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Importance of the GP Dipeptide of the "Antiporter Motif" and other Membrane-Embedded Proline and Glycine residues in Tetracycline Efflux Protein Tet(L)[†]

Magdia De Jesus[§], **Jie Jin**[¶], **Arthur A. Guffanti**, and **Terry A. Krulwich**[*]

Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine New York, New York 10029

Abstract

Proline and glycine residues are well represented among functionally important residues in hydrophobic domains of membrane transport proteins and several critical roles have been suggested for them. Here, the effects of mutational changes in membrane-embedded proline and glycine residues of Tet(L) were examined, with a focus on the conserved $GP^{155,156}$ dipeptide of Motif C, a putative "antiporter motif". Mutation of Gly^{155} to cysteine resulted in a mutant Tet(L) that bound its tetracycline-divalent metal ($Tc-Me^{2+}$) substrate but did not catalyze efflux or exchange of $Tc-Me^{2+}$ or catalyze uptake or exchange of Rb^+ which was used to monitor the coupling ion. These results support suggestions that this region is involved in the conformational changes required for translocation. Mutations in Pro^{156} resulted in reduction (P156G) or loss (P156A or C) of $Tc-Me^{2+}$ efflux capacity. All three Pro^{156} mutants exhibited a K^+ leak (monitored by $^{86}Rb^+$ fluxes) that was not observed in wild type Tet(L). A similar leak was observed in a mutant in a membrane-embedded proline residue elsewhere in the Tet(L) protein (P175C) as well as in a P156C mutant of related antiporter Tet(K). These findings are consistent with roles proposed for membrane-embedded prolines in tight helix packing. Patterns of Tc-resistance conferred by additional Tet(L) mutants indicate important roles for another GP dipeptide in transmembrane segment (TMS) X as well as for membrane-embedded glycine residues in TMS XIII.

Almost all transport proteins have membrane-embedded proline residues in some of the multiple α-helices that are a general feature of transporters (1). These residues are preferentially paired with glycine residues that are also found in abundance in TMS[1] of transporters (2,3). Membrane-embedded proline and glycine residues of transporters are hypothesized to promote helix kinks and swivels that play crucial roles in the conformational flexibility required for transport mechanisms (2-6). They are further hypothesized to have roles in helix packing and association (7-9). These hypotheses have been stunningly supported for the largest family of membrane transport proteins, the MFS, a family of structurally related transporters of prokaryotes and eukaryotes that includes uniporters, antiporters and symporters. The MFS comprises about a quarter of all transport proteins (10,11). The first three high resolution

[1]Abbreviations: BTP, bis-[tris(hydroxymethyl)methylamino]-propane; CCCP, cyanide-*m*-chlorophenylhydrazone; Δp, electrochemical proton gradient (inside negative and alkaline); Δψ, transmembrane electrical potential (inside negative); LBK, Luria Broth medium with KCl replacing NaCl; MFS, Major Facilitator Superfamily of membrane transporters; MIC, minimal inhibitory concentration; RSO, right side out membrane vesicles; Tc, tetracycline; $Tc-Me^{2+}$, tetracycline-divalent metal complex; TMS, transmembrane segment.

structures of MFS members, the oxalate/formate antiporter OxlT (from *Oxalobacter formigenes*) (12), the lactose/proton symporter LacY (from *Escherichia coli*) (13) and the inorganic phosphate/glycerol-3-phosphate antiporter GlpT (from *E. coli*) (14), show clearly that many of the 12-TMS of these transporters are highly curved, with several of them having evident kinks. The positions of kinking in LacY are associated with 6 prolines, 3 glycines and 2 alanines (15).

Antiporters of the MFS have a particular proline- and glycine-rich motif in TMS V that is designated Motif C and is one of several motifs that have been identified in 12-TMS and/or 14-TMS members of the superfamily (16-19) (Figure 1). Motif C has been a focus of interest in structure-function studies of both 12-TMS and 14-TMS tetracycline (Tc) antiporters, which are Tet proteins that catalyze efflux of a Tc-Me^{2+} complex from the cytoplasm in exchange for external H^+ (20-22). Active Tc-Me^{2+} efflux by Tet proteins depends upon the Δp established across the cytoplasmic membrane by ATP- or respiration-dependent proton pumping. Complete cysteine-scanning mutagenesis of the 12-TMS TetA(B) antiporter from Gram-negative transposon Tn*10* demonstrates that proline and glycine residues constitute a large proportion of the essential residues. The mutagenesis analysis further suggests that the conserved GP dipeptide of Motif C is located at a "permeability barrier" where conformational switching is likely to occur during the transport cycle (23-26). The importance of the GP peptide and several additional glycine residues of Motif C for Tc transport has also been shown in the Tet(K) protein by assays of mutants with diverse substitutions at these positions (27). Tet(K) and closely related Tet(L) form a class of 14-TMS Tet proteins in the MFS (28,29). Both genes are found in plasmids of Gram-positive bacterial species, e.g. *Staphylococcus aureus*, with Tet (L) also being encoded in the chromosome of some *Bacillus subtilis* strains (19,30,31). Tet(L) and Tet(K) share sequence similarity to the 12-TMS Tet proteins in the N-terminal domain comprising the first six TMS, but structure-function differences in the two groups are evident even in this domain, including the presence of an essential acidic residue in the Motif C of Tet (L) and Tet(K) that is not conserved in the Motif C of 12-TMS Tet proteins (17).

The 12- and 14-TMS Tet proteins are hypothesized to have evolved independently from each other on the basis of genomic considerations (32-34). They also have apparent functional differences. Only Tc-Me^{2+}/H^+ antiport has been demonstrated for 12-TMS Tet proteins and the antiport is electroneutral, with no flux of charge occurring during an antiport turnover (35). By contrast, Tet(L) and Tet(K) proteins catalyze $Na^+(K^+)$/H^+ antiport, that supports alkaline pH homeostasis and Na^+-resistance, in addition to the Tc-Me^{2+}/H^+ antiport that supports antibiotic-resistance. All the antiport reactions catalyzed by Tet(L) and Tet(K) are electrogenic i.e. more than one H^+ is translocated inward per Na^+, K^+ or mono-cationic $Tc^{(-1)}$-Me^{2+} complex translocated outward (36-39) in contrast to the finding with the 12-TMS Tn*10* Tet protein (35). Both Tet(L) and Tet(K) can also substitute K^+ for part of the H^+ coupling ion complement, especially at high pH, thus catalyzing both net K^+ and H^+ uptake under particular conditions of pH and K^+ status (40-42). This use of K^+ makes it possible to track the movement of the coupling ion (via inward fluxes of $^{86}Rb^+$) in a direct manner that is not possible when only H^+ is used as coupling ion (40).

In the present study, we sought to apply the experimental advantage of the inward $^{86}Rb^+$ flux assay to complement Tc-Me^{2+} efflux assays in probing the role of the Motif C GP dipeptide in 14-TMS Tet proteins. In addition, we examined the effects on Tc-Me^{2+} efflux of two new mutations at another important residue of Tet(L) Motif C, E152D and E152C, and one new mutation at a site in the periplasmic half of TMS IV that is thought to interact with Motif C, R110D (24,25). E152Q and R110C mutations were earlier shown to result in complete loss of tetracycline-resistance (41). Finally, we tested the effect on tetracycline-resistance of Tet(L) mutations in membrane-embedded glycines and prolines of two additional regions that are in the C-terminal domain of Tet(L): (i) a distinct GP dipeptide in TMS X that is in a functionally

important region of a *B. subtilis* multi-drug resistance protein (43); and (ii) a glycine-rich region of TMS XIII, another helix that contains an essential acidic residue in Tet(K) and Tet(L) (41, 44). The residues studied are highlighted in Figure 1.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following *E. coli* strains were used: *E. coli* DH5α [F⁻ ϕ80Δ*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*] (GIBCO-BRL)(45) was used for cell and vesicles assays of Tc resistance and transport, respectively; *E. coli* JM109 and CJ236 (New England Biochemical) were used for steps of the site-directed mutagenesis as described previously (40); and *E. coli* TK2420 (*trkD1* Δ(*trkA*) Δ(*kdpABC*)5 *kup*), a K⁺-uptake-deficient triple mutant (46), was used for assays of Rb⁺ uptake by right-side-out (RSO) vesicles. *E. coli* strains were routinely maintained and grown in LBK (47).

Construction and cloning of tet(L) and tet(K) genes with site-directed mutations

Mutations were introduced into the chromosomal *tet*(L) gene from *B. subtilis* and the *S. aureus* plasmid pT181 *tet*(K) gene which had earlier been cloned into the bacteriophage vector M13mp19 (40); phage DNA was used as template for site-directed mutagenesis by the method of Kunkel *et al.* (48). The mutants used in the study and the primers employed in their construction are shown in Table 1. Each construct was cloned into the shuttle vector pBK15 (obtained from K. Zen), which contains Em^{r}Cm^r markers, a pBD42-pBR322 joint replicon and a multiple cloning site. The *tet* genes were cloned under control of the *ermC* promoter; experiments were conducted without added Em using the modest level of basal expression. The sequence of each construct was verified by sequencing of the whole *tet* ORF in the institutional DNA Core Facility.

Assays of Tc-resistance of E. coli transformants

For assays of Tc-resistance, 10 μl of stationary phase cultures of *E. coli* DH5α transformants (with control or recombinant pBK15 plasmids) were inoculated into 2 ml of LBK containing various Tc concentrations in the range of 0-32 μg/ml. The A_{600} after 15 hours was plotted for determination of MIC (49). All assays were carried out in duplicate in at least two independent experiments. MIC values in this study are taken to represent Tc-resistance that is: negligible at ≤ 4 μg/ml; significantly defective at 5-12 μg/ml; and substantial at > 15 μg/ml. MIC values for Tc assayed *in vivo* do not correlate precisely with transport levels assessed *in vitro* (49, 50), but the semi-quantitative correlation between the lowest and highest group of values is strong.

Preparation of membrane vesicles

Everted vesicles were prepared from *E. coli* transformants in 10 mM BTP at pH 7.5 using a French Pressure cell method of Rosen (51) as described previously (40). RSO membrane vesicles were prepared by the lysozyme-method of Kaback (52) and were pre-loaded with either 100 μM choline chloride or KCl (42). Protein assays were conducted by the method of Lowry *et al.* (53) using egg white lysozyme as the standard.

Western analyses of membranes from E. coli transformants

After everted membrane samples (50 μg protein) were fractionated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes, Western analyses were carried out using antibodies raised to synthetic peptides corresponding to the N-terminus of Tet(L) or Tet(K), as described earlier (49). The signals were quantified by using ImageQuant software (Molecular Dynamics).

Transport assays in membrane vesicles of E. coli.

(i) Assessment of Tc-Co^{2+} efflux capacity by assays of energy (D-lactate)-dependent uptake of Tc-Co^{2+} into everted (inside-out) vesicles—Tc uptake was assayed at 30 C. in reaction mixtures containing 50 μg of vesicle protein in 10 mM BTP buffer, pH 7.5, 25 μM [^{3}H]Tc and 100 μM CoCl$_2$. Energized reactions also contained 5 mM Tris-D-lactate (an electron donor to the respiratory chain via membrane associated D-lactate dehydrogenase); this was included in the reaction mix so that the vesicles were energized when the substrate was added. The reaction was initiated by addition of [^{3}H]Tc-CoCl$_2$ from a 50x pre-mixed stock (1.25 mM Tc, 5 mM CoCl$_2$) that allowed formation of the substrate complex. At intervals after initiation of the reaction, samples were filtered GSWP 02500 filters (Millipore), washed with cold reaction buffer, dried and assayed by liquid scintillation spectrometry. Parallel de-energized control reactions were conducted for each type of transformant membrane preparation in the absence of Tris-D-lactate and with addition of the uncoupler CCCP to a final concentration of 10 μM. Substrate binding under these de-energized conditions was subtracted from values obtained in the presence of D-lactate and absence of CCCP. Vector controls were also conducted routinely.

(ii) Assays of [^{3}H]Tc-Co^{2+}:Tc-Co^{2+} and K$^+$-^{86}Rb$^+$: K$^+$-Rb$^+$ exchange in RSO vesicles—RSO vesicles were prepared in 10 mM BTP buffer, pH 7.5. For Tc-Co^{2+}:Tc-Co^{2+} exchange they were passively loaded with 50 μM CoCl$_2$ and 25 μM Tc, or with 50 μM cholineCl. Samples were diluted 1:10 in buffer containing 50 μM CoCl$_2$ and 25 μM [^{3}H]Tc. At various times, 50 μl samples, each containing 50 μg of vesicle protein, were filtered onto HAWP (0.45 μm) filters (Millipore) and radioactivity was monitored as described above. For K$^+$-^{86}Rb$^+$: K$^+$-Rb$^+$ exchange, the vesicles were loaded with either 100 μM KCl or 100 μM cholineCl. They were diluted and assayed as for the other exchange except that the dilution buffer contained 100 μM K$^+$-^{86}Rb$^+$. The binding control assays were conducted identically to the two exchange reactions except that the reaction mixtures contained 2% toluene to permeabilize the vesicles. Correction was made for the choline controls at each point of the assay and the data for the vector control are shown.

(iii) Assays of energy-dependent uptake of ^{86}Rb$^+$ into RSO vesicles and its dependence on the presence of a Tet(L) or Tet(K) efflux substrate inside the vesicle—RSO vesicles from *E. coli* TK2420 transformants were prepared in 10 mM BTP buffer, pH 7.5, and were passively loaded with either 100 μM cholineCl (non-efflux substrate for Tet(L) or Tet(K)) or 100 μM KCl (an efflux substrate). Assays of Tet(L)- and Tet(K)- ^{86}Rb$^+$-K$^+$ uptake were initiated by dilution of 25 μl of the vesicle suspension into 500 μl of 100 mM Tris-HCl (pH 7.5), containing 10 mM Tris-D-lactate and 100 μM ^{86}Rb$^+$-K$^+$. The final membrane concentration was 500 μg membrane protein/reaction mixture. Binding controls were carried out without added Tris-D-lactate and with the addition of 5% butanol; butanol was empirically found to yield more consistent control values in this particular assay than the 2% toluene used for the exchange assays. At intervals, samples were filtered and processed as described under (i) except that HAWP (0.45 μm) filters (Millipore) were used.

RESULTS

Mutants of Arg^{110} in TMS IV and of Glu^{152} and Gly^{155} in the Motif C GP dipeptide

Earlier indications of the importance of Arg^{110} and Glu^{152} (41) were followed up with new mutants before the effort focused on the Motif C GP dipeptide and other Gly and Pro residues in TMS domains. A new R110K mutation of Tet(L), a more conservative change than the original R110C mutation, allowed greater membrane assembly than the original mutation, but no significant Tc-resistance was conferred (Table 2). Transport assays showed that the initial rate of [^{3}H]Tc-Co^{2+} uptake by Tet(L) R110K vesicles was indistinguishable from that of wild

type Tet(L), but was followed by cessation of uptake after 30 seconds, which was not observed in the wild type (data not shown). This uptake deficit probably accounts for the negligible Tc-resistance conferred by this mutant and might result from a coupling defect. By contrast, a charge-preserving mutation of Glu[152] in Motif C, E152D, conferred significant Tc-resistance that was still defective relative to wild-type but also exhibited less membrane assembly than wild type; E152C and E152Q mutants of Tet(L) did not confer Tc-resistance. Transport assays showed that the $[^3H]Tc-Co^{2+}$ uptake activity of Tet(L) E152D in everted membrane vesicles and $^{86}Rb^+$ uptake activity in RSO vesicles were comparable to the wild type activity, whereas the Tet(L) E152C mutant exhibited no Tet(L)-dependent transport in either assay (data not shown). Presumably a negative change is required at this position.

Three Tet(L) proteins with mutations of Gly [155] of the GP[155,156] dipeptide (G155A, C, or S), all assembled at wild type levels or greater in the membranes of *E. coli* when expressed from a multi-copy plasmid (Table 2). Tet(L) G155A conferred substantial Tc-resistance while the G155C and G155S mutants were more deficient by this assessment (Table 2). Assays of $[^3H]$ Tc-Co^{2+} uptake by energized everted vesicles, the surrogate assay of Tc efflux by right-side-out cell or vesicle systems, confirmed that the Tet(L) G155A retained significant, low activity whereas the G155C and G155S mutants did not (Figure 2). None of the mutants exhibited any activity in assays of $^{86}Rb^+$ uptake in RSO membrane vesicles; in these assays, K^+-$^{86}Rb^+$ is used as part of the extravesicular coupling ion complement that exchanges for an intravesicular substrate of the transporter (Figure 2). The values shown in Figure 2 were corrected for binding controls conducted for each vesicle preparation. A consistent difference in the magnitude of $[3H]Tc-Co^{2+}$ binding was observed between the two inactive Gly[155] mutants, G155C and G155S, and the wild type Tet(L); there was no significant difference between the binding control of G155A and wild type. The binding assessed using uncoupler (CCCP)-treated vesicles averaged 0.05 ± 0.04 nmoles Tc/mg membrane vesicle protein in the wild type vesicles whereas the G155C vesicles averaged 0.25 ± 0.04 nmoles Tc/mg membrane protein; the same binding difference was observed when butanol treatment was used in addition to CCCP-treatment. Two experiments were conducted to assess the nature of this binding. First, the binding of $[^3H]Tc$ alone, under the same conditions except for the absence of Co^{2+}, was assayed and found to be identical and in the low 0.05 nmoles/mg membrane protein in both wild type and G155 mutant membranes. Second, experiments were conducted in which the vesicles were incubated in a standard reaction mix containing $[^3H]Tc-Co^{2+}$ and then washed with buffer containing 100 μM non-radioactive Tc-Co^{2+} using a protocol described by others (54); no reduction in the membrane-associated $[^3H]Tc$ was found. No such difference in binding, as compared to wild type, was observed with any other Tet(L) mutant in this study.

The profile of two Tet(L) Gly[155] mutants suggested that these mutant Tet(L) forms were able to bind the cytoplasmic efflux substrate but were unable to carry out a subsequent step in the transport cycle. Assays of both $^{86}Rb^+$:Rb$^+$ exchange and $[^3H]Tc-Co^{2+}$: Tc-Co^{2+} exchange were conducted on wild type Tet(L) and the Tet(L) G155C mutant. These two exchange reactions, respectively, involve flux across the membrane of the radiolabeled coupling ion (into RSO vesicles) or substrate (into everted vesicles), dissociation of the coupling ion or substrate from Tet(L) and then transport of unlabelled coupling ion or substrate across in the return direction. Each exchange reaction therefore assays a segment of the complete reaction cycle that includes a conformational switch and that can support bi-directional flux of either the coupling ion or of the substrate across the membrane (in the absence of a complete substrate efflux cycle). As depicted in the model in Figure 3 (left), the $[^3H]Tc-Co^{2+}$: Tc-Co^{2+} exchange and $^{86}Rb^+$:Rb$^+$ exchanges respectively assay back and forth flux through steps 1-5 and 6-10. As shown in Figure 3 (middle and right), wild type Tet(L) exhibited activity in both exchange reactions but Tet(L) G155C exhibited neither exchange activity.

Mutants of Pro[156] in the Motif C GP dipeptide

Mutation of Tet(L) Pro[156] led to significantly reduced Tc-Co^{2+} efflux in the P156G mutant and to complete loss of efflux relative to the control for the P156A and P156C mutants (Figure 4). There was no indication of increased substrate binding in the assays. In the assays of ^{86}Rb$^+$ uptake, a remarkable the pattern was observed for all three Pro[156] mutants. In this assay, ^{86}Rb$^+$ uptake into the RSO vesicles by wild type Tet(L) depends upon both the addition of the electron donor and on the presence of an efflux substrate for Tet(L) inside the vesicles; no Tet(L)-dependent ^{86}Rb$^+$ uptake was observed in the absence of added D-lactate (the electron donor) and or intravesicular K$^+$ (the efflux substrate loaded inside the vesicles in this experiment) (Figure 4). Earlier work showed that the net Rb$^+$-K$^+$ uptake in this assay is a mode of the antiport that is electrogenic and it could be supported by the presence of either K$^+$ or Tc-Co^{2+} inside the vesicles (40,42). It was therefore notable that ^{86}Rb$^+$ uptake by the mutant Tet(L) P156A,G or C forms was as high or higher under conditions in which the electron donor was present but the intravesicular K$^+$ was absent. This suggested that these mutant Tet(L) proteins promote leakage of K$^+$ into the vesicles in response to the generation of the transmembrane potential, $\Delta\Psi$, while such a leak is not observed in wild type Tet(L) vesicles or with other Tet(L) mutants (e.g. see Figure 3) (40,41,49). The activity in the complete assay, with both electron donor and intravesicular K$^+$, was lower than wild type in each Pro[156] mutant (Figure 4).

Mutations in Gly[155] and Pro[156] of Tet(K)

In the study of Motif C mutants of Tet(K) by Ginn *et al.* (27), G155A, S and T mutants and P156A,V,G, and S mutants were all found to have residual but greatly reduced Tc-Co^{2+} transport activity. In assays of [^{3}H]Tc-Co^{2+} uptake into everted vesicles under our conditions of expression and binding controls, the G155S mutant of Tet(K) exhibited 65% of wild type Tet(K) activity whereas the G155C and P156C mutants exhibited no activity (data not shown). Assays of ^{86}Rb$^+$ uptake by the P156C mutant of Tet(K) were carried out in comparison with wild type Tet(K) and the G155C Tet(K) mutant to assess whether mutations in Pro[156] result in an apparent K$^+$ leak as observed with Tet(L) mutants at this position. As shown in Figure 5, wild type Tet(K) exhibited a significantly greater rate of ^{86}Rb$^+$ uptake into RSO vesicles than Tet(L), as consistently observed before (40,42), and this uptake was dependent upon energization and intravesicular K$^+$. By contrast, Tet(K) G155C exhibited no ^{86}Rb$^+$ uptake under any condition. Tet(K) P156C exhibited a lower rate of ^{86}Rb$^+$ uptake than wild type in the complete assay mix and exhibited a significantly higher rate in the absence of intravesicular K$^+$, consistent with a $\Delta\Psi$-dependent K$^+$ leak.

Assessment of the K$^+$-Rb$^+$ leak vs antiport-dependent uptake in a Pro[175] mutant

The apparent K$^+$ leak conferred by mutations in Pro[156] raised the question of whether such a leak would result from mutational change in a TMS proline residue outside of Motif C. Assays were conducted on Tet(L) P175C a mutant Tet(L) in a membrane-embedded proline residue of Motif H of proton-coupled drug efflux transporters (TMS VI, see Figure 1 for position) (17); this mutant Tet(L) had earlier been shown to be incorporated into the membrane and confer significant Tc-resistance (41). In assays of Tc-Co^{2+} uptake into everted vesicles, Tet(L) P175C vesicles had 43% of the wild type level of activity. Assays of ^{86}Rb$^+$ uptake by RSO vesicles expressing wild type Tet(L) *vs* Tet(L) P175C showed distinct differences in pattern that were similar to those observed for mutants in Pro[156] (Figure 6). As always, ^{86}Rb$^+$ uptake by wild type Tet(L) depended upon both electron donor addition and the presence of intravesicular K$^+$. By contrast, the Tet(L) P175C vesicles exhibited electron donor-dependent ^{86}Rb$^+$ uptake that was at least as rapid in the absence as in the presence of intravesicular K$^+$ and the rate of transport was reduced relative to wild type Tet(L).

Mutations in additional membrane-embedded Gly and Pro residues

The PG[301,302] dipeptide of TMS X is modeled to be in a comparable position near the periplasmic end of the helix to the position of GP[155,156] of TMS V in Tet(L) (Figure 1). Mutations Gly[302] and neighboring Thr[303] significantly reduced the Tc-resistance conferred by Tet(L), whereas two different mutations in Pro[301] resulted in opposite effects, with P301G conferring greater Tc-resistance than wild type Tet(L) and P301S conferring no Tc-resistance (Table 2). Single cysteine replacements in a pair of similarly located glycines, Gly[405,406], as well as of Gly[400] in the Motif F of TMS XIII, greatly reduced Tc-resistance where as a mutant in Gly[398] was less impaired.

DISCUSSION

The functional importance of Pro and Gly residues in several TMS domains of Tet(L) reinforces the finding of Tamura *et al.* (24) that among the thirteen essential residues that are in the TMS domains of the TetA(B) protein there are eight Gly and 2 Pro residues. The specific hypothesis that Motif C is important in antiporters of the Drug/H^+ antiporter families (18) and that the conserved GlyPro[155,156] dipeptide is involved in the "permeability barrier" (23) is supported by the effects of Gly[155] mutations in the current study. The elevated binding of Tc by the de-energized transformant membranes of Tet(L) G155C and S, only when Co^{2+} was also present, indicates that substrate binding occurred in this mutant and that some subsequent step was arrested by the mutation. The absence of [^{3}H]Tc-Co^{2+}:Tc-Co^{2+} and $^{86}Rb^+$:Rb^+ exchange activity for the G155C mutant Tet(L) further suggests that the arrested step is the conformational switching in which movements in the region of the permeability barrier allow the substrate and the coupling ions to be translocated, in a ping-pong fashion (40), to the opposite sides from where they start. Apart from these structure-function inferences, a mutant that binds substrate but does not undergo major conformational movements could be useful for structural studies, as shown by the successful pursuit of a high resolution structure of LacY using a mutant, C154G, that binds substrate but is blocked in the conformational changes needed for translocation (13,55).

As with Tet(L) G155C, de-energized control vesicles in transport assays of the TetA(B) G145C mutation (the equivalent position to Tet(L) G155 in the 12-TMS Tc-Me^{2+}/H^+ antiporter) as well as mutants in two other Motif C Gly residues of TetA(B), showed elevated binding compared to controls for other mutants (23). Tet(L) G155C or S retained a modest capacity to confer resistance in the absence of Tc-Co^{2+} efflux activity, just as Iwaki *et al.* (23) found for the similarly located G145C mutation of TetA(B) G145C. It is not known whether these findings represent an effect of these mutant Tet proteins on the *E. coli* host (e.g. increasing expression of *E. coli* transporters with Tc transport capacity or reducing Tc entry by modulating membrane properties) or whether these mutant Tet proteins may actually retain modest transport capacity under *in vivo* conditions of ion composition etc. that are not reproduced in vesicles. Both here and in the study of Tet(K) Motif C by Ginn *et al.* (27), significant transport activity was observed in a G155S mutant (and several other Gly[155] mutants in the latter study) in contrast to the absence of activity in Tet(L) G155S. Probably, the closely related Tet(L) and Tet(K) have sufficient differences in conformation to account for small differences in mutational profiles. We note that although the Tet(L) G155A mutant retained some transport activity, no $^{86}Rb^+$ uptake by that mutant was observed, suggesting that the mutational change may have lowered the preference for Rb^+-K^+ *vs.* H^+ so that higher pH values would be required to observe participation of Rb^+-K^+ as coupling ion (Figure 2) (40).

The second major finding of the current study is that mutations in the conserved Pro[156], whether they completely abolish Tc-Co^{2+} efflux or not, produce an electrogenic K^+ leak, i.e. a Tet(L)-dependent, electron donor-dependent flux of $^{86}Rb^+$ into RSO membrane vesicles in the absence of a pre-loaded efflux substrate (K^+, Na^+ or Tc-Co^{2+}) inside the vesicles. This flux is thus not

coupled to efflux of an intravesicular substrate, a coupling that is characteristic of antiport and is observed for such fluxes in wild type Tet(L) (40,42). In assays of Tet(L) Pro^{156} RSO vesicles that were loaded with K^+, enhancement of $^{86}Rb^+$ uptake by the electron donor was observed with all three of the Pro^{156} mutants, and was particularly evident in the P156A mutant in the early time points (compare data shown with closed triangles with open triangles in right panels of Figure 4). The P156A and C mutants may retain some electrogenic monovalent cation/proton antiport activity although they were negative for Tc-Co^{2+} efflux. Several earlier mutants led to the hypothesis that although the efflux substrates share a binding site and translocation pathway, the most complicated substrate, the Tc-Co^{2+} complex, required more elements to position it properly than the simpler monovalent cation efflux substrates (40,41,49). Together, the current observations suggest that Pro^{156} is not essential for antiport but is very important in tight packing and "leak-proofing" in this critical region in both Tet(L) and Tet(K).

The packing function of Pro^{156} is likely to be a general feature of membrane-embedded proline residues since Tet(L) P175C exhibited a similar leak to the P156C mutants of Tet(L) and Tet(K). Prolines are not generally accommodated within membrane helices without deleterious effects and thus are likely to be functionally important when retained (56). It has been hypothesized that membrane-embedded proline residues are required for the initial formation of helix kinks but are often replaced during evolution by other residues, e.g. alanine, concomitant with adaptations that retain the kink and its function in the protein (57). Interestingly, Buurman et al. (58) suggested that multiple "aberrant" K^+ fluxes function as electrogenic uniport activities in E. coli and may together comprise an observed low affinity K^+ uptake capacity that has been termed TrkF. Perhaps some of these fluxes represent evolving transporters that are in the process of closing up small K^+ leaks created as membrane-embedded proline residues were replaced. Part of this aggregate, low affinity K^+ flux in E. coli is also likely to be comprised of antiporters that, like Tet(L), CzcD and others, are able to use both H^+ and K^+ as part of their coupling ion complement (42,59,60).

The data collected here on effects of mutations in residues of the Tet(L) PGT$^{301-303}$ tripeptide of TMS X and Gly residues of TMS XIII indicate the importance of many of these residues in Tc-resistance. The results with Pro^{301} are particularly intriguing, since the Tet(L) P301G mutant and P301S mutants conferred, respectively, greater than wild type resistance and no resistance to Tc. These findings suggest that the PGT tripeptide of TMS X does not function identically to Motif C but that it is likely be involved in different conformational states of the transporter that require flexibility in the Pro^{301} position. Both this region and Motif F merit further study; Motif F is confined to 14-TMS Tet proteins (17) and has not yet been studied in detail in any Tet(L) or Tet(K). The significant defect in Tc-resistance that results from mutations of glycines in this motif, especially Gly^{405}, suggest that this region too may be involved in the different conformational states of the transporter. Finally, the new mutations of Tet(L) R110K mutation and E152D mutation indicate that conservative mutations that preserve the original charge are tolerated better at the Glu^{152} position. Among Tet proteins, the Motif C Glu^{152} is only conserved in the Tet(L) and Tet(K) antiporters and even this may not be the rule. A new member of this group of Tet resistance proteins, Tet38, lacks Glu^{152} and also Pro^{156} (61); the catalytic properties of this protein will be of considerable interest.
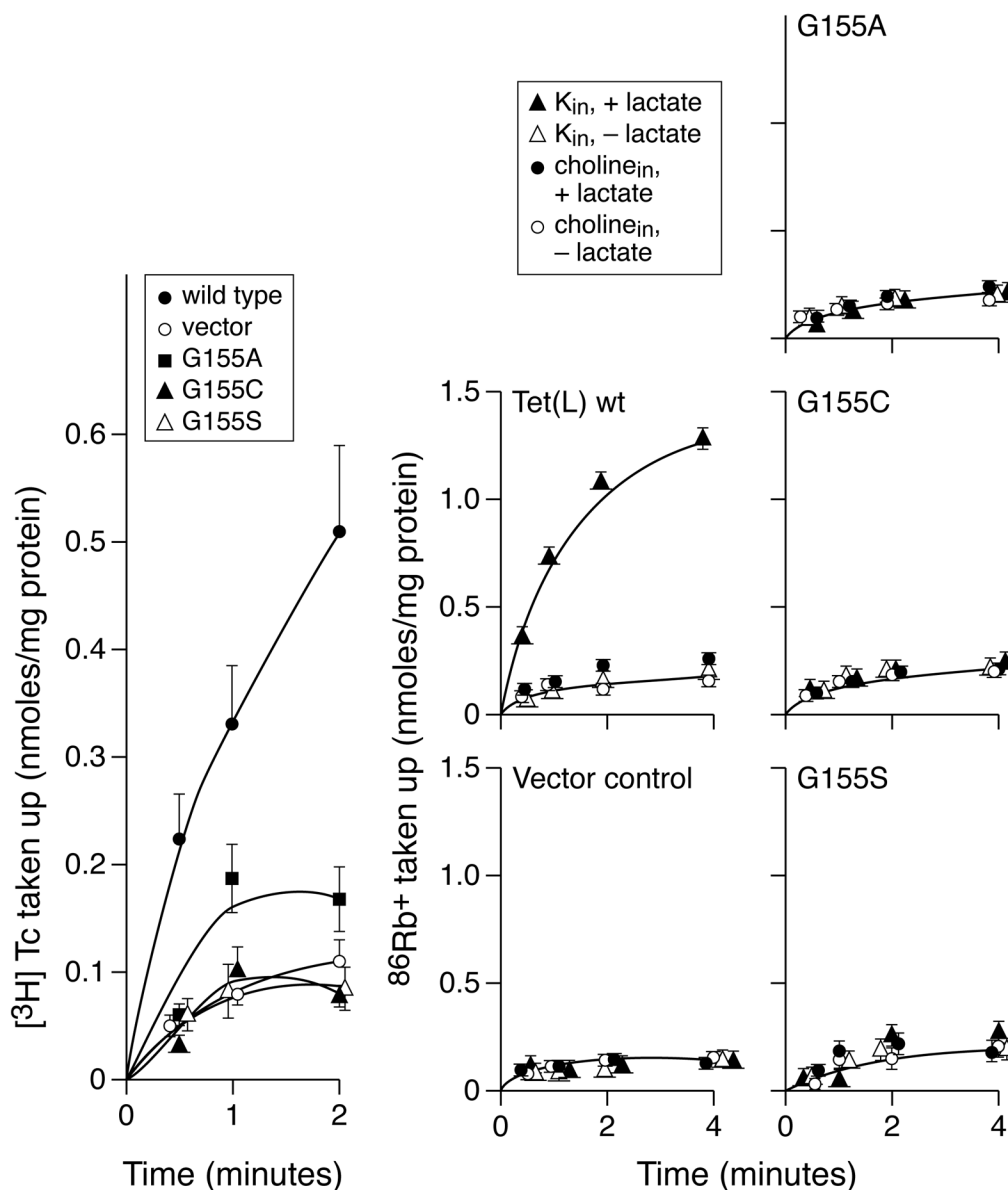
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Figure 1.
A topological model of Tet(L) highlighting the residues functionally probed in this study. The topology is based on the experimental work of others on Tet(K) (28,29). The gray bars indicate the positions of Motifs described by Paulsen *et al.* (17) that are conserved either in both 12-TMS and 14-TMS Drug/H$^+$ families (Motifs A,B,C) or conserved only in 14-TMS (Motifs D1, H, E, F).

Figure 2.
Transport assays of Tet(L) Gly[155] mutants in comparison with wild type Tet(L). Left panel: Uptake of [^{3}H]Tc-Co^{2+} into everted vesicles from the indicated transformants of *E. coli* DH5α were carried out as described under Materials and Methods. The values shown, with standard deviations, are the averages of duplicate determinations on at least two independent vesicle preparations. The values shown were corrected for the binding control of de-energized (CCCP-treated) vesicles from each of the same strains. Right panels: Uptake of [86]Rb^{+} was assayed in RSO vesicles of transformants expressing the same plasmids used in assays of [^{3}H] Tc-Co[2] uptake but the bacterial strain was *E. coli* TK2420. The results for each transformant are shown in separate panels because assays were conducted under several different conditions that distinguish an electrogenic leak from antiport (the conditions are listed in the box above the wild type panel).
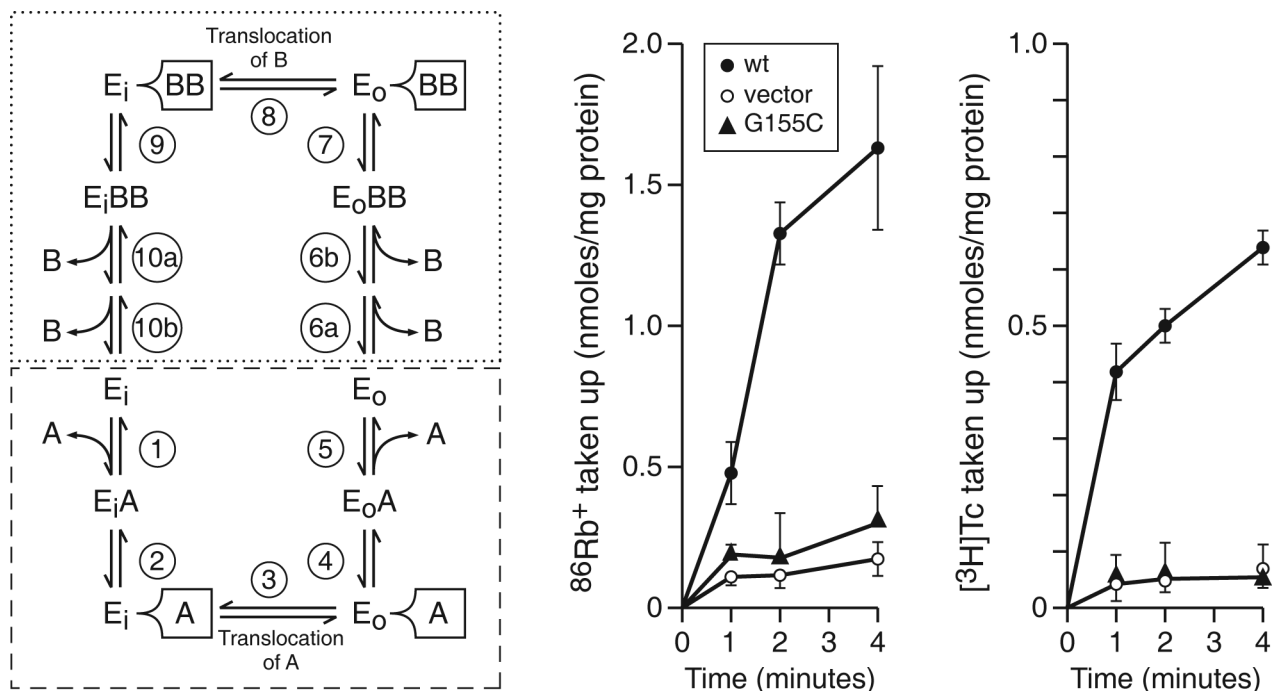
Figure. 3.
Assays of $[^3H]Tc$-Co^{2+}:Tc-Co^{2+} and K^+-$^{86}Rb^+$: K^+-Rb^+ exchange in RSO vesicles expressing Tet(L) G155C. Left: A diagram of the reaction cycle for Tet(L): E_i and E_o respectively represent the inward and outward facing conformations of the transporter; A is the efflux substrate (e.g. Tc-$Co2^+$) and B is a coupling ion which could be either H^+ or Rb^+-K^+. The B:A ratio is greater than unity (42); the precise stoichiometry is not known and is shown here as 2B:A for diagrammatic purposes. The reaction cycle illustrates an alternating access or ping-pong model in which efflux substrate A binds to E_i in step 1 followed by hypothetical tight binding (step 2) that may be detected in the G155C mutant because of a defect in subsequent translocation (step 3) that usually occurs immediately after tight binding. This is followed by hypothesized release from tight binding (step 4) and final substrate release (step 5). The Tc:Co^{2+} exchange reaction assesses this set of reactions (steps 1-5), starting with unlabeled Tc:Co^{2+} inside and labeled Tc:Co^{2+} outside and proceeding forward and reverse. A comparable half reaction, going in both directions, with the top half cycle (reactions 6-10, dotted box) is assessed by the K^+-$^{86}Rb^+$: K^+-Rb^+ exchange. Middle and Right: the experimental data for the two exchange reactions. The assays were carried out as described under Materials and Methods in comparison with wild type Tet(L) and control membranes. The data are the averages from two independent preparations and are shown with standard deviations.
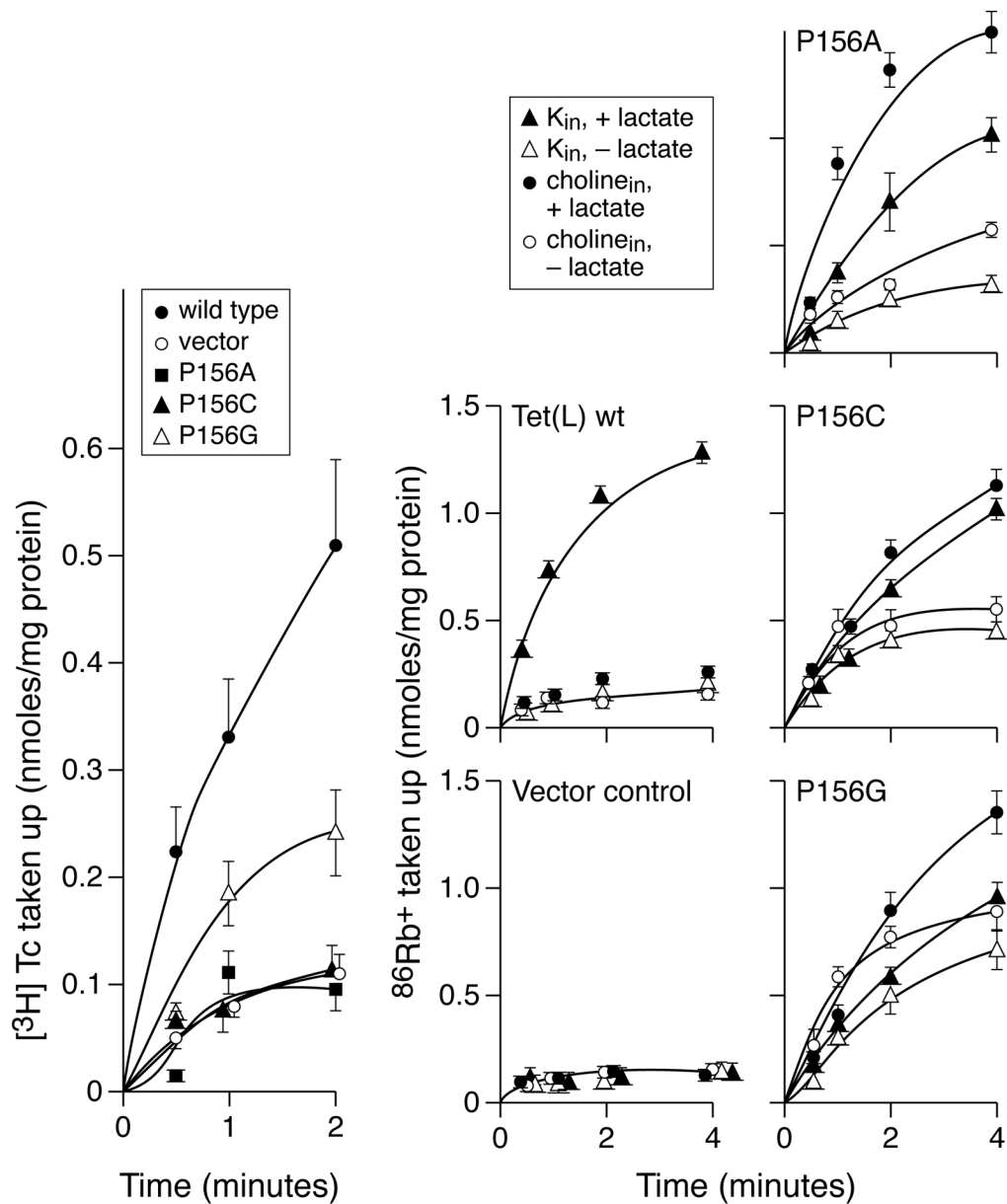
Figure 4.
Transport assays of Tet(L) Pro[156] mutants in comparison with wild type Tet(L). The assays were precisely as described in the legend to Figure 2.
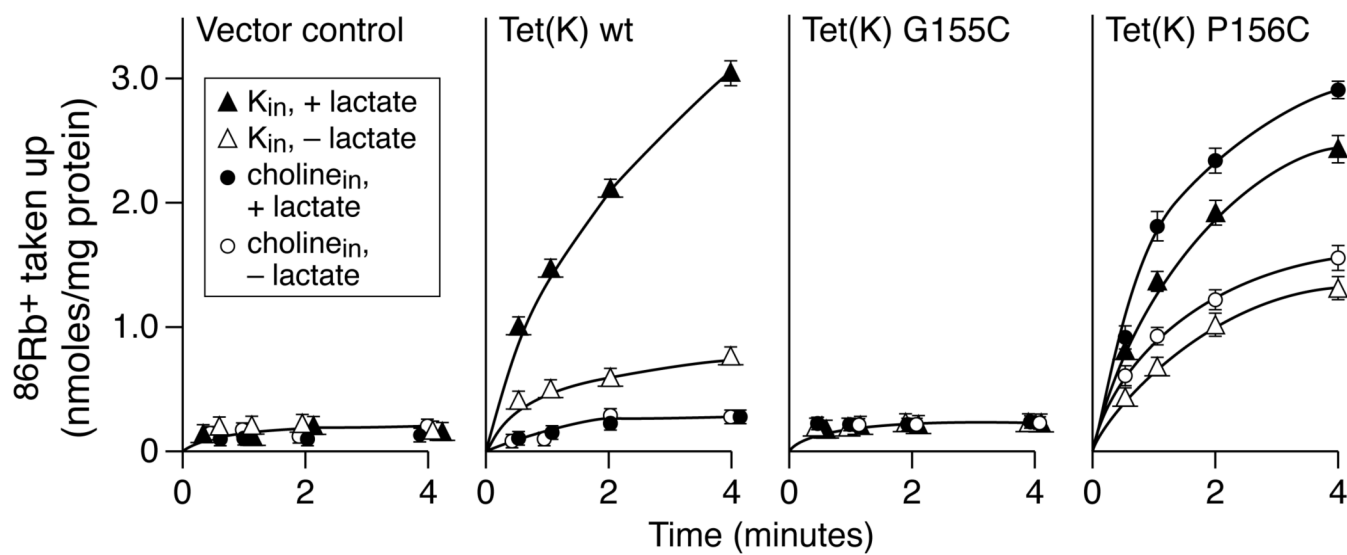
Figure 5.
Assays of ^{86}Rb$^+$ uptake by RSO vesicles of *E. coli* TK2420 expressing wild type Tet(K) or Tet(K) mutants G155C or P156C. Assays were conducted as described for the right-hand panels of Figure 2.
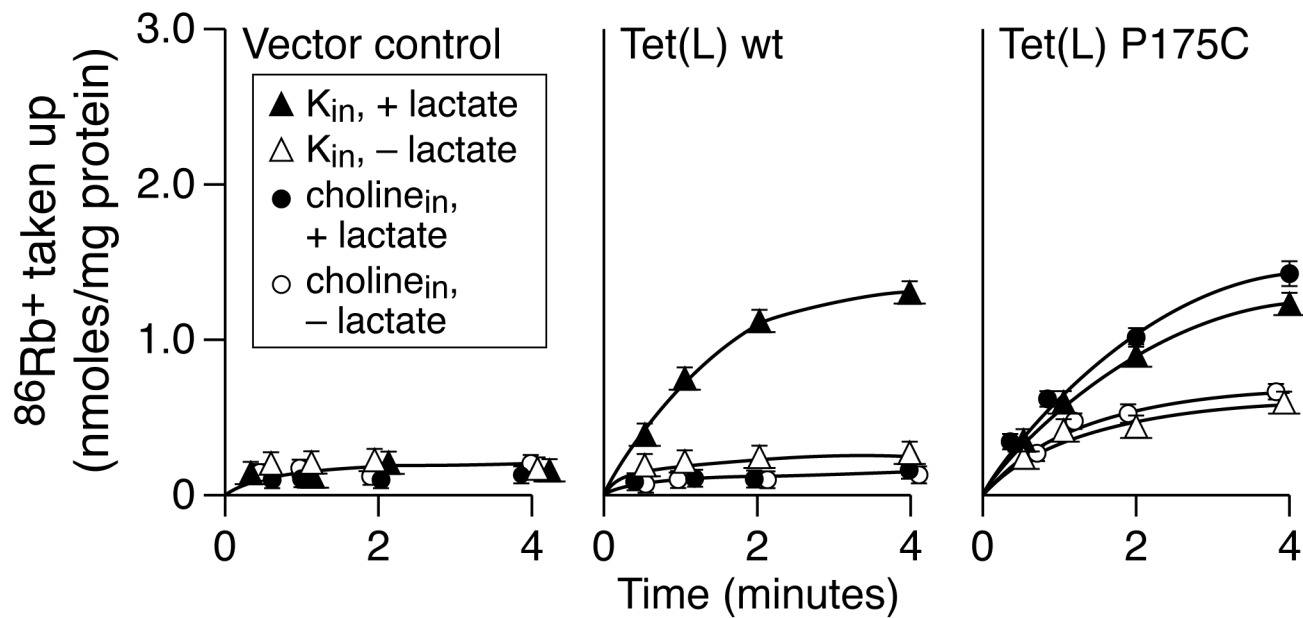
Figure 6.
Assays of $^{86}Rb^+$ uptake by RSO vesicles of *E. coli* TK2420 expressing wild type Tet(L) or Tet (L) P175C. Assays were conducted as described for the right-hand panels of Figure 2.

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Table 1

New mutational changes made in Tet(L) and Tet(K)[a]

Protein	Mutant	Primer Sequence 5'-3'
Tet(L)	E152D	CTGGTAGCAATGGGA**GAT**GGTGTTGGGCCAGCT
	G155A	ATGGGAGAAGGTGTT**GGC**CCAGCTATTGGCGGA
	G155C	ATGGGAGAAGGTGTTT**TGC**CCAGCTATTGGCGGA
	G302A	GGCATTATTTTCCCC**GCA**ACAATGAGTGTCATCA
	G302C	GGCATTATTTTCCCC**TGT**ACAATGAGTGTCATCA
	G398C	AGCTTTTTATCAGAG**TGT**ACGGGTATTGCGA
	G400C	TTATCAGAGGGAACG**TGT**ATTGCGATTGTAGGC
	G405C	GGTATTGCGATTGTA**TGC**GGTTTATTATCTATCGG
	G406C	ATTGCGATTGTAGGC**TGT**TTATTATCTATCGGCTTC
	P121C	GCAGCCGCATTC**TGC**GCTCTTGTGATGGTT
	P156A	GGAGAAGGTGTTGGG**GCA**GCTATTGGCGGAATG
	P156C	GGAGAAGGTGTTGGG**TGT**GCTATTGGCGGAATG
	P156G	GGAGAAGGTGTTGGG**CCA**GCTATTGGCGGAATG
	P175A	TATTTGCTGCTTATT**GCA**ACTGCAACAATTATC
	P175G	TATTTGCTGCTTATT**GGA**ACTGCAACAATTATC
	P183C	TATTTGCTGCTTATT**TGC**ACTGCAACAATTATC
	P301S	AGCGGCATTATTTCC**TCC**GGAACAATGAGTGTC
	R110K	ATTCTCATTCTAGCC**AAA**TTTATTCAAGGAATTGG
	T303A	CATTATTTTCCCCGGA**GCA**ATGAGTGTCATCATCTT
Tet(K)	G155C	CTTTAGGTGAAGGGTTA**TGT**CCTTCAATAGGGGGA
	G155S	CTTTAGGTGAAGGGTTA**AGT**CCTTCAATAGGGGGA
	P156C	GGTGAAGGGTTAGGT**TGT**TCAATAGGGGGAATA

[a] The bold letters in the primer sequence indicate the mutation introduced.

Table 2

Effects of the Tet(L) and Tet(K) mutations on protein assembly into the membrane and Tc-resistance in *E. coli* DH5α[a]

Tet(L) or Tet(K)	Location of mutation	Protein property	% Membrane Assembly[b]	MIC of Tc[c] (μg/ml)
-	-	None (vector control)	-	2
Tet(L)	None	Wild-type	100	30
	Motif B, TMS IV	R110C[d]	18	2
		R110K	82	4
	Motif C, TMS V	E152C	30	2
		E152D	49	12
		E152Q[d]	98	3
		G155A	134	16
		G155C	100	8
		G155S	152	8
		P156A	60	2
		P156C	94	2
		P156G	121	2
	Motif H, TMS VI	P175A	89	8
		P175C[d]	88	16
		P175G	74	16
	TMS X	P301G	ND[e]	>32
		P301S	86	2
		G302A	76	8
		G302C	62	2
		T303A	66	8
	TMS XIII	G398C	80	16
		G400C	73	8
		G405C	60	2
		G406C	65	8
Tet(K)	Motif C, TMS V	Wild-type	100	12
		G155C	105	4
		G155S	88	8
		P156C	98	2

[a] All values are the average of duplicate determinations in at least two independent experiments, with standard deviations ≤15% of the mean.

[b] Percentage of the wild-type values (set at 100) as evaluated by Western analyses.

[c] MIC is minimal Tc concentration at which there was no growth of the *E. coli* DH5α transformant after a 15-h incubation in rich medium (41).

[d] These values have been reported previously , and are shown here for comparison.

[e] Not determined.