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Decreased Function of the Class B Tetracycline Efflux Protein Tet with Mutations at Aspartate 15, a Putative Intramembrane Residue

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The aspartate 15 residue within the first predicted intramembrane helix of the tetracycline efflux protein Tet has been conserved in four tetracycline resistance determinants from gram-negative bacteria. Its replacement in class B Tet by tyrosine, histidine, or asparagine resulted in a 60 to 85% loss of tetracycline resistance and a similar loss of tetracycline-proton antiport. The tyrosine and histidine substitutions lowered the V_{max} of the efflux system by some 90% but did not alter the K_m. The asparagine substitution raised the K_m over 13-fold, while the V_{max} was equal to or greater than that of the wild type. Therefore, although the nature of its role is unclear, aspartate 15 is important for normal Tet function.

The structural (*tetA*) genes of three different tetracycline resistance determinants from gram-negative bacteria (17) show a high degree of amino acid sequence similarity (class A and C Tet proteins are 78% identical, while the class B protein is 45% identical to the other two). These cytoplasmic membrane proteins use proton motive force to efflux a tetracycline-cation complex (10, 21).

Tet proteins appear to have two sets of six transmembrane α helices connected by a large cytoplasmic loop (5, 6, 14, 15, 19). There are four conserved potentially ionic residues predicted to be well within the membrane: Asp-15, Asp-84, His-257, and Asp-285 (for class B; for classes A and C, the residue numbers are greater by two). Asp-15, at least, is also conserved in class D (12). The maintenance evolutionarily of these ionic residues suggests an important role, since their presence in the membrane would be energetically costly. Moreover, because the substrate for the Tet protein is believed to be a tetracycline-magnesium ion complex with a net +1 charge which exchanges for a proton (20, 21), a potentially negatively charged residue within the membrane, such as Asp, might be involved in binding substrate during transport. His-257 appears to assist in proton exchange rather than in substrate binding (18). The present study focused on the Asp-15 residue of the class B Tet by use of site-directed mutagenesis in *Escherichia coli*.

Preparation of site-directed mutations. Oligonucleotide-directed mutagenesis of the class B *tetA* gene cloned on pLR1068 (Fig. 1) was performed with double-stranded DNA as described previously (7). Briefly, pLR1068 was linearized by a single *BalI* cut in the chloramphenicol resistance gene. Following alkaline phosphatase treatment, this fragment was hybridized with a 5.47-kb *NdeI-MluI* fragment of pLR1068 (missing the sequence for the first 126 amino acids of Tet) in the presence of the mutagenic oligonucleotide CGTTAC TCXATGCCATG, where X was T, C, or A (position 1 of the Asp-15 codon). The wild-type Asp-15 (G in position 1) was thereby mutated to Tyr, His, or Asn respectively. HB101 (2) was transformed with these hybrid fragments, and chloramphenicol-resistant transformants were isolated. Transformants whose DNA hybridized at 45°C with the end-radiolabeled oligonucleotide used for mutagenesis (about 2% of the total) were selected, and the mutation was verified by sequencing in the region (Sequenase; U.S. Biochemical Corp.). The mutant plasmids were named pMS45 and pMS108 (both Tyr), pMS202 (His), and pMS208 (Asn).

Tetracycline resistance specified by mutant Tet proteins. The *tetA* gene is under negative transcriptional regulation by *tetR* (Fig. 1) and can be induced by subinhibitory levels of tetracycline (1 μg/ml) or the noninhibitory inducer autoclaved chlortetracycline (50 μg/ml, autoclaved in growth medium [1]). Susceptibility to tetracycline was determined in L broth-grown induced cultures on gradient plates after 15 to 18 h at 37°C as described previously (3), using strain HB101 as host. The tetracycline resistance of the strain bearing wild-type Tet was greater than 135 ± 35 μg/ml (mean ± standard deviation, five experiments). The upper limit of this resistance was ≤250 μg/ml. The resistances of the Tyr, His, and Asn mutants and the plasmidless strain were 45 ± 13, 53 ± 12, 40 ± 4, and less than 1 μg/ml, respectively. The mutations therefore decreased resistance by 60 to 85% but did not eliminate Tet function.

Active transport of tetracycline in everted membrane vesicles. Lactate-dependent equilibrium accumulation of radiolabeled tetracycline by everted membrane vesicles (10) prepared from induced cells grown in minimal medium was used initially to assay the active efflux function of the Tet proteins (Fig. 2). After equilibration with 4.5 μM [^{3}H]tetracycline, vesicles from cells bearing wild-type Tet had actively accumulated 0.69 ± 0.1 nmol/mg of vesicle protein (mean ± standard deviation, three determinations with two vesicle preparations). Vesicles from the host strain (ML308-225) without a *tet*-bearing plasmid had no active uptake. Everted vesicles from the three mutants prepared and assayed at the same time as wild-type vesicles showed a 78 to 90% loss of active transport. Therefore, the reduction in resistance was reflected in a reduction of active efflux measured at equilibrium.

The tetracycline uptake system appears to exhibit Michaelis-Menten saturation kinetics (10, 21). To determine whether K_m and V_{max} were altered in the mutants, we

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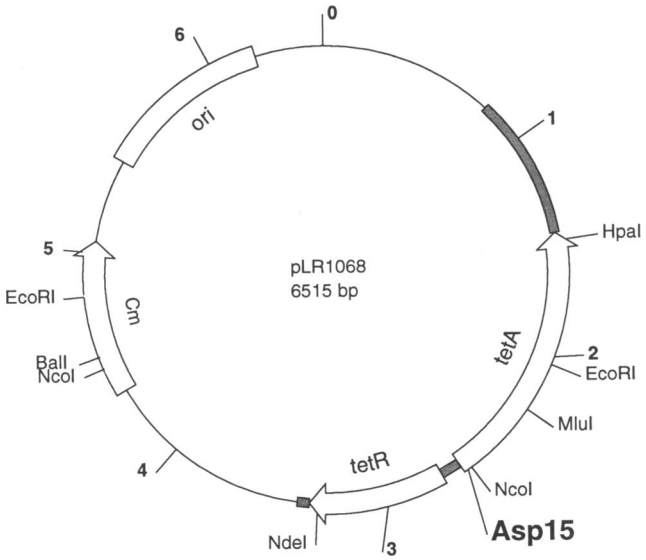

FIG. 1. Restriction map of pLR1068. The darkened area as well as *tetA* (efflux protein) and *tetR* (repressor protein) comprise the region derived from Tn*10*. The origin (ori) is from p15A. Cm, chloramphenicol resistance gene. Numbers indicate kilobases. The location of aspartate 15 in the TetA protein is shown.

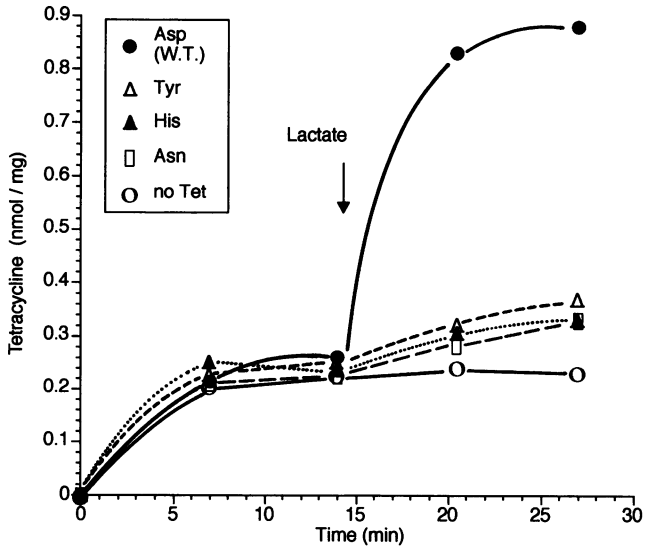

FIG. 2. Uptake of tetracycline by everted membrane vesicles of strain ML308-225 bearing wild-type, mutant, or no Tet protein. Assays were performed at 30°C at pH 7.5 with 50 mM potassium phosphate–10 mM $MgSO_4$ at 0.5 mg of vesicle protein per ml by filtration (10). [^{3}H]tetracycline (New England Nuclear; 0.6 Ci/mmol) was added to 4.5 μM at zero time; lithium lactate was added at time indicated by the arrow to energize the vesicles. Equilibrium active uptake was defined as the uptake attributable to lactate 13 min after its addition. The amino acid at residue 15 of Tet for each strain is shown. W.T., wild type Tet; no Tet, no Tet protein (vesicles from plasmidless strain).

assayed the uptake rate with 50 to 150 μl of vesicles at 1 mg of protein per ml. After incubation with or without 20 mM lithium lactate at 30°C for 3 to 5 min, an equal volume of buffer containing [^{3}H]tetracycline was added. Thirty seconds later, 6 to 8 ml of cold 100 mM LiCl–50 mM potassium phosphate (pH 7.5) was added, and the sample was filtered and washed with the same volume. K_m and V_{max} were determined from the net lactate-dependent rate of uptake at different tetracycline concentrations by Hofstee plots. For vesicles bearing the Asp (wild type), Tyr, and His proteins, the K_m was 11 to 14 μM. The V_{max} for the Tyr and His proteins was 8 to 12% of that of the wild type (which was 4 nmol/min/mg). For vesicles with the Asn protein, the rate (at external tetracycline concentrations below the wild-type K_m) was only a few percent of the wild-type rate, so assays in quadruplicate were required. Little saturation was observed; the K_m was greater than 200 μM. The V_{max} was 6 nmol/min/mg or possibly greater.

The observation that a change from Asp to Asn in Tet protein greatly changed the K_m while a seemingly more radical change, to Tyr or His, did not raised a concern that this low-level tetracycline transport was actually due to the host itself. We compared the rate of transport in vesicles from uninduced and induced cells bearing the Asn mutant gene with that in vesicles from susceptible (i.e., host) cells; the susceptible and induced cells had been grown with autoclaved chlortetracycline. The assay was done at 80 μM tetracycline. No lactate-dependent uptake was seen in vesicles from either the susceptible or the uninduced cells, while vesicles from the induced cells showed the usual, low active uptake. Therefore, the Asn mutant protein was indeed responsible for the active uptake seen.

We also examined the Asn mutant plasmid (pMS208) for an unexpected second mutation at another location in the *tetA* gene which might be responsible for the high K_m. We replaced the larger (4.6-kb) *Nco*I fragment, bearing all of the *tetA* gene except for that part encoding the first 17 amino acids, with the corresponding wild-type fragment. The resulting recombinant plasmid (pLY11) behaved like pMS208 with respect to tetracycline resistance, K_m, and V_{max}. The nucleotide sequence of pLY11 corresponding to the first 38 amino acids of Tet showed only the single mutation at residue 15. Therefore, the single replacement of Asp-15 by Asn indeed was responsible for the great increase in K_m.

In summary, the V_{max} of tetracycline transport for the Tyr and His mutant Tet proteins was lowered by ca. 90%, while the K_m was unaltered. For the Asn mutant, the V_{max} was relatively unaffected, while the K_m was increased more than 13-fold.

Analysis of proton antiport. When the fluorescent base acridine orange is concentrated in everted membrane vesicles in response to a pH gradient across the membrane (acidic inside), its fluorescence is quenched (11). We found that tetracycline added to such a quenched system led to "dequenching" if the vesicles contained active wild-type Tet protein. The interpretation (see also reference 18) was that as a tetracycline-cation complex was transported in, H^+ came out, via antiport, lowering the ΔpH.

None of the residue 15 mutants showed as much fluorescence dequenching as did the wild type (Fig. 3). In all cases, however, dequenching could be detected, with either tetracycline (Fig. 3) or its analog doxycycline (data not shown) as the substrate. For vesicles bearing the Tyr and Asn mutant proteins, dequenching caused by tetracycline (21 to 42 μM) was about 8% of that of the wild type, and it was 16% for vesicles bearing the His mutant protein (average of three determinations with the same two vesicle preparations used for equilibrium active-transport studies). Tetracycline caused no dequenching in vesicles from susceptible cells (Fig. 3). Although the degree of quenching is not quantita-

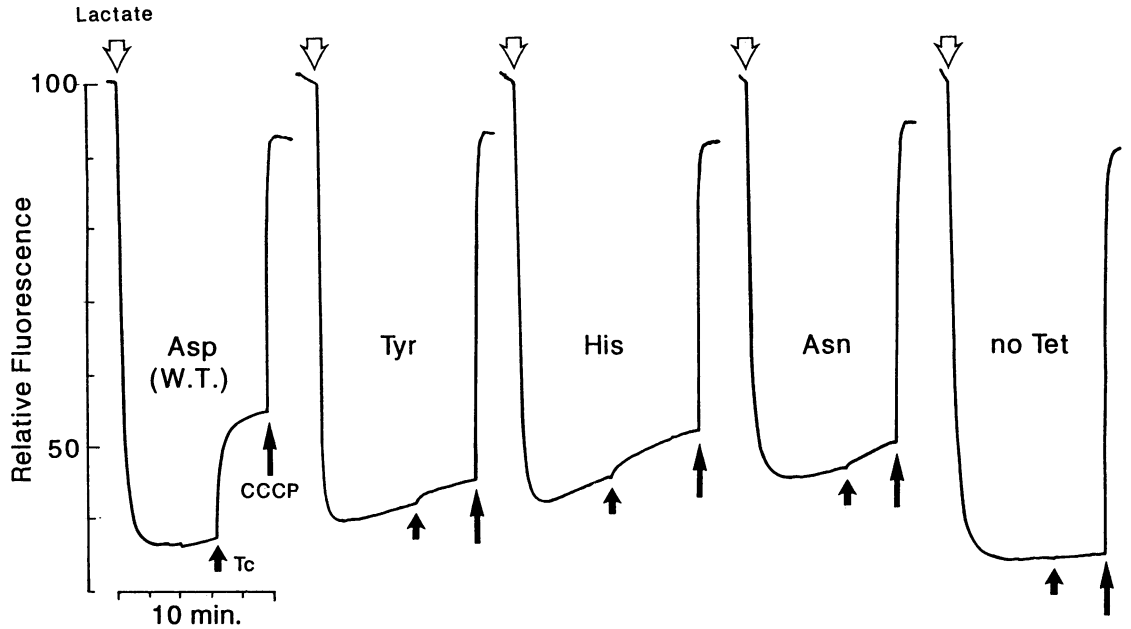

FIG. 3. Fluorescence dequenching (antiport) by tetracycline in everted membrane vesicles bearing wild-type, mutant, or no Tet protein. Fluorescence (Perkin Elmer LS-5 spectrofluorometer; excitation, 490 nm; emission, 530 nm) of acridine orange was measured at 30°C at pH 7.5 with 50 mM potassium phosphate–150 mM KCl–10 mM $MgSO_4$–2 μM acridine orange, using the same preparation of vesicles used for Fig. 2 at 0.1 mg of protein per ml. At the time indicated by the first arrow, lactate (10 mM) was added, causing fluorescence quenching. At the time indicated by the second arrow, tetracycline was added to 42 μM, and net dequenching (at equilibrium) was quantified 3 min later, taking into account the rate of dequenching before addition of tetracycline. At the time indicated by the third arrow, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (20 μM) was added to deenergize the vesicles. KCl was used in the buffer to increase the lactate-dependent fluorescence quenching; this increase is presumably due to an enhanced pH gradient accompanying a chloride-induced diminution of electrical potential (11).

tively proportional to the pH gradient (9), as an approximation, the dequenching (antiport) and active-transport activities at equilibrium were lowered to similar extents in the mutants. These results suggest that in the mutants, substrate transport is still coupled to proton antiport.

Amounts and mobilities of Tet proteins. The decrease in resistance and in transport V_{max} might be explained trivially by lower amounts of mutant Tet protein in cells or vesicles. In whole cells or membranes prepared following sonication, the amount of the Tyr mutant protein was sometimes greater than that of the wild type, but otherwise no notable differences in amounts were seen (Fig. 4). In the everted vesicles used for transport assays, the amount of the various Tet proteins per milligram of protein was somewhat variable, but the lowered transport activity in vesicles from mutants could not be attributed to less Tet protein (data not shown). For unknown reasons, the Tet protein of the Tyr mutant ran as a single band, while that of the wild type and the other mutants often ran as a doublet (Fig. 4).

The Tyr mutant protein of pMS45 (as well as that of pMS108) migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular mass 1 to 2 kDa less than that of the wild-type protein (Fig. 4). The His mutant protein also migrated slightly faster than the wild type (Fig. 4). To be certain that the pMS45 phenotype did not result from more than one mutation, we replaced the 4.6-kb (larger) *Nco*I fragment of pMS45 (bearing all of the *tetA* gene except for the region encoding the first 17 amino acids) with the corresponding wild-type fragment. The Tet protein encoded by the resulting recombinant plasmid (pLY10) was not distinguishable from that of pMS45 in rate of migration or amount (Fig. 4, lane 5) or in the tetracycline resistance conferred on cells (data not shown). The pLY10 nucleotide sequence corresponding to the first 39 amino acids of Tet confirmed that all amino acids except residue 15 were wild type. Thus, a single substitution of Tyr for Asp at residue 15 caused faster migration of Tet protein

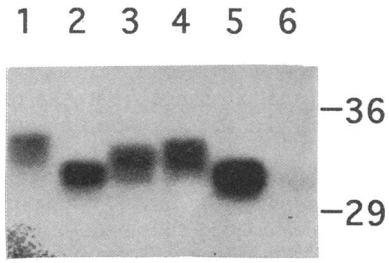

FIG. 4. Immunoblot of membrane proteins from HB101 cells containing wild-type, mutant, or no Tet protein. Cells were grown in L broth with 0.1% glucose and 25 μg of autoclaved chlortetracycline per ml and lysed by sonication, and crude membranes were sedimented at 265,000 × *g* for 25 min. They were solubilized in gel buffer (8) at 20°C, separated by SDS–10% PAGE (8), and electroblotted onto Immobilon P (Millipore). The blot was probed with anti-Ct_{14}, a rabbit antiserum to the carboxy-terminal 14 amino acids of Tet (19; gift of A. Yamaguchi), followed by ^{125}I-protein A (70 to 100 mCi/mg; ICN). Only the Tet region of the blot is presented. Protein molecular weight standards are shown in thousands. Lanes: 1, pLR1068 (wild type, Asp); 2, pMS45 (Tyr); 3, pMS202 (His); 4, pMS208 (Asn); 5, pLY10 (reconstructed Tyr); 6, no plasmid.

in SDS-PAGE and the decreased resistance phenotype. The faster migration might result from greater SDS binding to the altered amino acid; it might also result from a different Tet conformation conducive to enhanced mobility in SDS and hence might be indicative of a role for Asp-15 in structure. Single mutations which altered mobility in SDS-PAGE have also been described for other proteins (13, 16).

Resistance to tetracycline analogs. If Asp-15 were part of a substrate-binding site, its replacement by other amino acids might lead to altered substrate specificity. We did find differences in the relative resistance to tetracycline and its analogs minocycline and doxycycline among cells bearing wild-type and residue 15 mutant Tet proteins. However, such differences were also found in a Tet mutant of lowered resistance that lacked 14 carboxy-terminal amino acids, as well as in seven of nine other (unsequenced) Tet mutants from our collection. Therefore, these differences are probably not specific.

Concluding remarks. A simple model for Tet function could involve binding of a tetracycline-magnesium cationic complex to Tet on its cytoplasmic side, with concomitant proton release, followed by a rate-limiting translocation efflux step involving a conformational change in Tet. Replacement of Asp by Tyr or His did not alter the apparent K_m for tetracycline transport. Therefore, either (i) the (potentially) negative charge or other chemistry of Asp was not important for normal substrate binding or (ii) the K_m as determined is not really a measure of substrate binding. The significance of the high K_m of the Asn mutant is not clear. The lower V_{max} seen for the His and Tyr (but not the Asn) substitutions may have resulted from a retardation of a translocation step. Therefore, as suspected from its conservation evolutionarily, Asp-15 is important for Tet efflux function, although its precise role is uncertain.

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ADDENDUM

While this work was in review, an article was published which suggested that a negative charge at the residue 15 position in Tet was obligatory and that this residue may participate in a substrate-binding site (A. Yamaguchi et al., J. Biol. Chem. **267**:7490–7498, 1992).

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