Role of the Charge Interaction between Arg<sup>70</sup> and Asp<sup>120</sup> in the Tn10-encoded Metal-Tetracycline/H<sup>+</sup> Antiporter of Escherichia coli

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We reported that the positive charge of Arg<sup>70</sup> is mandatory for tetracycline transport activity of Tn10-encoded metal-tetracycline/H<sup>+</sup> antiporter (TetA(B)) (Someya, Y., and Yamaguchi, A. (1996) Biochemistry 35, 9385–9391). Arg<sup>70</sup> may function through a charge-pairing with a negatively charged residue in close proximity. Therefore, we mutated Asp<sup>66</sup> and Asp<sup>120</sup>, which are only two negatively charged residues located close to Arg<sup>70</sup> in putative secondary structure of TetA(B) and highly conserved throughout transporters of the major facilitator superfamily. Site-directed mutagenesis studies revealed that Asp<sup>66</sup> is essential, but Asp<sup>120</sup> is important for TetA(B) function. Surprisingly, when Asp<sup>120</sup> was replaced by a neutral residue, the R70A mutant recovered tetracycline resistance and transport activity. There was no such effect in the Asp<sup>66</sup> mutation. The charge-exchanged mutant, R70D/D120R, also showed significant drug resistance and transport activity (about 50% of the wild type), although the R70D mutant had absolutely no activity, and the D120R mutant retained very low activity (about 10% of the wild type). Both the R70C and D120C mutants were inactivated by N-ethylmaleimide. Mercuric ion (Hg<sup>2+</sup>) gives a positive charge to SH group of a Cys residue through mercaptide formation, had an opposite effect on the R70C and D120C mutants. The activity of the R70C mutant was stimulated by Hg<sup>2+</sup>; however, on the contrary, the D120C mutant was partially inhibited. On the other hand, the R70C/D120C double mutant was almost completely inactivated by Hg<sup>2+</sup>, probably because the side chains at positions 70 and 120 are bridged with Hg<sup>2+</sup>. The close proximity of positions 70 and 120 were confirmed by disulfide cross-linking formation of the R70C/D120C double mutant when it was oxidized by copper-(1,10-phenanthroline). These results indicate that the positive charge of Arg<sup>70</sup> requires the negative charge of Asp<sup>120</sup> for neutralization, probably for properly positioning transmembrane segments in the membrane.

Tetracycline resistance in bacterial cells is mediated mainly by the integral membrane protein designated TetA<sup>1</sup> (1), which catalyzes antiport of a divalent cation-tetracycline complex and a proton (2, 3). The class B TetA protein (TetA(B)) encoded by transposon Tn10 is composed of 401 amino acids (4, 5) and believed to have 12 membrane-spanning segments (6–8). Its structural and functional features are distinctly those of secondary transporters widely spread in many organisms (9, 10). Therefore, this protein is a good model system for studying the molecular mechanism of membrane transport.

Several highly homologous amino acid sequence motifs are conserved in equivalent positions of proton-coupled symporters, antiporters, and uniporters (9, 10), suggesting the common role(s) of these motifs in transport phenomena. Three motifs, GXXDRXGXR in loop 2–3, DXXXXX in loop 4–5, and PESPR in loop 6–7, are also found in the cytoplasmic face of the N-terminal half of TetA(B). We have already focused on the loop 2–3 motif itself (11) and on several charged residues in these motifs (12–15), and we found Asp<sup>66</sup> and Arg<sup>70</sup> in loop 2–3, Asp<sup>120</sup> and Arg<sup>127</sup> in loop 4–5, and Glu<sup>181</sup> in loop 6–7 to be residues essential or important for function, which was based on the loss of transport activity and altered kinetic constants. However, we have not yet identified the precise role(s) of these residues, except that the negative charge of Asp<sup>66</sup> may first interact with a monocationic substrate, tetracycline-divalent cation complex (16).

Possible salt bridges in the transmembrane region were reported on Escherichia coli lactose permease (17–21) and a rat vesicular monoamine transporter (VMAT2) (22) and seem to be important for transport activity and substrate recognition. However, such salt bridges in the transmembrane region are not always found in all transporters, because two charged amino acid residues participating in them are not conserved in most of the secondary active transporters. Therefore, we focused on several charged residues (Asp<sup>66</sup>, Arg<sup>70</sup>, and Asp<sup>120</sup>) in the cytoplasmic loops of TetA(B) and analyzed the salt bridge formation. Interestingly, because these three residues are all conserved in the secondary transporters, the results should be applicable to all secondary transporters belonging to the large family of proton-coupled symporters, antiporters, and uniporters.

EXPERIMENTAL PROCEDURES

Materials—[7-<sup>3H</sup>]Tetracycline was purchased from NEN Life Science Products. Restriction and modifying enzymes were obtained from Takara, Toyobo, and New England Biolabs. o-Phenanthroline was purchased from Sigma. All other chemicals were of reagent grade and were obtained from commercial sources.

Bacterial Strains—E. coli W3104 (23) was used for the preparation of inverted membrane vesicles and measurement of tetracycline resist-

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A Charge Pair in the Tetracycline Transporter

**Results**

The Effect of Mutation of Asp<sup>66</sup> and Asp<sup>120</sup> on the Arg<sup>70</sup> Mutant—Asp<sup>66</sup> is located in the same loop region as Arg<sup>70</sup>, and Asp<sup>120</sup> is located at the adjacent cytoplasmic loop in the putative secondary structure of TetA(B) (Fig. 1). They are the only negatively charged residues in each loop. The site-directed mutagenesis of these residues was performed previously (12, 13), and in this study, additional mutations were constructed as described under “Experimental Procedures.” The resulting mutants were subcloned into a low copy number plasmid, pLGT2, and expressed in *E. coli* W3104 cells harboring the Asp<sup>66</sup>-Arg<sup>70</sup>-Asp<sup>120</sup> mutant plasmid. No plasmid was recovered in either the charge-neutralized (D66N/D120N) and charge-exchanged (D120N/D66R) mutants. The R70A mutant showed almost no transport activity, whereas the D120N mutant retained about two-thirds of the wild-type activity; Fig. 2B), which was less than that of the D120N single mutant under these conditions. The R70A/D120N double mutant showed almost no drug resistance, whereas the D120N/D120R double mutant showed significant transport activity (about 25% of the wild-type activity; Fig. 2B). The positive charge at position 70 is not essential for the transport function when Asp<sup>120</sup> was replaced with a neutral residue. The Arg<sup>70</sup> mutant showed no drug resistance but almost no tetracycline transport, whereas the R70D mutant showed absolutely no drug resistance (Table I). When Asp<sup>120</sup> of the R70A or R70D mutant was replaced by site-directed mutagenesis, the resulting charge-neutralized (R70A/D120N) and charge-exchanged (R70D/D120R) mutants clearly recovered drug resistance in comparison with either the Arg<sup>70</sup> or Asp<sup>120</sup> single mutant (Table I). The resistance of the charge-exchanged mutant (50 μg/ml) is higher than that of the charge-neutralized mutant (38 μg/ml).

**Tetracycline Transport Activities of the Arg<sup>70</sup> and Asp<sup>120</sup> Single or Double Mutants**—Tetracycline transport activities were measured in the inverted membrane vesicles prepared from *E. coli* W3104 cells expressing mutant tetA(B) genes. Under normal assay conditions (50 mM MOPS-KOH, pH 7.0, in the presence of 0.1 mM KCl, 50 μM Co<sup>2+</sup>Cl<sub>n</sub> and 10 μM [H]<sup>3</sup>tetracycline), the R70A mutant showed almost no transport activity, whereas the D120N mutant retained about two-thirds of the wild-type activity (Fig. 2A). Surprisingly, the R70A/D120N double mutant showed significant transport activity (about 50% of the wild-type activity; Fig. 2A), indicating that the positive charge at position 70 is not essential for the transport function when Asp<sup>120</sup> was replaced with a neutral residue. The transport activity of the double mutant was slightly less than that of the D120N single mutant under these conditions.

When Arg<sup>70</sup> was replaced with Asp, the transport activity was completely lost (Fig. 2B). On the other hand, the D120R mutant had significant transport activity (about 25% of the wild-type activity; Fig. 2B), which was less than that of the...
D120N single mutant. Surprisingly, the charge-exchanged R70D/D120R mutant showed high tetracycline transport activity (up to more than 70% of the wild-type activity; Fig. 2C), whereas the Asp120 single mutant showed very low transport activity (Fig. 2D). The tetracycline uptakes for NADH-energized vesicles are shown. C, the non-energized control.

**Kinetics of the Tetracycline Transport Mediated by the Arg70 and Asp120 Single or Double Mutants**—Fig. 2, C and D, shows the tetracycline transport by everted membrane vesicles under the conditions in which TetA(B) was almost saturated by the tetracycline-Co⁴⁺ chelation complex. Under these conditions, the R70A mutant showed very low transport activity (Fig. 2C), whereas the R70D mutant had absolutely no activity (Fig. 2D). The relative transport activity of the D120N and D120R mutants (Fig. 2C and D) compared with the wild-type, was significantly lower than that under normal conditions (Fig. 2, A and B). Both the charge-neutralized and charge-exchanged mutants showed clearly higher activity (Fig. 2C and D) than either the Arg70 or Asp120 single mutant.

The signs in parentheses indicate a positive charge (+), negative charge (−), or no charge (0) at positions 70 and 120, respectively. Thus, it seems preferable for tetracycline transport function that the sum of the charges at positions 70 and 120 be equal to 0. Net negative or positive charge(s) in this pair hinders the transport function.

On the other hand, **[Km](#)** values reveal a perspective different from **[Vmax](#)** values as to the relationship between the side chains at positions 70 and 120. As shown in Table II, all of the Arg70 single mutants and Arg70/Asp120 double mutants showed **[Km](#)** values similar to those of the wild-type; however, only the Asp120 single mutants showed significantly reduced **[Km](#)** values (values were reduced by a factor of about 4). The reduction of **[Km](#)** values in the D120N and D120R mutants is probably due to the apparent reduction of the dissociation constant of the substrate from the carrier caused by the decrease in the substrate translocation. If this is the case, why is the **[Km](#)** value of the R70A mutant not reduced, despite its very low translocation rate (**[Vmax](#)** value)? One reasonable answer to this question may be that the reduction of the substrate binding affinity that occurred in the R70A mutant covers the reduction of the apparent dissociation constant, resulting in the unaltered **[Km](#)** value.

**The Effect of SH Reagents and Mercuric Ions on the Cys Mutants at Positions 70 and 120**—Single or double Cys mutants at positions 70 and 120 were constructed by site-directed mutagenesis in order to investigate the effect of site-specific chemical modification at these positions. Fig. 3 shows the N-ethylmaleimide (NEM) concentration dependence of tetracycline transport activity mediated by the inverted membrane vesicles containing mutant TetA(B) proteins. In our previous study (12), we reported that the transport activity of wild-type TetA(B) was not affected by NEM at all. The R70C single mutant and the R70C/D120C double mutant were almost completely inactivated by NEM, whereas the D120C mutant was only partially inactivated (about 40% under the saturated NEM concentration). As described in our previous study (30), the chemical modification of the water-exposed Cys residue is saturated at about 2 mM NEM. Therefore, it seems that the partial inactivation of the D120C mutant is not due to the low reactivity of the Cys residue at position 120. The results indicate that each molecule of the ethylmaleimidyli-D120C mutant retains 60% of the activity before modification. A similar result was obtained when the S65C mutant was inactivated by methyl methanethiosulfonate (12). The incomplete inactivation indicates that the inactivation is due to steric hindrance but not to the masking of the functional group.

When Arg70 was replaced with Cys, the resulting mutant retained low but significant activity, probably due to the fact that the weak mercaptide formation between Cys70 and Co⁴⁺
proximity, Hg$^{2+}$ significantly stimulated the transport activity of the R70C mutant by a factor of about 2.5 because Hg$^{2+}$ forms stable mercaptide with Cys$^{70}$, which confers a positive charge that is fully functional during the transport process (14). On the other hand, the D120C mutant is partially inhibited by Hg$^{2+}$ by a factor of about 2 (Fig. 4B). As a result, the R70C and D120C single mutants showed similar levels of transport activity. The transport activity level of the D120C mutant in the presence of Hg$^{2+}$ was also similar to that of the D120R mutant (Fig. 2B).

On the contrary, the R70C/D120C double mutant was almost completely inactivated by Hg$^{2+}$ (Fig. 4C). The effect of Hg$^{2+}$ on the double Cys mutant was not due to the sum of the effects on each single mutant. If Cys$^{70}$ and Cys$^{120}$ are located in close proximity, Hg$^{2+}$ is expected to bridge these two Cys residues. The complete inactivation of the R70C/D120C double mutant by Hg$^{2+}$ is probably due to the mercaptide linkage between the side chains of positions 70 and 120.

The Formation of Disulfide Linkage between Cys$^{70}$ and Cys$^{120}$—To examine whether or not the distance between the side chains of Cys$^{70}$ and Cys$^{120}$ is small enough to form a mercaptide linkage, we investigated the formation of disulfide cross-linking between these two SH groups when the proteins were oxidized. The everted membrane vesicles containing the single or double Cys mutants at positions 70 and 120 were oxidized with Cu$^{2+}$/o-phenanthroline. The electrophoretic distance of the oxidized proteins was then analyzed by Western blotting (Fig. 5). Both the wild-type and single Cys mutants showed no alteration of the electrophoretic distance under all conditions examined. On the other hand, when the R70C/D120C mutant was oxidized, the electrophoretic distance was significantly elongated, indicating that the apparent molecular volume shrunk. When the SH groups of the double mutant were modified by NEM before oxidation, the distance was not changed by oxidation. In addition, when the oxidized proteins were reduced with β-mercaptoethanol, the electrophoretic distance returned to the normal value. These results indicate that the SH groups of Cys$^{70}$ and Cys$^{120}$ make a disulfide linkage by oxidation, resulting in the conformational change of the proteins.

**DISCUSSION**

In our previous study (14), we raised the possibility that the ionic interaction of Arg$^{70}$ with an acidic residue contributes to the switching of the opening/closing of the entrance transport path. Therefore, in this study, Asp$^{66}$ or Asp$^{120}$ was mutated as a candidate in combination with an Arg$^{70}$ mutation. Our results supported the observation that the positive charge of Arg$^{70}$ interacts with the negative charge of Asp$^{120}$, whereas the charge pairing between Arg$^{70}$ and Asp$^{120}$ is not essential for function. When both Arg$^{70}$ and Asp$^{120}$ were neutralized, and when the charges of Arg$^{70}$ and Asp$^{120}$ were exchanged, the tetracycline resistance level (Table I) and the $V_{\text{max}}$ value of the tetracycline transport (Table II) were much higher than those of the respective single mutants. It seems preferable that the net charge between positions 70 and 120 is equal to 0. One possibility is that the net negative charge between positions 70 and 120 prevents proper positioning of the transmembrane segments in the membrane. It was interesting that the tetracycline transport activity of the Cys$^{70}$/Cys$^{120}$ double mutant was almost completely inhibited by Hg$^{2+}$ (Fig. 4). This suggests that Hg$^{2+}$ bridges two SH groups from positions 70 and 120. Oxidative disulfide cross-linking experiments using Cu$^{2+}$/o-phenanthroline clearly indicated cross-linking between the SH groups of Cys$^{70}$ and Cys$^{120}$ (Fig. 5). Thus, it is concluded that Arg$^{70}$ is located close to Asp$^{120}$. It has been estimated that the average distance between the α-carbons of the cross-linked cysteine residues is 5–6 Å (31). However, in the normal (non-oxidized) state, Cys$^{70}$ and Cys$^{120}$ were not cross-linked because the effect of NEM on the tetracycline transport was additive (Fig. 3), and the electrophoretic mobility of the non-oxidized Cys$^{70}$/Cys$^{120}$ mutant protein was the same as that of the NEM-treated Cys$^{70}$/Cys$^{120}$ mutant protein (Fig. 5). Therefore, it is likely that the distance between Cys$^{70}$ and Cys$^{120}$ is greater than average. It is known that the maximum distance at which two SH groups can be cross-linked is 7 Å (31).

Both Arg$^{70}$ and Asp$^{120}$ of TetA(B) are well conserved in transporters of major facilitator superfamily (9, 10), a fact that is therefore suggestive of a common role in the mechanism of
transport and/or protein folding in the membrane. We previously concluded that the positive charge of Arg70 was important for TetA(B) activity because the Lys70 mutant retained the highest activity (11) and the Cys70 mutant was activated by Hg^{2+} (14). In a mutagenesis study of E. coli \( a \)-ketoglutarate permease, Seol and Shatkin (32) reported that Arg92, which corresponds to Arg70 of TetA(B), was necessary for activity. On the contrary, the corresponding Arg73 residue of lactose permease does not seem to be critical for activity (33, 34). However, the motif in loop 2–3 of lactose permease is slightly modified; that is, an extra Leu residue is inserted before the Arg73 residue. The difference between lactose permease and TetA(B) (and also \( a \)-ketoglutarate permease) may be due to the difference in the amino acid sequence of the motif. However, TetA(B) and lactose permease share the property that the loop 2–3 region of two transporters is important for conformational change of the protein during the transport cycle (33, 35–39).

Asp120 of TetA(B) was also important for activity because the kinetics of tetracycline transport was corrupted (Ref. 13 and this study). To date, the effects of mutation of the residue corresponding to Asp120 of TetA(B) were tested on lactose permease (40) and the myo-inositol/H\(^{+}\) transporter from Leishmania donovani (41). Both Glu126 of lactose permease and Glu121 of the myo-inositol/H\(^{+}\) transporter played a critical role in transport function, whereas Glu120 in lactose permease is thought to form a charge pair with Arg144. In any case, the steady-state level of substrate transport was drastically decreased.

We emphasize that the interaction between the positive charge of Arg70 and the negative charge of Asp120 may contribute to the conformational change of the protein and/or proper positioning of the transmembrane segments in the membrane. The latter idea is consistent with the conclusion of a recent study on Glu1 (42) that the conserved motif (RXGRR) is important for the correct topogenesis. This is thought to be the feature common in members of the major facilitator superfamily.

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