We previously reported the crystal structure of the major multidrug exporter AcrB in *Escherichia coli* (Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002) *Nature* 419, 587–593). The extramembrane headpiece of the AcrB trimer contains a central pore composed of three α-helices. Each pore helix belongs to a different monomer. In this study, we constructed cysteine-scanning mutants as to the residues comprising the pore helix. Of the 21 mutants (D99C to P119C), 5 significantly reduced drug resistance and drug-exporting activity. These residues are localized on one side of the pore helix, i.e. on the wall of the pore. These observations strongly indicate the important role of this pore in the drug transport process. A N-ethylmaleimide binding experiment revealed that the pore is in the closed state, and the thickness of the permeability barrier in the middle of the pore corresponds to 2.5 α-helical turns. Two mutants (V105C and Q112C), which showed the greatest loss of activity of all of the pore mutants, were detected in the form of disulfide cross-linking dimers under normal conditions, suggesting that a conformational change of the pore is indispensable during the transport process.

AcrB is an inner membrane transporter that cooperates with a membrane fusion protein, AcrA, and an outer membrane channel, TolC (1, 2), and exports a wide variety of drugs directly out of cells, bypassing the periplasm (3). The AcrAB-ToLC system exports drugs not only from the cytoplasm but also from the periplasm (4). Homologues of the AcrAB-ToLC system are widely distributed in Gram-negative organisms and confer intrinsic drug resistance (5, 6). AcrB belongs to a resistance-nodulation division family (7), the members of which are composed of 12 transmembrane segments and 2 large periplasmic loops (8). The substrate specificity is determined by the periplasmic large loops of AcrB (9, 10) and its homologues (11, 12).

We determined the crystal structure of AcrB (1), which is not only the first structure of a multidrug exporter but also the first structure of a proton-coupled solute transporter elucidated. AcrB comprises a homotrimer, each monomer being composed of a 40-Å-thick transmembrane region and a large headpiece protruding 70 Å into the periplasm. At the center of the headpiece, there is a central pore composed of three α-helices. The distal end of the pore connects with a funnel-like opening at the top of the headpiece, and the proximal end connects to the central cavity located between the headpiece and the transmembrane region. The central cavity opens into the periplasm via three vestibules between the monomers. Substrates in the outer leaflet of the plasma membrane can enter the cavity via these vestibules and might be actively transported through the central pore into outer membrane channel TolC (1). This is the mechanistic basis of the periplasmic active transport by the AcrAB-ToLC system. It has been reported that multiple substrates bind to the central cavity (13), supporting this presumed mechanism.

The central pore seems to be closed in the resting condition, because there is not enough space for substrate molecules to pass through (1). Therefore, the pore is presumed to play an important role in the drug export function of AcrB not only as a substrate translocation pathway but also as a valve for active transport. To investigate such important roles of the central pore, we constructed and analyzed cysteine-scanning mutants as the pore-forming helices in this study.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—** *Escherichia coli* strains TG1 (14), JM109 (15), CJ236 (16), and W3104 (17) were used for DNA manipulation, DNA sequencing, site-directed mutagenesis with the Kunkel method (18), and investigation of the phenotypes of the mutants, respectively. Strains KAM3 (19) and W3104ΔacrAB (20) are *acrB* (KAM3) and *acrAB* (W3104ΔacrAB) gene-deletion derivatives of *E. coli* TG1 and W3104, respectively. KAM3 was kindly provided by Tomofusa Tsuchiya and Yuji Morita (Okayama University, Okayama, Japan). Plasmids pACBH, a derivative of pUC118 that carries the *acrR*, *acrA*, and His-tagged *acrB* genes, and pACBHCL are derivatives of pACBH that contains His-tagged Cys-less *acrB* in the wild-type His-tagged *acrB* gene, were prepared previously (8). For use as templates for site-directed mutagenesis, the DNA fragments encoding the N- and C-terminal halves of Cys-less AcrB were separately subcloned into pUC118 previously, i.e. pACBN and pACBHCL carry the N- and C-terminal halves, respectively (6).

**Site-directed Mutagenesis—** To facilitate DNA recombination, five unique restriction enzyme sites were introduced into the *acrB* gene by silent site-directed mutagenesis. The resulting plasmid, pACBHLLR, encoding the cassetted *acrB* gene is a derivative of pACBHCL containing five new restriction sites: XbaI at position 167, BamHI at position 257, NotI at position 303, AarII at position 491; and Smal at position 833. All of these restriction sites were introduced by silent mutagenesis; thus, the amino acid sequence of AcrB was not changed. The cassetted...
versions of the plasmids encoding the N- and C-terminal halves of AcrB were also subcloned from pACBHLR and named pACBRN and pACBHLRC, respectively. The mutagenesis for constructing 21 Cys mutants (i.e. D99C, A100C, D101C, I102C, A103C, Q104C, V105C, Q106C, V107C, Q108C, N109C, K110C, L111C, Q112C, L113C, A114C, M115C, P116C, L117C, L118C, and P119C) was performed with mutagenic oligopeptides containing mismatches for the desired amino acid replacements and silent mismatches producing new restriction enzyme sites by the method of Kunkel et al. (18). Mutations were first detected by restriction enzyme analysis and then verified by DNA sequencing. After verification of the mutations, adequate restriction fragments of pACBRN were introduced into pACBHLR by means of corresponding fragment exchange.

Detection of Mutant AcrB Expression—E. coli W3104acrAB was transformed with the mutant plasmids, and then the expression of the mutant AcrB protein was detected by Western blotting with an anti-AcrB rabbit antibody, which was prepared by Medical Biological Laboratories Co., Ltd (Nagoya, Japan) using purified AcrB protein as an antigen.

Drug Resistance Determination—The minimum inhibitory concentrations of drugs and toxic compounds were determined as the concentrations that greatly prevented bacterial growth on YT-agar (0.8% tryptone, 0.5% yeast extract, and 0.5% NaCl) plates with sequential 2-fold dilutions as described previously (21).

DASPEI (2-(4-dimethylamino)styryl-N-ethylpyridinium iodide) Transport Assay—E. coli KAM3 cells were transformed with the plasmids encoding the wild-type and mutant AcrB. Cells were harvested from 2-ml cell culture (A_{610} = 0.6) and suspended in 2 ml of 50 mM phosphate buffer, pH 7.5, containing 20 μM DASPEI (22) and 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP). To prelabel the cells with DASPEI, the mixture was incubated at 37 °C for 5 min followed by the collection of the cells by brief centrifugation and resuspension in the same buffer without CCCP. While monitoring the fluorescence (excitation, 461 nm; emission, 560 nm) of the cell suspension with a fluorescence spectrophotometer (PerkinElmer LS55), glucose (final concentration of 0.2%) was added to start DASPEI extrusion. At 4 min after glucose addition, CCCP was added to dissipate the energy.

Assaying of the Reaction of [14C]N-ethylmaleimide with AcrB Proteins—The [14C]NEM binding experiment was performed as follows. A membrane suspension (200 μg of protein) prepared by brief sonication of E. coli W3104acrAB cells carrying pACBHLR or one of its derivatives was incubated with 0.5 mM [14C]NEM in 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 mM KCl for 5 min at 30 °C. The membrane protein was solubilized with 1% Triton X-100 and 0.1% SDS in phosphate-buffered saline containing 5 mM unlabeled NEM, and then His-tagged AcrB proteins were precipitated with nickel-chelating-Sepharose beads (Amersham Biosciences). After washing the beads twice with 50 mM imidazole, the AcrB proteins were eluted with 500 mM imidazole.

Drug Resistance Determination—The minimum inhibitory concentrations of drugs and toxic compounds were determined as the concentrations that greatly prevented bacterial growth on YT-agar (0.8% tryptone, 0.5% yeast extract, and 0.5% NaCl) plates with sequential 2-fold dilutions as described previously (21).

RESULTS

Construction of Cysteine-scanning Mutants as to the Pore Helices and Expression of the Mutants—The central pore is composed of three α-helices (Fig. 1). Each helix belongs to a different monomer. The pore-forming helix is the second α-helix (Na2) in the N-terminal large loop between transmembrane helices 1 (TM1) and 2 (TM2) (1) that is composed of 21 amino acid residues Asp-99 to Pro-119 (Fig. 2). The amino acid sequence of this region is highly conserved among AcrB homologues (Fig. 2). In particular, in AcrB, AcrF, and MexB, 19 of the 21 residues are completely identical, whereas the sequences of AcrD and MexD contain three and five more charged residues, respectively, in this region than the former three homologues. This discrepancy might be related to the fact that the substrate specificity of the latter two homologues is rather different from that of the former three (21, 23, 24).

The 21 amino acid residues of the pore-forming α-helix were replaced with cysteine one by one by site-directed mutagenesis.

1 The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid; NEM, N-ethylmaleimide.
Drugs resistance of E. coli W3104 cells lacking acrAB genes harboring plasmids carrying a mutant acrB gene with the wild type acrA gene

Drugs resistance against 16 representative drugs and toxic compounds.

**Table I**

| Drug             | W3104 | W3104 ΔacrAB | W3104Δacr harboring plasmid carrying mutated acrB |
|------------------|--------|--------------|---------------------------------------------------|
|                   |       |              | pACBHLR | D101C | V105C | N109C | Q112C | P116C | Others |
|                   | µg/ml |              |         |       |       |       |       |       |        |
| Chloramphenicol   | 6.25  | 0.78         | 3.13    | 0.78  | 0.78  | 1.56  | 0.78  | 1.56  | 1.56-3.13 |
| Tetracycline      | 2.65  | 1.56         | 3.13    | 1.56  | 1.56  | 1.56  | 1.56  | 1.56  | 1.56-3.13 |
| Minocycline       | 25    | 6.25         | 12.5    | 12.5  | 6.25  | 12.5  | 6.25  | 12.5  | 12.5 |
| Erythromycin      | 100   | 6.25         | 100     | 25    | 25    | 25    | 25    | 50    | 50-100 |
| Nalidixic acid    | 1.33  | 0.78         | 1.33    | 0.78  | 0.78  | 0.78  | 0.39  | 0.78  | 0.78-1.56 |
| Norfloxacin       | 0.1   | 0.05         | 0.1     | 0.05  | 0.05  | 0.05  | 0.05  | 0.05  | 0.1    |
| Enoxacin          | 0.2   | 0.05         | 0.2     | 0.1   | 0.05  | 0.1   | 0.05  | 0.05  | 0.1-0.2 |
| Novobiocin        | >400  | 25           | >400    | 400   | 200   | 400   | 400   | >400  | >400 |
| Acriflavine       | 200   | 6.25         | 100     | 25    | 12.5  | 25    | 12.5  | 25    | 50-100 |
| Crystal violet    | 25    | 0.78         | 3.13    | 1.56  | 0.78  | 1.56  | 0.78  | 3.13  | 3.13 |
| Ethidium Bromide  | >400  | 12.5         | >400    | 400   | 100   | 400   | 50    | 400   | >400 |
| Rhodamine 6G      | 400   | 3.13         | 200     | 100   | 25    | 50    | 25    | 400   | 400 |
| TPP               | >400  | 12.5         | 400     | 400   | 50    | 200   | 25    | 400   | 400 |
| Benzalkonium      | 50    | 3.13         | 25      | 12.5  | 6.25  | 12.5  | 3.13  | 12.5  | 12.5-25 |
| SDS               | >400  | 50           | >400    | 400   | 100   | 400   | 100   | >400  | >400 |
| Deoxycholate      | >40000| >40,000      | >40,000 | 2500  | 40000 | 20,000| 20,000| >40,000 | >40,000 |

**Fig. 3.** Western blotting of AcrB proteins from E. coli W3104ΔacrAB cells carrying plasmids encoding Cys mutant AcrB proteins. Cells were harvested at A600 = 0.9, and membrane fractions were prepared by brief sonication and ultracentrifugation. Aliquots of the membrane fractions (5 µg of total protein) were subjected to SDS-polyacrylamide electrophoresis. AcrB proteins were detected by Western blotting using anti-AcrB rabbit antibodies and alkaline phosphatase-labeled anti-rabbit IgG goat antibodies as the first and second antibodies, respectively. Lane 1, W3104; lane 2, W3104ΔacrAB; lane 3, pACBHLR (wild); lane 4, D99C; lane 5, A100C; lane 6, D101C; lane 7, N109C; lane 8, A103C; lane 9, Q104C; lane 10, V105C; lane 11, Q106C; lane 12, V107C; lane 13, Q108C; lane 14, N109C; lane 15, K110C; lane 16, L111C; lane 17, Q112C; lane 18, L113C; lane 19, A114C; lane 20, M115C; lane 21, P116C; lane 22, L117C; lane 23, L118C; lane 24, P119C; and lane 25, purified AcrB (wild).

**Fig. 4.** Helical wheel projection of the residues in the pore-forming helix. White letters on a black background indicate the functionally important residues.

as described under “Experimental Procedures.” The expression of these Cys mutants was detected by Western blotting with anti-AcrB antibodies (Fig. 3). All of the mutants were well expressed, and no significant differences in the expression level were observed.

**Drug Resistance Patterns of Cys-scanning Mutants**—The drug resistance against 16 representative drugs and toxic compounds of E. coli W3104ΔacrAB cells was measured as described under “Experimental Procedures” (Table I). In comparison with wild-type W3104 cells, the acrAB gene-deletion strain showed hypersensitivity to all of these 16 compounds by a factor of 4–128. Multicopy plasmid pACBHLR encoding wild-type AcrAB restored the intrinsic drug resistance of E. coli with the exception of that to crystal violet, whereas the resistance level of the cells carrying the multicopy acrAB gene was not higher than that of the wild-type E. coli strain, indicating that the resistance level is not proportional to the gene dosage.

Most of the plasmids encoding Cys mutants restored the intrinsic resistance of E. coli W3104ΔacrAB to all of the compounds tested, similar to that encoding the wild-type AcrB, with the exception of five mutants (Table I). Of the 21 mutants, only 5 showed decreased resistance to several or all of the compounds tested in comparison with the wild type. In particular, the resistance levels of the cells expressing the V105C and Q112C mutants were decreased or lost for all of the compounds tested. In the case of the D101C, N109C, and P116C mutants, the drug resistance was partially lost, i.e. the resistance to some drugs was lost but that to the other drugs remained, indicating that the substrate specificity changed to become narrower than that of the wild type.

Among the functionally important residues, Asp-101 and Asn-109 are completely conserved in the five AcrB homologues listed in Fig. 2; however, Val-105, Gln-112, and Pro-116 are not conserved in MexD and/or AcrD. These three non-conserved residues may be related to substrate specificity. Interestingly, all of the functionally important residues are located on the same side of the pore-forming helix (Fig. 4).

**DASPEI Export Activity**—DASPEI is an amphiphilic compound that fluoresces in a hydrophobic environment (Fig. 5A)
When intact cells carrying the plasmid encoding the wild-type AcrB were incubated with DASPEI under starved conditions (in the presence of CCCP), DASPEI was accumulated in the plasma membrane and the cells fluoresced (22). After removal of CCCP, when glucose was added as an energy source to drive the AcrAB-TolC system, the fluorescence drastically decreased, whereas the addition of CCCP again rapidly restored the fluorescence (Fig. 5B). The rapid decrease in fluorescence of energized cells indicates the active extrusion of DASPEI from the plasma membrane due to the “membrane vacuum cleaner” function of the AcrAB-TolC system. The host KAM3 cells lacking AcrB did not show such a rapid fluorescence decrease. The small decrease in fluorescence of KAM3 cells might be the result of trace amounts of exporters other than AcrAB.

Fig. 5C shows the fluorescence trace of DASPEI-preloaded cells carrying mutant plasmids. The D99C mutant is an active control, which shows wild-type drug resistance and DASPEI extrusion activity. On the other hand, the V105C and Q112C mutants showed a very low decrease in fluorescence, which was almost the same as the background level in KAM3 cells without a plasmid, indicating that these mutants had lost the DASPEI export activity. The N109C, D101C, and P116C mutants showed an intermediate fluorescence decrease, the degree of the fluorescence decrease increasing in this order. The order of the DASPEI export activities of the N109C, D101C, and P116C mutants was consistent with the widths of the drug resistance spectra of these mutants (Table I).

**Accessibility of NEM to Cysteine Residues of the Cys-scanning Mutants**—The binding of [14C]NEM to the Cys-scanning mutants was measured as described under “Experimental Procedures.” As shown in Fig. 6, NEM bound well to the four N-terminal (cavity-side) mutants (D99C, A100C, D101C, and I102C) and the five C-terminal (funnel-side) mutants (Q112C, L113C, M115C, P116C, and L117C), whereas it hardly or only weakly bound to the middle nine mutants (A103C, Q104C, V105C, Q106C, V107C, Q108C, N109C, K110C, and L111C). Although position 114 is located on the funnel side, the A114C mutant did not bind to NEM at all. In addition, the two C-terminal-end mutants (L118C and P119C) did not bind to NEM (data not shown). These observations suggest that: 1) the N-terminal end of the pore helix projects into and is exposed to the central cavity; 2) the pore is closed and that the residues in the middle of the pore are occluded in the protein interior; and 3) the C-terminal side of the pore is open to the funnel. The crystal structure of AcrB (1) supports these conclusions. In addition, the side chain of Ala-114 protrudes opposite to the pore (1). The pore helix bends at the C-terminal end and is connected to short helix No2’ (Fig. 7B), which is inserted into the core of the protein. Leu-118 and Pro-119 are located between the pore helix and No2’. The side chains of these two residues also protrude into the protein core.
Competitive inhibition of $[^{14}C]$NEM binding with a membrane-impermeable sulfhydryl reagent, AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid), was also measured. As a result, NEM binding to all of the NEM-reactive mutants was found to be more or less inhibited by AMS pretreatment of intact cells (data not shown). Therefore, it is clear that AMS can gain access to the central cavity from the periplasm via vestibules as well as to the funnel.

Because the SDS electrophoresis shown in Fig. 6 was performed in the absence of $\beta$-mercaptoethanol, a significant amount of the disulfide cross-linking dimer was detected for the V105C and Q112C mutants. These residues face the pore interior, and in the trimer, they are close to the corresponding residues in the next monomer (Fig. 7). The greatest loss of transport activity of these two mutants among all of the pore mutants might be because of the conformational fixation by these S-S cross-links, suggesting the importance of a conformational change of the pore during the transport process. Besides, cross-linking was also observed in the D99C, D101C, I102C, and N109C mutants but the degree of linking was too low to affect the activity.

**DISCUSSION**

One of the striking facts revealed by determination of the crystal structure of the multidrug efflux transporter AcrB was the fact that active drug export seems to occur at the extramembrane pore (1). In the presumed substrate translocation pathway through the AcrB trimer, only the central pore is closed, whereas the pathways to the central cavity from the periplasm via vestibules and from the cytoplasm via trans-
membrane grooves are open for substrates (1). Because the proton motive force might be captured in the transmembrane region, probably at the Asp-Lys ion pair (1), the energy might be transmitted via a remote conformational change to open the extramembrane pore. This finding supports the important role of the extramembrane pore in the AcrB function.

Fig. 7 shows the side chains of the functionally important residues determined in this study on the three-dimensional structural model of AcrB around the extramembrane central pore. The side chains of the functionally important residues, Asp-101, Val-105, Asn-109, Gln-112, and Pro-116, are depicted on a ribbon representation of the AcrB structure. It is clear that all of the functionally important residues face the inside of the pore (Fig. 7A) and that all of the residues facing the inside of the pore are functionally important (Fig. 7B), indicating that the pore is a strong candidate for the substrate translocation pathway.

The results of the NEM binding experiment supported that the pore is closed. The thickness of the permeability barrier corresponds to 2.5 turns of a-helices (9 residues). The cysteine-scanning mutants also confirmed the importance of a conformational change of the pore during the transport process, that is, the mutants in which the pores are fixed in the closed state by disulfide cross linking showed an almost complete loss of the transport activity. The crystal structure of TolC, which is an outer membrane channel coupled with AcrB, is also closed (27), supporting that the pore domain including the central pore is closed. The thickness of the permeability barrier pathway.

The role of an extramembrane Pore of AcrB

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Extramembrane Central Pore of Multidrug Exporter AcrB in *Escherichia coli* Plays an Important Role in Drug Transport

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