Mercaptide Formed between the Residue Cys70 and Hg^{2+} or Co^{2+} Behaves as a Functional Positively Charged Side Chain Operative in the Arg70 → Cys Mutant of the Metal−Tetracycline/H^+ Antiporter of *Escherichia coli*†

Yuichi Someya and Akihito Yamaguchi*

Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567, Japan

Received April 30, 1996⊗

ABSTRACT: The bacterial tetracycline/H^+ antiporter (TetA) mediates active efflux of a chelation complex of tetracycline with a divalent cation such as Mg^{2+}, Co^{2+}, or Mn^{2+} [Yamaguchi, A., Udagawa, T., & Sawai, T. (1990a) *J. Biol. Chem. 265*, 4809−4813]. The positive charge of Arg70 in the antiporter is important for the transport function [Yamaguchi, A., Someya, Y., & Sawai, T. (1992c) *J. Biol. Chem. 267*, 19155−19162]. Out of six site-directed mutants of Arg70, only the Lys70 mutant retained moderate transport activity, whereas the Ser70, Ala70, Trp70, Leu70, and Asp70 mutants had no or extremely low transport activity. In this study, we constructed the Cys70 mutant and found that the Cys70 mutant showed, unexpectedly, a significant activity comparable to that of the Lys70 mutant in the presence of Co^{2+} ions, whereas it showed very low activity as well as the Ala70 mutant in the presence of Mg^{2+} or Mn^{2+} ions. Hg^{2+}, which is known to be a cysteine specific modifier but has no ability to form a complex with tetracycline, caused a dramatic increase in the V_{max} value of Co^{2+}-dependent tetracycline transport mediated by the Cys70 mutant without affecting the K_m value, whereas activities of the wild-type and the Lys70 and Ala70 mutants were not affected by Hg^{2+}. Hg^{2+} alone without Co^{2+} could not support the transport activity at all, because Hg^{2+} does not form a chelation complex with tetracycline. These observations suggest that a mercaptide formed between the SH group of Cys70 and Hg^{2+} or Co^{2+} works as a positively charged side chain like that of Arg or Lys. When the SH group of the Cys70 mutant was masked with modification by sulfhydryl reagents, the residual activity was no longer affected by Hg^{2+}. Inversely, when the Cys70 mutant was preincubated with Hg^{2+}, it was protected from the inactivation by sulfhydryl reagents. These observations also confirm the mercaptide formation between the Cys70 and a divalent cation as a functional side chain.

The metal−tetracycline/H^+ antiporter [TetA(B)] encoded by transposon Tn10 (McMurry et al., 1980; Yamaguchi et al., 1990a) mediates high-level resistance to tetracyclines (Mendez et al., 1990). This is composed of 401 amino acid residues (Hillen & Schollmeier, 1983; Nguyen et al., 1983), which was estimated to have 12 membrane-spanning α-helices (Eckert & Beck, 1989; Allard & Bertrand, 1992), and belongs to a major facilitator superfamily (Marger & Saier, 1993). Extensive site-directed mutagenesis studies on this protein have been performed (Yamaguchi et al., 1990b, 1991, 1992a−d, 1993a−c; McMurry et al., 1992), and now this protein is a paradigm not only of bacterial drug exporters (Neyfakh et al., 1991; Levy, 1992) but also of antiporters and symporters.

It is well-known that transporters belonging to the major facilitator superfamily have a unique conserved sequence motif on their putative cytoplasmic loop region ($loop_{2-3}$) between transmembrane helices 2 and 3 (Maiden et al., 1987; Henderson, 1990), which is represented as GXXXDRXGRR. The precise role of this motif still remains unknown. We first constructed a complete set of site-directed mutants of all 10 amino acid residues composing the $loop_{2-3}$ of TetA (Yamaguchi et al., 1992c). As a result, it was revealed that only 4 residues, Gly62, Asp66, Gly69, and Arg70, out of 10 residues were important for the function or the maintenance of the protein structure. In spite of high conservation among the major facilitator superfamily, mutations of Arg67 and Arg71 caused no significant decrease in activity (Yamaguchi et al., 1992c). As for Asp66, the negative charge is essential for function because only the Glu66 mutant retained very low but significant transport activity (Yamaguchi et al., 1990b).

The $loop_{2-3}$ of TetA contains four positively charged residues and only one negatively charged residue. Among four positively charged residues, the importance of Arg70 was strikingly conspicuous. When Arg70 was replaced with Ser, Ala, Trp, Leu, or Asp, the transport activity was almost lost. Only the Lys70 mutant retained the transport activity, which was about 30% of the wild-type activity (Yamaguchi et al., 1992c). Thus, it seemed that a positive charge at this position was essential.

The example other than TetA for site-directed mutagenesis studies of the $loop_{2-3}$ region of membrane transporters is the α-ketoglutarate permease (KgtP). Seol and Shatkin (1992) confirmed our findings as to the importance of the fifth acidic residue and the ninth basic residue in this motif by constructing corresponding site-directed mutants of KgtP. Very recently, Jessen-Marshall et al. (1995) performed the

† This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan. Y.S. is a Research Fellow of the Japan Society for the Promotion of Science.

* To whom correspondence should be addressed. Telephone: 81-6-879-8545. Fax: 81-6-879-8549. E-mail: akihito@sanken.osaka-u.ac.jp.

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

S0006-2960(96)01026-4 CCC: $12.00

site-directed mutagenesis study for the $loop_{2-3}$ region of the lactose permease, which also confirmed the importance of this region although none of the basic residues was essential for lactose transport.

The Cys70 mutant of the TetA protein was constructed at first in order to determine whether the side chain is exposed to the medium on the basis of the reactivity of the SH group with *N*-ethylmaleimide. As a result, the SH group of the Cys70 mutant showed high reactivity to *N*-ethylmaleimide (manuscript in preparation), confirming that position 70 is located on the surface of the membrane, exposed to the medium. In the course of this experiment, we noticed that the Cys70 mutant had unexpectedly significant tetracycline transport activity in spite of the side chain having no positive charge. In this study, we report the reason why the Cys70 mutant of TetA shows such significant transport activity.

EXPERIMENTAL PROCEDURES

Materials. [7-^{3}H]Tetracycline was purchased from Du Pont-New England Nuclear. [α-^{32}P]dCTP was purchased from Amersham. All other chemicals were of reagent grade and from commercial sources.

Bacterial Strains and Plasmids. *Escherichia coli* TG1 (Taylor et al., 1985) and W3104 (Yamamoto et al., 1981) were used for plasmid isolation and preparation of inverted membrane vesicles, respectively. pER (Yamaguchi et al., 1992a) is a subclone of the *tetA* gene used for mutagenesis. Low-copy number plasmid pLGT2 (Yamaguchi et al., 1992a), which was derived from pLG339 (Stoker et al., 1982), contains the entire *tetR* and *tetA* genes.

Site-Directed Mutagenesis. Mutagenesis for the Arg70 → Cys (R70C) substitution was performed using the Amersham kit (oligonucleotide-directed in vitro mutagenesis system, Ver. 2.1) (Taylor et al., 1985). The nucleotide sequence of the mutagenic primer was 5′-GATTTGGTTGCAGGCCT-GTGCTGT-3′ (the codon for Cys70 is underlined), and single-stranded DNA of pER was used as a template. The mutation was detected by the appearance of the *Stu*I site and confirmed by DNA sequencing (Sanger et al., 1977) using an Amersham kit (Sequenase version 2.0). The *Eco*RV-*Eco*RI fragment containing the R70C mutation was cut out from the mutant pER plasmid and exchanged with the corresponding region of pLGT2 to construct pLGR70C.

Preparation of Inverted Membrane Vesicles. Inverted membrane vesicles were prepared by passing *E. coli* W3104 cells harboring plasmids carrying the wild-type or mutant *tetA* gene through a French pressure cell, suspended in 50 mM MOPS−KOH[1] buffer (pH 7.0) containing 0.1 M KCl, and stored at −80 °C (Yamaguchi et al., 1992c).

Transport Assay. [^{3}H]Tetracycline uptake by inverted vesicles was assayed as described previously (Yamaguchi et al., 1992c). Ten microliters of inverted vesicles (3.5 mg of protein/mL) was energized by adding 0.5 μL of 250 mM β-NADH (for background uptake, this step was omitted) at 30 °C. After 1 min, 40 μL of 50 mM MOPS−KOH (pH 7.0), 0.1 M KCl containing 12.5 μM [^{3}H]tetracycline, and 62.5 μM $CoCl_2$ (which gave final concentrations of 10 and 50 μM, respectively) were added, incubated at 30 °C for the indicated times, and filtrated through a Millipore filter (0.45 μm). The filter radioactivities were counted with a liquid scintillation counter. In some assays, 12.5 μM $CoCl_2$ was substituted with 1.25 mM $CoCl_2$, 12.5 μM $HgCl_2$, or a mixture of 62.5 μM $CoCl_2$ and 12.5 μM $HgCl_2$. The kinetic constants for tetracycline transport were measured in the presence of 1 mM $CoCl_2$ and various concentrations (5−100 μM) of [^{3}H]tetracycline.

H^+-Translocation in Inverted Membrane Vesicles. H^+ translocation across membranes was measured by monitoring the change in fluorescence of acridine orange (excitation wavelength, 490 nm; emission wavelength, 530 nm) or quinacrine (excitation wavelength, 440 nm; emission wavelength, 500 nm). Typically, 20 μL of inverted vesicles (2 mg of protein/mL) was diluted with 1 mL of 50 mM MOPS−KOH (pH 7.0) containing 0.1 M KCl, 10 mM $MgSO_4$, and 1.25 μM acridine orange or 0.8 μM quinacrine. Membranes were energized with a final concentration of 625 μM NADH. At the end of one experiment, NH_4Cl was added to a final concentration of 2.5 mM.

Other Methods. Measurement of the tetracycline resistance level and Western blotting were performed as described previously (Yamaguchi et al., 1990b, 1992c).

RESULTS

Construction of the Arg70 → Cys Mutant of the TetA Protein. The Cys70 mutant of TetA was constructed by site-directed mutagenesis described in Experimental Procedures. *E. coli* cells carrying a low-copy number plasmid, pLGR70C, encoding the Cys70 mutant TetA showed a moderate level of tetracycline resistance (minimum inhibitory concentration, 12.5 μg/mL), which was about half of the resistance level of the Lys70 mutant (25 μg/mL). The expression of the mutant *tetA* gene was detected by Western blotting using anti-TetA-C-terminal-peptide antiserum. There was no significant difference in the amounts of the mutant and wild-type TetA proteins (data not shown).

Tetracycline Transport Activity of Inverted Membrane Vesicles. Figure 1A shows the tetracycline uptake by inverted membrane vesicles containing the mutant or wild-type TetA proteins under our standard condition in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. Under this condition, the initial rate of the active uptake of tetracycline by the wild-type vesicles was 2.7 nmol of tetracycline per milligram of membrane protein per 30 s. The inverted vesicles prepared from the Ala70 and Ser70 mutant cells retained less than 4% of the wild-type transport activity (<0.1 nmol per milligram of protein per 30 s), while the Leu70 mutant vesicles had completely lost the transport activity. In contrast, the Lys70 mutant vesicles retained the transport activity of 1.0 nmol per milligram of protein per 30 s, which corresponded to about 40% of the wild-type activity. These observations were essentially the same as our previous observation, which indicated the importance of a positive charge at position 70 (Yamaguchi et al., 1992c). However, although apparently the Cys70 mutant has no positive charge at position 70, the Cys70 mutant vesicles showed significant transport activity of 0.54 nmol milligram of protein per 30 s, which corresponded to about 20% of the wild-type activity and 54% of the Lys70 mutant activity.

This observation was confirmed in the presence of high concentrations of $CoCl_2$ (1 mM) (Figure 1B). Under this condition, the initial rates of tetracycline transport by the wild-type and the Lys70 mutant vesicles were similarly increased by a factor of about 2−2.5, whereas the activity

[1] Abbreviations: NEM, *N*-ethylmaleimide; MMTS, methyl methanethiosulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

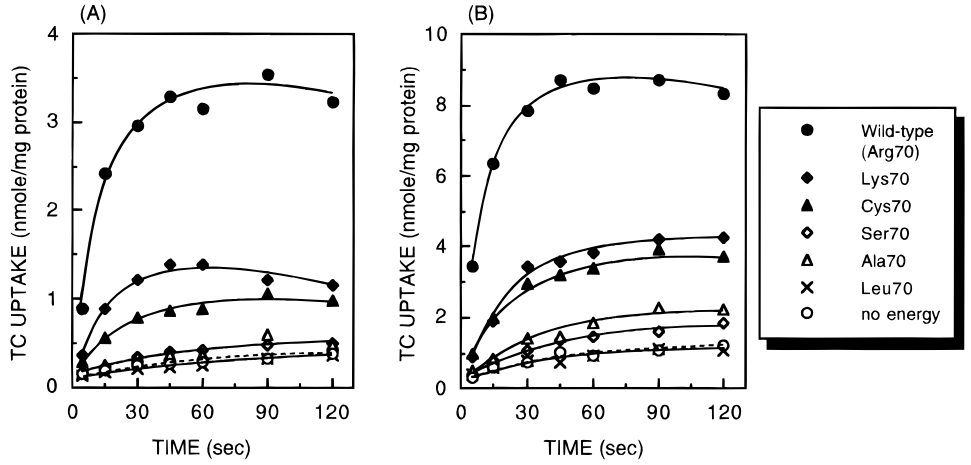

FIGURE 1: Co^{2+}-dependent tetracycline (TC) uptake by inverted membrane vesicles from *E. coli* W3104 cells expressing the Arg70 mutant TetA proteins. Transport assays were performed in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ (A) or 1 mM $CoCl_2$ (B). Solid lines and broken lines represent the uptake in the presence and absence of NADH, respectively.

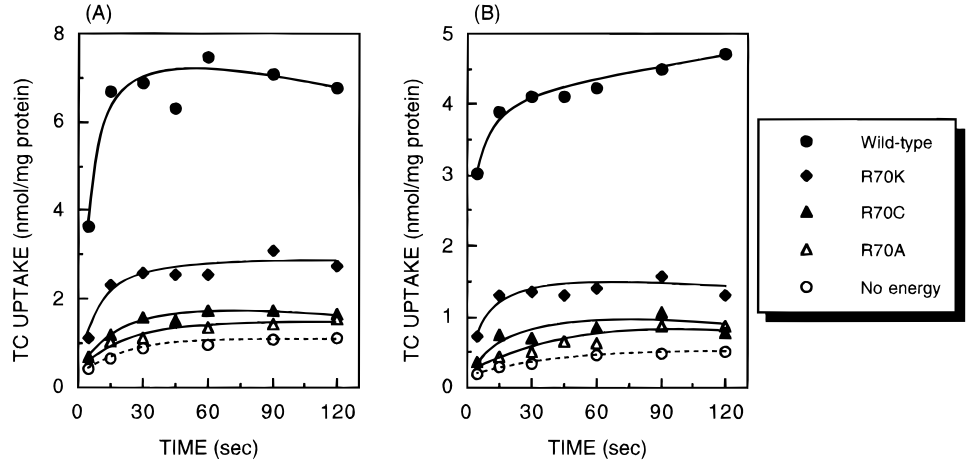

FIGURE 2: Mn^{2+}- and Mg^{2+}-dependent tetracycline (TC) uptake by inverted membrane vesicles from *E. coli* W3104 cells expressing the Arg70 mutant TetA proteins. Transport assays were performed in the presence of 10 μM [^{3}H]tetracycline and 1 mM $MnCl_2$ (A) or 1 mM $MgSO_4$ (B). Other assay conditions were the same as in Figure 1.

of the Cys70 mutant was increased more than 4-fold compared with that in the presence of 50 μM $CoCl_2$. As a result, the rate of transport by the Cys70 mutant (2.1 nmole per milligram of protein per 30 s) corresponded to about 30% of the wild-type activity and about 80% of the Lys70 mutant activity (2.7 nmol per milligram of protein per 30 s).

In order to determine the affinity of these mutants for the tetracycline−Co^{2+} chelation complex, initial rates of tetracycline transport were measured in the presence of excess (1 mM) $CoCl_2$ and various concentrations of [^{3}H]tetracycline. The K_m and V_{max} values were calculated from nonlinear regression (Table 1). All the position 70 mutants showed K_m values comparable to the wild-type values, suggesting that Arg70 is not involved in the direct interaction with the cationic substrate. The Cys70 mutant had a V_{max} value significantly higher than those of other neutral mutants (Ala and Ser) and seemed to have a property similar to that of the Lys70 mutant having a positive charge at position 70.

The chelation complex of tetracycline with Mn^{2+} or Mg^{2+} also acts as a substrate for TetA (Yamaguchi et al., 1990a). However, the unexpectedly high activity of the Cys70 mutant was not observed in the presence of Mn^{2+} or Mg^{2+} (Figure 2). In the presence of these two divalent cations, the transport activity of the Cys70 mutant was very low, comparable to that of the Ala70 mutant. Therefore, it seems

Table 1: Kinetic Constants for Tetracycline Uptake by Inverted Membrane Vesicles[a]

mutations	Hg^{2+}	K_m (μM)	V_{max} [nmol (mg of protein)$^{-1}$ min^{-1}]
none (wild-type)	(−)	24.8 ± 7.3	43.1 ± 4.8
	(+)	31.4 ± 3.5	46.1 ± 2.0
Arg70 → Cys	(−)	26.6 ± 5.7	10.7 ± 0.8
	(+)	19.7 ± 6.0	22.5 ± 2.2
Arg70 → Lys	(−)	18.6 ± 2.7	19.2 ± 1.0
	(+)	10.3 ± 4.2	17.5 ± 1.9
Arg70 → Ala	(−)	22.7 ± 3.1	4.4 ± 0.3
Arg70 → Ser	(−)	23.8 ± 9.8	2.3 ± 0.4

[a] The rates of active [^{3}H]tetracycline uptake for the initial 30 s were measured in the presence of various concentrations of tetracycline and 1 mM $CoCl_2$ (−) or 1 mM $CoCl_2$ plus 10 μM $HgCl_2$ (+). The kinetic constants were calculated from nonlinear regression.

that the Cys70 side chain behaves like a positively charged side chain only in the presence of Co^{2+} ion.

Effect of Hg^{2+} on the Transport Activity of the Cys70 Mutant. One possibility for such an unexpectedly high activity of the Cys70 mutant is that the mercaptide (CH_2-SCo^+) formed between the SH group and a cobalt ion (Weissbecker, 1956) behaves like a positively charged side chain. Hg^{2+} is known to have high reactivity with the SH group, to produce a mercaptide (CH_2SHg^+). Contrary to Co^{2+}, Mn^{2+}, and Mg^{2+}, Hg^{2+} did not form a chelation complex. This was confirmed by the following experiments.

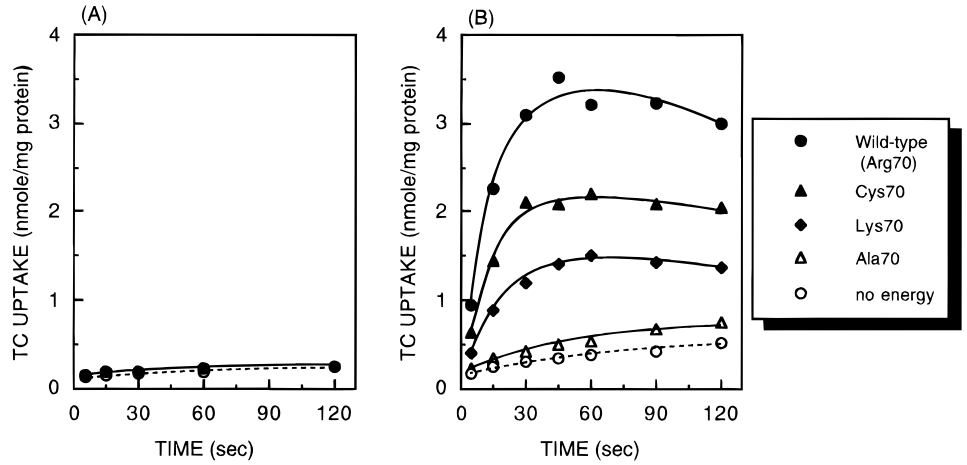

FIGURE 3: Tetracycline (TC) uptake by inverted membrane vesicles in the presence of 10 μM $HgCl_2$. Tetracycline uptake was measured in the presence of 10 μM [^{3}H]tetracycline and 10 μM in $HgCl_2$ in the absence (A) or presence (B) of 50 μM $CoCl_2$. Other assay conditions were the same as in Figure 1.

When tetracycline forms a chelation complex with a divalent cation (Co^{2+}, Mn^{2+}, or Mg^{2+}), there is a bathochromic shift from 360 to 395 nm (Yamaguchi et al., 1990a). However, there is no such bathochromic shift of the absorption spectum of tetracycline even in the presence of 5 mM $HgCl_2$ (data not shown), indicating the inability of Hg^{2+} to form a chelation complex with tetracycline. Therefore, Hg^{2+} is useful to distinguish between the effect of the formation of a mercaptide with an SH group and the effect of the formation of the chelation complex with tetracycline. We tested the effect of $HgCl_2$ on the tetracycline transport activity (Figure 3). In the absence of other divalent cations, $HgCl_2$ did not support the tetracycline transport, mediated not only by the Cys70 mutant but also by the wild-type TetA and the Lys70 and Ala70 mutants (Figure 3A), due to the inability of Hg^{2+} to form a chelation complex with tetracycline. On the contrary, in the presence of 50 μM $CoCl_2$, 10 μM $HgCl_2$ caused a large stimulation, which was specific to the Cys70 mutant-mediated transport (Figure 3B). Under this condition, the initial rate of tetracycline uptake by the Cys70 mutant was 1.8 nmol per milligram of protein per 30 s, which was about 3-fold higher than that in the absence of $HgCl_2$ and corresponded to about 65% of the wild-type transport activity (Figure 3B). In contrast, the transport activities of the other TetA proteins, including the wild-type and the Lys70 and Ala70 mutants, were not affected by 10 μM $HgCl_2$ at all (Figure 3B). As a result, the transport activity of the Cys70 mutant became almost 2-fold higher than that of the Lys70 mutant under this condition, indicating that the Hg^{2+}–mercaptide side chain at position 70 works better than Lys70. $HgCl_2$ increased the V_{max} value of the Cys70 mutant without affecting the K_m value (Table 1).

Hg^{2+} is a potent inhibitor of the bacterial respiratory chain so that we need to check the effect of $HgCl_2$ on the energized state of the inverted vesicles. The dissipation of the respiration-dependent ΔpH by addition of $HgCl_2$ was detected by the acridine orange fluorescence-quenching method. There was no significant dissipation of ΔpH observed upon addition of less than 10 μM $HgCl_2$ (Figure 4).

Then we measured the $HgCl_2$ concentration dependence of tetracycline transport activities of the wild-type and the Cys70 mutant (Figure 5). The activity of the wild-type was not affected by $HgCl_2$ until it reached 10 μM. Upon addition of more than 10 μM, the wild-type activity was gradually inhibited, with an ID_{50} of about 100 μM. The inhibition of

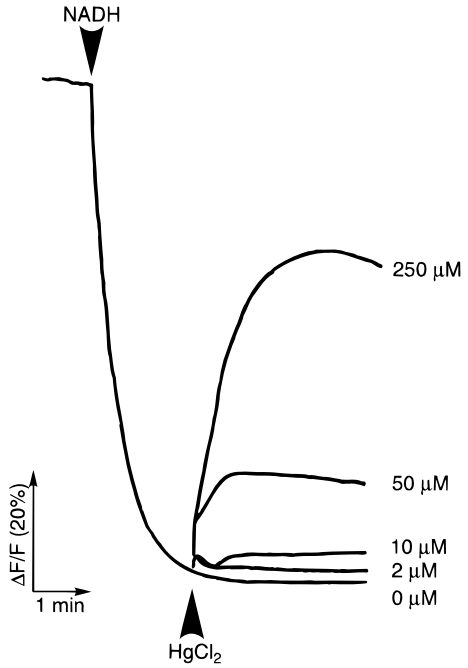

FIGURE 4: Effect of $HgCl_2$ on DpH formed by NADH-dependent respiration in inverted membrane vesicles. H^+ translocation across inverted vesicles was measured by monitoring the change in fluorescence of acridine orange. Twenty microliters of the vesicles (2 mg of protein/mL) prepared from *E. coli* W3104 cell sharboring pLGT2 (wild-type plasmid) was diluted into 1 mL of MOPS–KOH buffer (pH 7.0) and energized with 625 μM NADH. After 1 min, $HgCl_2$ was added, which gave the indicated final concentrations.

the wild-type activity by $HgCl_2$ was due to the dissipation of the energized state of the membrane. On the other hand, the activity of the Cys70 mutant was once activated around 10 μM $HgCl_2$ and then inhibited by higher concentrations of $HgCl_2$. Therefore, it is clear that the inhibitory effect of $HgCl_2$ on the energized state of the membrane and the stimulation effect on the transport activity of the Cys70 mutant were independent of one another.

Effects of Sulfhydryl Reagents on Tetracycline Transport. Out of seven site-directed cysteine mutants in the putative cytoplasmic $loop_{2-3}$ region hitherto constructed in our previous paper (Yamaguchi et al., 1992c), only the Ser65 → Cys mutant was inhibited by *N*-ethylmaleimide (NEM), whereas the other six Cys mutants (at positions 63, 64, 67–69, and 71) were not affected by NEM at all. The

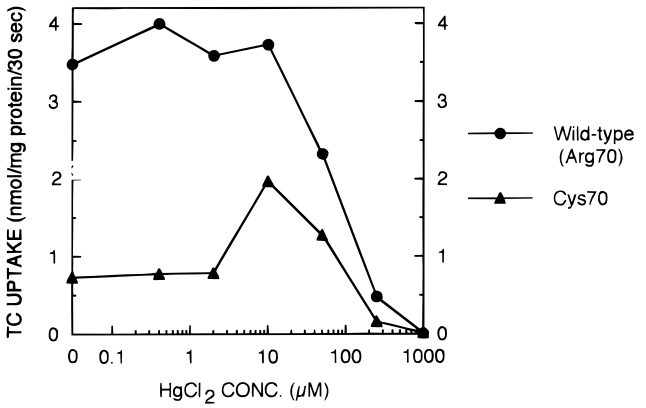

FIGURE 5: $HgCl_2$ concentration dependence of [^{3}H]tetracycline transport activity of the wild-type and the Cys70 mutant TetA proteins in inverted membrane vesicles. The net active uptake of tetracycline during the initial 30 s in the assay solution containing 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ in the presence of the indicated concentrations of $HgCl_2$ was plotted against $HgCl_2$ concentration.

inactivation of the Cys65 mutant was due to steric hindrance, which was dependent upon the volume of the substituent (Yamaguchi et al., 1994). We have now tested the effect of sulfhydryl reagents on the newly constructed Cys70 mutant.

As shown in Figure 6, NEM and methyl methanethiosulfonate (MMTS), which gives a thiomethyl substituent smaller than NEM, caused a similar degree of inactivation of the Cys70 mutant. After the treatment of excess sulfhydryl reagents, the residual activity was only about 10−15% of its original level before treatment with SH reagents. MMTS caused maximum inactivation at a lower concentration than NEM, suggesting higher reactivity of MMTS with an SH group. When the mutant was at first treated with 2 mM MMTS, further treatment with NEM no longer caused any effect on the activity, suggesting that the reaction of Cys70 with MMTS was complete. Thus, we concluded that the thiomethyl- or NEM-substituted Cys70 mutant still had a remaining low activity.

The substituent volume independence of the Cys70 mutant inactivation from sulfhydryl reagents was a completely different feature from that of the Cys65 mutant inactivation (Yamaguchi et al., 1994). In the latter case, MMTS caused only 40% inactivation, whereas NEM caused complete

Table 2: Effects of Sulfhydryl Modification on Hg^{2+} Activation for the Arg70 → Cys Mutant[a]

mutants	SH reagents	initial rate of tetracycline uptake [nmol (mg of protein)$^{-1}$ per 30 s] none	initial rate of tetracycline uptake [nmol (mg of protein)$^{-1}$ per 30 s] $+Hg^{2+}$	activation (+Hg/−Hg)[b]
Arg70 → Cys	none	0.7	1.9	2.7
	2 mM MMTS	0.3	0.3	1.0
	5 mM NEM	0.2	0.2	1.0
wild-type	none	2.9	2.9	1.0
	2 mM MMTS	2.9	2.6	0.9
	5 mM NEM	2.5	2.2	0.9

[a] Inverted vesicles were pretreated with the indicated SH reagents for 5 min at 30 °C, and NADH-dependent 30 s uptake of tetracycline was measured in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ (none) or 50 μM $CoCl_2$ and 10 μM $HgCl_2$ ($+Hg^{2+}$). [b] Activation by Hg^{2+} was calculated as a ratio of the initial rates in the presence and absence of $HgCl_2$.

inactivation (Yamaguchi et al., 1990b). Thus, the inactivation of the two Cys mutants occurs by different mechanisms. The volume independence of the Cys70 inactivation suggests that the inactivation is not due to steric hindrance but based upon the masking of the SH group. The fact that the level of the residual activity of the thiomethyl- or NEM-substituted Cys70 mutant was compatible to the activity of the Ala70 and Ser70 mutants supported this assumption.

Table 2 shows the effect of $HgCl_2$ on the activity of the wild-type and the Cys70 mutant before and after the treatment of sulfhydryl reagents. When the Cys70 mutant was treated with NEM (5 mM) or MMTS (2 mM), residual activity was no longer stimulated by $HgCl_2$. This observation clearly confirmed that the stimulation effect of $HgCl_2$ was due to the mercaptide formation with a free SH group at position 70.

E*ffect of Hg^{2+} on the Inhibition of the Cys70 Mutant with NEM.* Quinacrine fluorescence in the energized Cys70 mutant vesicles was increased by addition of 20 μM tetracycline in the presence of 10 mM $MgSO_4$, indicating the tetracycline/H^+ antiport mediated by the Cys70 mutant (Figure 7A, trace 1). The degree of the tetracycline-induced fluorescence change was remarkably stimulated by 10 μM $HgCl_2$ (Figure 7A, trace 2), corresponding to the stimulation of the transport activity by Hg^{2+}. On the other hand, the

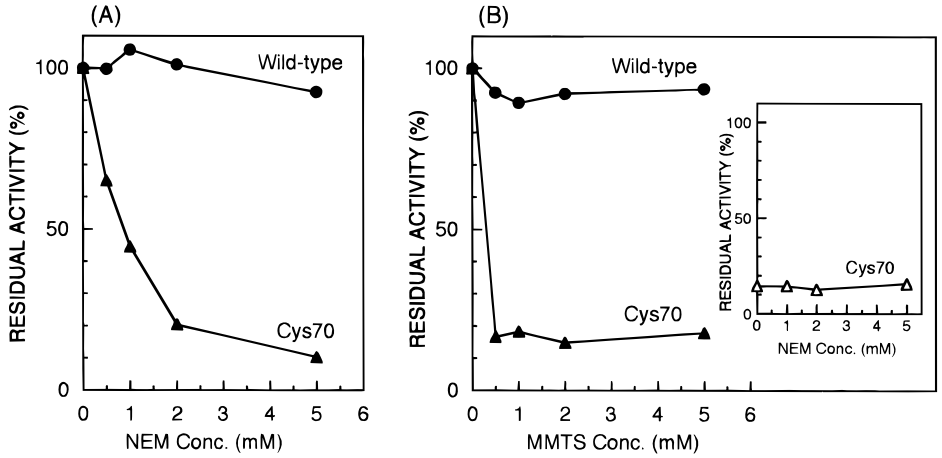

FIGURE 6: Effect of sulfhydryl reagents on tetracycline uptake by inverted vesicles from the Cys70 mutant. Inverted vesicles were preincubated with the indicated concentrations of *N*-ethylmaleimide (NEM) (A) or methyl methanethiosulfonate (MMTS) (B) for 5 min at 30 °C before the measurement of tetracycline uptake. Then the net active uptake was measured in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. Residual activity is expressed as a percentage of activity without sulfhydryl reagent. In the inset of panel B, the residual activities of the Cys70 vesicles, when they were first treated with 2 mM MMTS for 5 min at °C and then treated with the indicated concentrations of NEM for 5 min at °C, are shown.

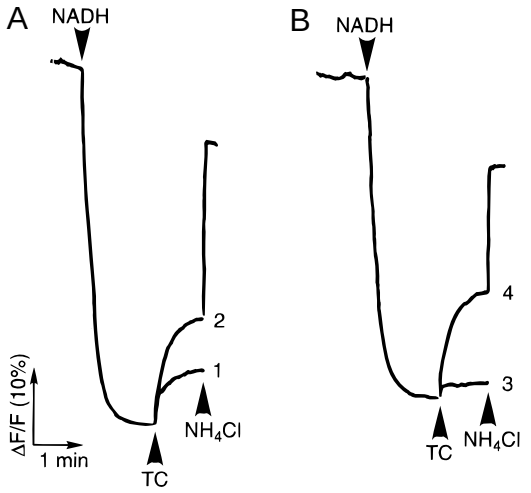

FIGURE 7: Effect of preincubation with Hg^{2+} on NEM inhibition of tetracycline-dependent H^+ translocation in the Cys70 vesicles. H^+ translocation across inverted vesicles was measured by monitoring the change in fluorescence of quinacrine. Twenty microliters of the Cys70 vesicles (2 mg of protein/mL) was diluted into 1 mL of 50 mM MOPS−KOH (pH 7.0), 0.1 M KCl, and 10 mM $MgSO_4$ and energized with 625 μM NADH. Neither $HgCl_2$ nor NEM was added in trace 1 of panel A. In trace 2 of panel A, the vesicles were preincubated with 10 mM $HgCl_2$ for 1 min prior to energization. In trace 3 of panel B, the vesicles were preincubated with 2 mM NEM for 3 min prior to energization. In trace 4 of panel B, the vesicles were preincubated with 10 mM $HgCl_2$ for 1 min followed by incubation with 2 mM NEM for 3 min prior to energization.

tetracycline-induced fluorescence change was remarkably inhibited by preincubation with 2 mM NEM (Figure 7B, trace 3) or 1 mM MMTS (data not shown). The inhibition of the tetracycline-induced fluorescence change with the SH reagents was not observed in the wild-type or the Lys70 mutant vesicles (data not shown); thus, it is clear that the inhibition of the fluorescence change was due to the modification of the Cys70 side chain by the SH reagents. However, when the Cys70 mutant vesicles were preincubated with 2 mM NEM in the presence of 10 μM $HgCl_2$, the tetracycline-induced fluorescence change in the Cys70 mutant vesicles was no longer affected by 2 mM NEM (Figure 7B, trace 4) or 1 mM MMTS (data not shown). This observation clearly indicated that a mercaptide formed between Cys70 and Hg^{2+} protected Cys70 from the chemical modification with SH reagents and the mercaptide side chain acted as a functional side chain.

Figure 8 shows the [^{3}H]tetracycline uptake by inverted membrane vesicles in the presence of 2 mM NEM. The uptake mediated by the Cys70 mutant vesicles was almost completely inhibited with 2 mM NEM, whereas that of the wild-type vesicles was not inhibited (Figure 8A). On the other hand, in the presence of 10 μM $HgCl_2$, the uptake by the Cys70 mutant was not inhibited by 2 mM NEM (Figure 8B), confirming that Hg^{2+} protects the Cys70 from the chemical modification with NEM.

DISCUSSION

Although a positive charge at position 70 in TetA(B) is important for the transport function (Yamaguchi et al., 1992c) and a cysteine residue has a small neutral side chain, the Arg70 → Cys mutant of TetA(B) showed unexpectedly significant tetracycline transport activity in the presence of Co^{2+} ions (Figure 1). In this study, we revealed that such behavior of the Cys70 mutant was due to the mercaptide formed between the SH group of Cys70 and a divalent cation, which provided a functional positively charged side chain operational in tetracycline transport.

Co^{2+} is able to form tightly bound complex with a thiol group of cysteine and glutathione (Maines & Kappas, 1976; Weissbecker, 1956) and a model protein (Regan & Clarke, 1990; Klemba & Regan, 1995), supporting the idea that a mercaptide formed between Co^{2+} and the SH group of the Cys70 residue works as a positively charged side chain. To the best of our knowledge, there was no report about whether Mg^{2+} and Mn^{2+} are able to form a mercaptide. As judged from our result, the efficiency of the mercaptide formation of these cations should be very low. The activity of the Cys70 mutant in the presence of Mn^{2+} or Mg^{2+} was very low, similar to or slightly higher than that of the Ala70 mutant. The most effective cation to form mercaptide is Hg^{2+}. However, Hg^{2+} does not form a chelation complex with tetracycline, and thus, Hg^{2+} alone did not confer TetA-mediated tetracycline transport as expected. When Hg^{2+} was added with Co^{2+}, Hg^{2+} highly stimulated the Co^{2+}-dependent tetracycline transport mediated by the Cys70 mutant but it did not affect the activity mediated by the wild-type and the Lys70 and Ala70 mutants. Surprisingly, the Hg^{2+}-stimulated activity of the Cys70 mutant was remarkably higher than that of the Lys70 mutant, indicating that the mercuric methyl mercaptide side chain at position 70 works better than the aminobutyl side chain.

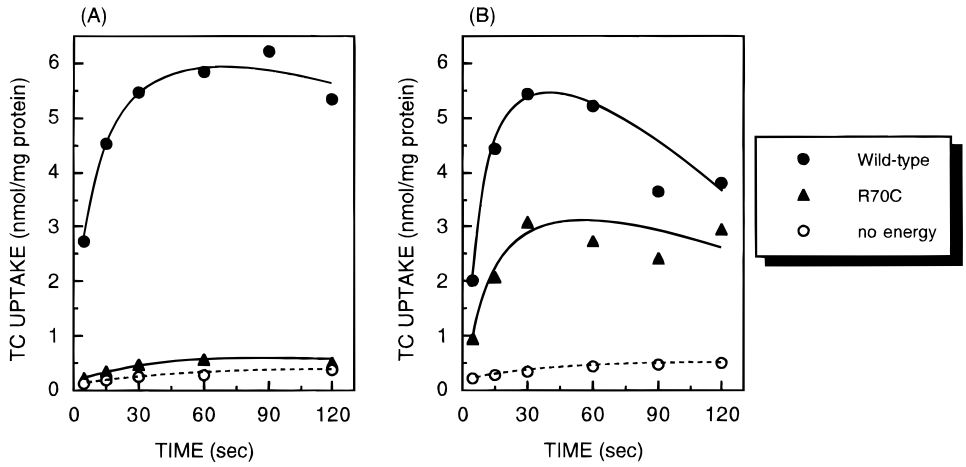

FIGURE 8: Tetracycline (TC) uptake by inverted membrane vesicles in the presence of 2 mM NEM (A) and 2 mM NEM plus 10 μM $HgCl_2$ (B). Tetracycline uptake was measured in the presence of 10 μM [^{3}H]tetracycline, 50 μM $CoCl_2$, and 2 mM NEM in the absence (A) or presence (B) of 10 μM $HgCl_2$. Other assay conditions were the same as in Figure 1.

The mercaptide formation between Hg^{2+} and the thiol group of the Cys70 mutant was also confirmed by the following observations. (1) After the masking of the SH group by modification with NEM or MMTS, the residual activity of the Cys70 mutant was no longer affected by Hg^{2+} (Table 2). (2) When the Cys70 mutant was preincubated with Hg^{2+}, the Cys70 mutant was protected from the inactivation by sulfhydryl reagents (Figures 7 and 8).

Some cases of functional re-creation of essential charged side chains by chemical modification of introduced Cys residues have been reported. As for a negatively charged residue, an artificially re-created carboxyl moiety by modification of a mutant cysteine residue can restore the function of original acidic residues in bacteriorhodopsin (Greenhalgh et al., 1992) and lactose permease (Dunten et al., 1993). In the case of bacteriorhodopsin (Greenhalgh et al., 1992), the proton-pumping activity of the Asp85 → Cys mutant was restored by modification with iodoacetic acid. In spite of the great difference in the side chain volume between the re-created carboxyl moiety and that of an aspartic acid, the former can transfer a proton from the Schiff base (Greenhalgh et al., 1992). On the other hand, a re-created carboxyl moiety in the lactose permease plays a structural role. Asp237 of the lactose permease contributes to the protein folding by forming a salt bridge with Lys358 in the transmembrane region. The replacement of Asp237 with Cys virtually abolished transport activity. The carboxymethylation of Cys237 restored full activity through reformation of the salt bridge (Dunten et al., 1993). As for a positively charged residue, chemical modification of the K358C mutant of the lactose permease with methanethiosulfonate ethylammonium (MTSEA) yields active permease through restoration of the salt bridge between Asp237 and the residue at position 358 (Sahin-Tóth & Kaback, 1993).

However, to the best of our knowledge, this study is the first report on functional re-creation of a positively charged side chain of membrane proteins by a mercaptide formation of the SH group of the introduced Cys residue with a divalent cation. Although putative cytoplsmic $loop_{2-3}$ contains four positively charged residues, three of which are highly conserved in the major facilitator superfamily (Yamaguchi et al., 1992c), Arg70 is the only functionally important basic residue in this loop (Yamaguchi et al., 1992c). The $loop_{2-3}$ also contains a functionally essential acidic residue, Asp66. We obtained some evidence that Asp66 contributed to the substrate−protein interaction and the following conformational change of the $loop_{2-3}$ region (Kimura et al., 1995). The reactivity of [^{14}C]NEM with the Ser65 → Cys mutant could be used as an indicator of a conformational change after the substrate−protein interaction; i.e., the reactivity was stimulated in the presence of tetracycline, indicating the substrate-dependent exposure of this region to the medium. The stimulation did not occur in the Asp66 → Ala/Ser65 → Cys double mutant probably because of a defect in the substrate−protein interaction. In contrast, the degree of stimulation of [^{14}C]NEM−protein reaction by the substrate increased in the Arg70 → Ala/Ser65 → Cys double mutant (Kimura et al., 1995). Thus, it seems that Arg70 does not directly contribute to the substrate−protein interaction as supported by the result shown in Table 1 which described no significant change in the apparent K_m values. The other possibility is that Arg70 contributes to the switching of the open/closed state of the entrance gate probably through an ionic interaction with Asp66 or some other acidic residues, just similar to Lys319 in the lactose permease (Lee et al., 1993).

REFERENCES

Allard, J. D., & Bertrand, K. P. (1992) *J. Biol. Chem. 267*, 17809−17819.

Dunten, R. L., Sahin-Toth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 3139−3145.

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

Greenhalgh, D. A., Subramaniam, S., Alexiev, U., Otto, H., Heyn, M. P., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 25734−25738.

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

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

Jessen-Marshall, A. E., Paul, N. J., & Brooker, R. J. (1995) *J. Biol. Chem. 270*, 16251−16257.

Kimura, T., Inagaki, Y., Sawai, T., & Yamaguchi, A. (1995) *FEBS Lett. 362*, 47−49.

Klemba, M., & Regan, L. (1995) *Biochemistry 34*, 10094−10100.

Lee, J.-I., Hwang, P. P., & Wilson, T. H. (1993) *J. Biol. Chem. 268*, 20007−20015.

Levy, S. B. (1992) *Antimicrob. Agents Chemother. 36*, 695−703.

Maiden, N. C. J., Davis, E. O., Baldwin, S. A., Moore, D. C. M., & Henderson, P. J. F. (1987) *Nature 325*, 641−643.

Maines, M. D., & Kappas, A. (1976) *Biochem. J. 154*, 125−131.

Marger, M. D., & Saier, M. H., Jr. (1993) *Trends Biochem. Sci. 18*, 13−20.

McMurry, L., Petrucci, R. E., Jr., & Levy, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3974−3977.

McMurry, L. M., Stephan, M., & Levy, S. B. (1992) *J. Bacteriol. 174*, 6294−6297.

Mendez, B., Tachibana, C., & Levy, S. B. (1980) *Plasmid 3*, 99−108.

Neyfakh, A. A., Bidenko, V. E., & Chen, L. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4781−4785.

Nguyen, T. T., Postle, K., & Bertrand, K. P. (1983) *Gene 25*, 83−92.

Regan, L., & Clarke, N. D. (1990) *Biochemistry 29*, 10878−10883.

Sahin-Tóth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 10027−10035.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Seol, W., & Shatkin, A. J. (1992) *Biochemistry 31*, 3550−3554.

Stoker, N. G., Fairweather, N. F., & Spratt, B. G. (1982) *Gene 18*, 335−341.

Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764−8785.

Weissbecker, L. (1956) *Beih. Med. Monatsschr. Z. Allgeme Med. Ther. 15*, 120−138.

Yamaguchi, A., Udagawa, T., & Sawai, T. (1990a) *J. Biol. Chem. 265*, 4809−4813.

Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T., & Sawai, T. (1990b) *J. Biol. Chem. 265*, 15525−15530.

Yamaguchi, A., Adachi, K., Akasaka, T., Ono, N., & Sawai, T. (1991) *J. Biol. Chem. 266*, 6045−6051.

Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., & Sawai, T. (1992a) *J. Biol. Chem. 267*, 7490−7498.

Yamaguchi, A., Nakatani, M., & Sawai, T. (1992b) *Biochemistry 31*, 8344−8348.

Yamaguchi, A., Someya, Y., & Sawai, T. (1992c) *J. Biol. Chem. 267*, 19155−19162.

Yamaguchi, A., Ono, N., Akasaka, T., & Sawai, T. (1992d) *FEBS Lett. 307*, 229−232.

Yamaguchi, A., Akasaka, T., Kimura, T., Sakai, T., Adachi, Y., & Sawai, T. (1993a) *Biochemistry 32*, 5698−5704.

Yamaguchi, A., Kimura, T., Someya, Y., & Sawai, T. (1993b) *J. Biol. Chem. 268*, 6496−6504.

Yamaguchi, A., Kimura, T., & Sawai, T. (1993c) *FEBS Lett. 322*, 201−204.

Yamaguchi, A., Kimura, T., & Sawai, T. (1994) *J. Biochem. (Tokyo) 115*, 958−964.

Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y., & Yamagishi, S. (1981) *J. Bacteriol. 145*, 808−813.

BI961026Z