Proximity of Periplasmic Loops in the Metal-Tetracycline/H⁺ Antiporter of Escherichia coli Observed on Site-directed Chemical Cross-linking

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Our previous study on second-site suppressor mutations of the Tn10-encoded metal-tetracycline/H⁺ antiporter suggested that Leu30 and Ala354, located in periplasmic loop 1–2 and 11–12, respectively, are conformationally linked to each other (Kawabe, T., and Yamaguchi, A. (1999) FEBS Lett. 457, 169–173). To determine the spatial proximity of these two residues, cross-linking gel-shift assays of the L30C/A354C double mutants were performed after the mutant had been oxidized with Cu²⁺/2-phenanthroline. The results indicated that Leu30 and Ala354 are close to each other but that Glx45, which is located in cytoplasmic loop 2–3, and Ala354 are distant from each other, as a negative control. Then, a single Cys residue was introduced into each of the six periplasmic loop regions (P1–P6), and eleven double mutants were constructed. Of these eleven double Cys mutants, the L30C/A354C and L30C/T235C mutants showed a mobility shift on oxidation, indicating that P1 is spatially close to P4 as well as P6. In contrast, the other nine mutants, L30C/S92C, L30C/S156C, L30C/S296C, S92C/S296C, S92C/T235C, S92C/A354C, S156C/T235C, S156C/S296C, and S156C/A354C, showed no mobility shift under oxidized conditions on intramolecular cross-linking. The S92C and S296C mutants showed dimerization on intermolecular cross-linking, indicating that P2 and P5 are located at the periphery of the helix bundle.

The Tn10-encoded metal-tetracycline/H⁺ antiporter (TetA(B)) is a typical bacterial drug export protein (2, 3), and its molecular mechanism and molecular structure have been studied as a paradigm of antiporter-type drug exporters including bacterial multidrug exporters (4–6). TetA(B) belongs to a major facilitator superfamily (MFS) (7); however, the direction of the coupling of substrate transport with protons is opposite to that in the case of MFS symporters such as lactose permease.

The transmembrane helix arrangement in lactose permease has been extensively studied by use of double Cys mutants as an alternative means of three-dimensional structure prediction without x-ray crystallographic analysis (8–10). It is of interest to find out whether antiporters and symporters are the same with regard to the fundamental molecular construction or whether the difference in the coupling direction reflects the helix arrangement. The twelve-transmembrane structure of TetA(B) has been experimentally confirmed (11), and the exact range of each transmembrane segment was determined on the basis of site-directed chemical modification of cysteine-scanning mutants (12–15). To evaluate the mutual proximity of transmembrane helices of TetA(B), we introduced a Cys residue into each periplasmic loop on the basis of a Cys-free mutant.

To obtain double Cys combinations, we first looked for a pair for which close proximity was expected. In our previous paper (1), we reported that both G62L, which is in cytoplasmic loop 2–3, and G332S, which is in cytoplasmic loop 10–11, are suppressed by the second-site mutations of both L30S and A354D, which are located in periplasmic loop 1–2 and loop 11–12, respectively. G62L and G332S mutations both have a similar conformational effect on positions 30 and 354, resulting in the embedding of these residues in the hydrophobic interior of the membrane. Second-site mutations at Leu30 and Ala354 prevent the remote conformational distortion caused by the G62L and G332S mutations (1, 16). These results indicate the possibility that positions 30 and 354 are spatially close to each other. Thus, the L30C/A354C double mutant was prepared and the cross-linking was examined, with the G62C/A354C double mutant as a negative control. As a result, we could detect intramolecular cross-linking as an SDS-PAGE mobility shift of the double mutant after oxidation; then we introduced a Cys residue into each of the six periplasmic loop regions and constructed double Cys mutants. The results of the cross-linking assay indicate that the two ends, P1 and P6, are close to each other and that one of the central loops, P4, is close to P1. Such a periplasmic loop arrangement is similar to that of lac permease (17), indicating that the MFS transporters may have a general three-dimensional structure despite the difference in the proton-coupling direction.

EXPERIMENTAL PROCEDURES

Single Cys Mutants—Single Cys mutants were constructed by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel (18). For the mutagenesis, plasmid p7TC77A (12) was used as a template, where the Cys377–Ala mutation was introduced into pCTI183 (19), which carries the 2.45-kilobase pair Tn10 tetA and tetR gene fragments. Mutations were detected as the appearance of a newly introduced restriction site and verified by DNA sequencing. To avoid unexpected additional mutations, a cassette of the mutated fragment

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after sequencing was exchanged with the corresponding DNA fragment of the wild-type plasmid. Low copy number mutant plasmids were constructed through exchange of the BglII-BamHI fragment of the cysteine-scanning mutant tetA gene with the corresponding fragment of the low copy number plasmid pLGT2 (20) and used for the construction of double Cys mutants.

**Double Cys Mutants**—Double Cys mutants were constructed by recombination of a DNA fragment of one single Cys mutant gene with the corresponding fragment of the other single Cys mutant gene. L30C-containing double Cys mutants were constructed by exchanging the BglII-EcoRV fragment of the L30C DNA with the corresponding fragment of the low copy number plasmid pLGT2 (20) and used for the construction of double Cys mutants.

**Disulfide Cross-linking**—Crude membranes containing double Cys mutants were prepared by brief sonication of Escherichia coli W3104 cells carrying a mutant plasmid. Disulfide cross-linking was carried out by incubation of the membrane suspension (1.6 mg of protein/ml) in 180 μM Cu²⁺/o-phenanthroline (CuPh) and 50 mM potassium phosphate buffer (pH 7.5) at 25 °C for 30 min. The reaction was terminated by the addition of EDTA (final concentration, 5.7 mM). The samples were mixed with β-mercaptoethanol-free SDS sample buffer, followed by SDS-polyacrylamide gel electrophoresis. The TetA(B) bands were visualized by Western blotting using anti-TetA(B) C-terminal antiserum (21). As a control, the sulfhydryl groups of the double Cys mutants were masked by preincubation with 33 mM N-ethylmaleimide (NEM) at 30 °C for 10 min prior to the cross-linking reaction. When the effect of

**TABLE I**

Tetracycline resistance levels of E. coli W3104 cells harboring a plasmid encoding double-Cys mutants

| Mutant       | MIC \( \mu g/ml \) |
|--------------|---------------------|
| no plasmid   | 1.6                 |
| pLGT2        | 200                 |
| C377A        | 200                 |
| L30C/S92C    | 150                 |
| L30C/S156C   | 100                 |
| L30C/T235C   | 100                 |
| L30C/S296C   | 150                 |
| S92C/T235C   | 100                 |
| S92C/S296C   | 150                 |
| S92C/A354C   | 150                 |
| S156C/T235C  | 25                  |
| S156C/S296C  | 50                  |
| S156C/A354C  | 50                  |
| G62C/A354C   | 6.3                 |

**FIG. 1.** Model of the membrane topology of TetA(B). Putative transmembrane helices are enclosed in boxes. This structure is predicted on the basis of the results of site-directed competitive chemical modification (11). The residues subjected to cysteine-introducing mutagenesis in this study are depicted as bold letters.

**FIG. 2.** Cu²⁺/o-phenanthroline-catalyzed mobility shift assay by SDS-PAGE. Sonicated membranes containing a Cys-free, single Cys, or double Cys mutant were oxidized with 180 μM CuPh at 25 °C for 30 min; then the membranes were subjected to SDS-PAGE. TetA(B) bands were visualized by Western blotting using anti-TetA(B) C-terminal antibodies. A, mobility shift assays of the Cys-free, L30C, A354C, and L30C/A354C mutants. The Cys-free (lane 1), L30C (lane 2), and A354C (lane 3) mutants were oxidized with CuPh. The L30C/A354C double Cys mutant was not oxidized (lane 4) or was oxidized with CuPh (lane 5). In lane 6, the SH groups of the double Cys mutant were masked by NEM prior to oxidation. In lane 7, the oxidized double Cys mutant was reduced with β-mercaptoethanol. B, a negative control for cross-linking. The G62C/A354C double Cys mutant was oxidized with CuPh as in A (+), or its SH groups were masked prior to oxidation (−).
tetracycline was examined, the membranes were preincubated with 1 mM tetracycline and 50 mM MgSO₄ for 5 min at 30 °C prior to the oxidation.

**Cross-linking by Dimaleimide Derivatives**—α-Phenylenedimaleimide (PDM), m-PDM, p-PDM (Wako Pure Chemical Ind., Co., Ltd., Osaka, Japan), and 1,6-bis(maleimido)hexane (BMH) (Pierce, Illinois) were used as cross-linking reagents. A membrane suspension (1.6 mg of protein/ml) was incubated with 0.5 mM cross-linking reagent in 50 mM potassium phosphate buffer (pH 7.5) at 25 °C for 30 min. Reactions were terminated by the addition of β-mercaptoethanol-free SDS sample buffer. The cross-linking bands were detected as described above.

**RESULTS**

**Drug Resistance Levels of the Double Cys Mutants**—Table I shows the drug resistance of *E. coli* W3104 cells carrying the plasmid encoding the double Cys mutant *tetA(B) gene. All of the double Cys mutants showed significant tetracycline resistance except for G62C/A354C, indicating that these mutants fundamentally maintained the normal conformation.

**Intramolecular Cross-linking of the L30C/A354C Double Cys Mutant**—Leu³⁰ and Ala³⁵⁴ are located in putative periplasmic loops P1 and P6 (Fig. 1). The topology shown in Fig. 1 was partially revised from a previous figure (11) on the basis of our recent results relating to the chemical modification of cysteine-scanning mutants around transmembrane segment 1 (TM1).² In the current topology, Leu³⁰ is at the middle of P1 whereas Ala³⁵⁴ is near the boundary between TM11 and P6. The L30C and A354C single mutants and the L30C/A354C double mutant showed the same mobility (34 kDa) as the Cys-free TetA(B) during SDS-PAGE (Fig. 2A); however, after oxidation of the membranes with Cu²⁺/o-phenanthroline, only the L30C/A354C double Cys mutant gave a new band (30 kDa) exhibiting significantly increased mobility in the gel (Fig. 2A) in addition to the non-oxidized band. The 30-kDa band was not observed when the sulfhydryl groups were masked by preincubation of the membranes with NEM (Fig. 2A). In addition, when oxidized L30C/A354C membranes were reduced with β-mercaptoethanol, the 30-kDa band was not observed (Fig. 2A). The 30:34-kDa band ratio depended on the temperature and the period of oxidation; however, the 34-kDa band remained under all con-

² S. Iwaki, T. Someya-Kimura, N. Tamura, and A. Yamaguchi, unpublished results.
This finding also supported the Cys296-Cys296 intermolecular cross-linking. The S156C single Cys mutant did not produce a 64-kDa band. The L30C/S156C double Cys mutant gave an oxidation-dependent 64-kDa band that drastically decreased with masking of SH groups by NEM (Fig. 4A). The S156C single Cys mutant did not produce a 64-kDa band. This finding also supported the Cys296-Cys296 intermolecular cross-linking. The S156C/T235C mutant gave an unidentified and oxidation-independent 71-kDa band similar to the L30C/S156C mutant.

As a negative control, the G62C/A354C double Cys mutant was oxidized under the same conditions as the L30C/A354C double Cys mutant. In our previous study, it was confirmed that Gly62 is located on the cytoplasmic surface of the membrane (16). As expected, the G62C/A354C double Cys mutant showed no mobility shift under oxidizing conditions (Fig. 2B).

Cross-linking of Double Cys Mutants Containing the L30C Mutation—Combinations of the L30C mutation and a Cys mutation in each periplasmic loop region were constructed. Of the four double Cys mutants, only L30C/T235C produced a 30-kDa band like the L30C/A354C mutant. Masking by NEM and reduction with β-mercaptoethanol completely prevented this mobility shift (Fig. 3). Thus, it is concluded that positions 30 (P1) and 354 (P6) are spatially close to each other.

On the other hand, the L30C/A354C double Cys mutant observed under oxidizing conditions (Fig. 2B). The double Cys mutants containing S92C combined with L30C (Fig. 3), T235C, S296C, and A354C (Fig. 4) all did not produce a 30-kDa band under oxidizing conditions. On the other hand, all of these double Cys mutants produced an oxidation-dependent 64-kDa band, confirming the Cys92-Cys92 intermolecular cross-linking. The double Cys mutants containing S92C combined with L30C, T235C, S296C, and A354C (Fig. 4) all did not produce a 30-kDa band under oxidizing conditions. The L30C/S296C double Cys mutant produced 98 and 118-kDa bands corresponding to a trimer and a tetramer, respectively, in addition to the 64-kDa band, indicating that both Cys92 and Cys296 in this mutant can undergo intermolecular cross-linking and confirming the location of these positions at the periphery of the helix bundle.

As to the S92C/S296C double Cys mutant, several cross-linking reagents for SH groups were tested. As shown in Fig. 4C, all of the cross-linking reagents tested here caused the formation of dimers, trimers, and tetramers; however, the degree of the multimer formation was in the following order: P-PDM > BMH > m-PDM > o-PDM. NEM pretreatment prevented the multimer formation with cross-linking reagents. These results supported the hypothesis that positions 92 and 296 are located at the periphery of the periplasmic helix bundle of TetA(B).

Effect of Tetracycline on Cross-linking—In our previous study, we found that conformational changes in the cytoplasmic and periplasmic loop regions were induced in the presence of tetraacycline.
of tetracycline (22). Thus, the effect of tetracycline on the cross-linking was examined. Among the eleven periplasmic double Cys mutants, only the cross-linking of the L30C/T235C mutant was significantly prevented in the presence of tetracycline (Fig. 5), suggesting that the distance between P1 and P4 and/or the orientation of the side chains could be changed by tetracycline.

**DISCUSSION**

In this study, we showed that intramolecular disulfide cross-linking could be detected as a mobility shift during SDS-PAGE of double Cys mutants under oxidizing conditions. On the basis of this mobility shift, we investigated the proximity of the periplasmic loop regions. As a result, it was revealed that P1 was close to P6 and P4. The P2 and P5 loops seem to be located on the outside surface of the helix bundle. These results indicate that in the periplasm, the terminal loops are proximal to each other in addition to one of the central loops, and the second two loops from the terminal are at the periphery of the helix bundle. Tetracycline caused alteration of the distance between and/or orientation of P1 and P4 but did not affect the distance between P1 and P6. Our site-directed chemical modification studies on the cysteine-scanning mutants of TetA(B)\(^3\) exhibited that helices 3, 6, 9 and 12 are completely embedded in the hydrophobic interior of the membrane whereas helices 2, 5, 8, and 11 face a water-filled channel throughout their length. On the other hand, helices 1 and 7 face the channel only at their periplasmic halves whereas helices 4 and 10 face the channel at their cytoplasmic halves; that is, these four helices tilt with respect to the channel. On the basis of the results of site-directed chemical modification studies and the current results of cross-linking studies, we presented a model of the helix arrangement of TetA(B) on the periplasmic surface of the membrane as shown in Fig. 6.

In this study, we examined the intramolecular cross-linking on the basis of a mobility shift during SDS-PAGE caused by a cross-linking-induced conformational change of TetA(B). This method is remarkable because it can be performed with intact membrane proteins retaining full transport activity; however, when the cross-linking-induced conformational change is small, the cross-linking may be missed in the gel shift assay. Split proteins (9) or protease digestion of protease-site-introduced proteins (17) has been generally used to confirm intramolecular cross-linking. Unfortunately, because split TetA(B) proteins exhibit reduced transport activity (23) and the efficiency of the membrane assembly by the Cys mutants of the split TetA(B) was very low, we could not use split TetA(B) proteins. Similarly, protease-introduced TetA(B) proteins could not be used in this experiment. Therefore, further investigation is required to determine whether the Cys residues in the double Cys mutants showing no mobility shift during oxidation are really distant from each other. However, the proximity of the Cys residues in the double Cys mutants showing a mobility shift is beyond doubt.

Cross-linking studies on membrane transporters have been extensively performed by Kaback et al. (8), with lactose permease, and were revealed to be very useful for estimating the helix arrangements of polytopic membrane proteins. For periplasmic loops of lactose permease, Sun and Kaback (17) reported that P1 is close to P4 and P6, and P2 and P5 are at the periphery. The cross-linking between P1 and P4 was affected by the ligand-induced conformational change (24). The results of the cross-linking studies on the periplasmic loops are strikingly similar to the current results for TetA(B). As for the transmembrane segments of lactose permease, TM1 is close to TM5, 7, and 11 (10, 25). TM5 is also close to TM7 and 8 at the periplasmic side (27). If cross-linking can be formed across the water-filled channel, our model for helix packing of TetA(B) was perfectly consistent with the cross-linking results for lactose permease. Goswitz and Brooker (26) presented a general model for the transmembrane helix arrangement of MFS transporters. When considering the tilting of helices 1, 4, 7, and 10, our model is consistent with the Goswitz and Brooker model (26), including the order of each helix arrangement. Our current study strongly supports the idea that MFS transporters have a common three-dimensional structure.

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