5′-CGGAGGCCTGTGCTGTTGTTGACATTAAT-3′ (*StuI*)	77	TCA→ACA	Ser→Thr
pS91A	5′-CTGGCTTTTGCTAGCGCTCTTTGG-3′ (*Eco47III*)	91	TCA→GCT	Ser→Ala
pS91T	5′-CTGGCTTTTACAAGCGCTCTTTGG-3′ (*Eco47III*)	91	TCA→ACA	Ser→Thr

The mutagenic primers contained two kinds of mismatches, mismatches to cause amino acid replacements (asterisks) and silent mismatches to cause new restriction site(s) (underlined).

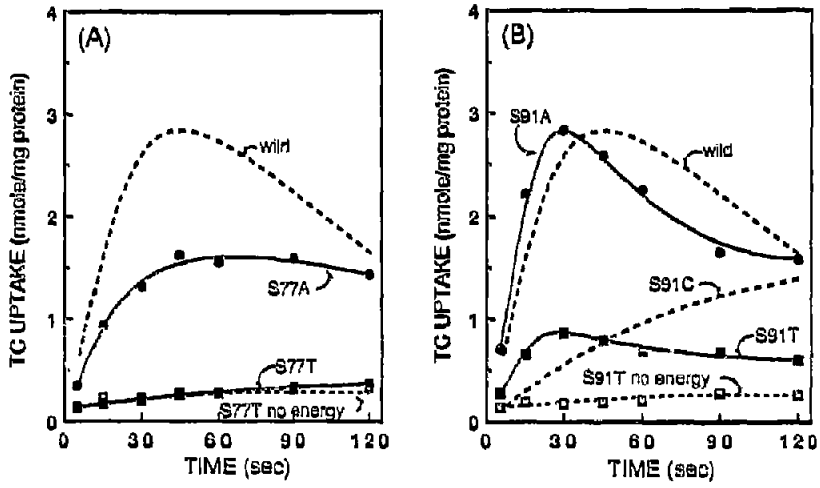

Fig. 4. Tetracycline uptake by inverted vesicles containing a mutant TET protein of which Ser-77 or Ser-91 were substituted by Ala or Thr. The assay conditions were the same as in Fig. 3. (A) Ser-77-substitution mutants; ●, S77A; ■, S77T. Open squares with broken line indicate the background uptake by the S77T vesicles in the absence of NADH. (B) Ser91-substitution mutants; ●, S91A; ■, S91T. Open squares with broken line indicate the background uptake by the S91T vesicles in the absence of NADH.

3.3. *Arrangements of the serine residues on the helical wheel projection of the helix 3*

Fig. 5 shows a helical wheel projection of helix 3, in which the positions of the Asp and Ser residues, denoted as three letter symbols, in the primary sequence of Tn10-TET are shown. An arrow indicates the direction of the hydrophobic moment of this helix, calculated using Kyte and Doolittle's hydropathy index [13], indicating that helix 3 is amphipathic. Such amphiphilic helices have been widely reported in membrane proteins [14] and are expected to form a membrane-spanning channel structure [14]. It is very interesting that the important serine residues, Ser-77 and Ser-91, are on a vertical stripe that includes another essential residue, Asp-84 [7], situated on the hydrophilic side of helix 3. Such a stripe-like arrangement of important residues has also been reported in *lac* permease [15,16]. The results in this report also support the gating-channel model for transport [17].

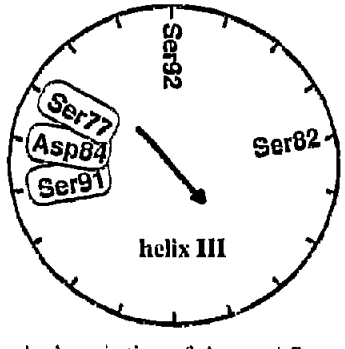

Fig. 5. Helical-wheel projection of Asp and Ser residues in putative helix 3. Residues important for or related to tetracycline transport function are shown enclosed. The arrow indicates the direction of the hydrophobic moment of this helix, calculated using Kyte and Doolittle's hydropathy indexes [13] for residues from Pro-72 to Met-96.

Acknowledgements: This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan and a Grant from Naito Memorial Foundation.

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