Site-Directed Disulfide Cross-Linking Shows that Cleft Flexibility in the Periplasmic Domain Is Needed for the Multidrug Efflux Pump AcrB of *Escherichia coli*[∇]

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Escherichia coli AcrB is a multidrug efflux transporter that recognizes multiple toxic chemicals having diverse structures. Recent crystallographic studies of the asymmetric trimer of AcrB suggest that each protomer in the trimeric assembly goes through a cycle of conformational changes during drug export. However, biochemical evidence for these conformational changes has not been provided previously. In this study, we took advantage of the observation that the external large cleft in the periplasmic domain of AcrB appears to become closed in the crystal structure of one of the three protomers, and we carried out *in vivo* cross-linking between cysteine residues introduced by site-directed mutagenesis on both sides of the cleft, as well as at the interface between the periplasmic domains of the AcrB trimer. Double-cysteine mutants with mutations in the cleft or the interface were inactive. The possibility that this was due to the formation of disulfide bonds was suggested by the restoration of transport activity of the cleft mutants in a *dsbA* strain, which had diminished activity to form disulfide bonds in the periplasm. Furthermore, rapidly reacting, sulfhydryl-specific chemical cross-linkers, methanethiosulfonates, inactivated the AcrB transporter with double-cysteine residues in the cleft expressed in *dsbA* cells, and this inactivation could be observed within a few seconds after the addition of a cross-linker in real time by increased ethidium influx into the cells. These observations indicate that conformational changes, including the closure of the external cleft in the periplasmic domain, are required for drug transport by AcrB.

It is now well known that multidrug efflux transporters cause problems in cancer chemotherapy, as well as in antibiotic treatment of bacterial infections. These transporters recognize many structurally dissimilar toxic compounds and actively extrude them from the cell. The *Escherichia coli* AcrB transporter (14, 15), which belongs to the resistance-nodulation-division family (32), is responsible for most of the intrinsic drug resistance of this organism (20, 21, 28) and is also perhaps the best-studied bacterial multidrug pump. It is a homotrimer in which each subunit contains 12 transmembrane (TM) helices and two large periplasmic domains, one between TM helix 1 (TM1) and TM2 and one between TM7 and TM8. The trimeric AcrB molecule in turn occurs as a multiprotein complex (30, 31, 36) together with the outer membrane channel protein TolC (8, 12) and the periplasmic linker protein AcrA (14). The top of the periplasmic domain of AcrB is thought to interact with the internal end of TolC, and this structure allows direct export of drugs to the external medium (20).

AcrB utilizes the proton motive force as energy for its transport function (14, 34, 35). Charged residues within the TM helices of this transporter, including Asp407, Asp408, and Lys940, have been identified as amino acid residues essential for activity (19), presumably functioning as components of the proton relay system. We previously used site-directed mutants with mutations in these residues, as well as Thr978, which was also shown to be a likely partner in the proton relay network,

in an attempt to obtain an AcrB conformation mimicking the transient conformation in the middle of drug extrusion (29, 27). Although the structures of the TM domains of the mutants showed that the mutations disrupt the close interaction among the four residues mentioned above, the conformation of the periplasmic domain, through which the drug molecules must be transported, was rather similar to that of the wild-type protein, in contrast to our expectations (27).

The recent elucidation of the crystallographic structures of the asymmetric trimers of AcrB (18, 26) resulted in important progress in our understanding of the transport process. In the asymmetric trimer, each protomer of AcrB has a conformation different from that of its neighbors. Murakami et al. (18) further succeeded in solving the structure of a trimer containing a bound drug molecule in one of the protomers and thus proposed that each protomer corresponds to one of the three functional states of the transport cycle, namely, “access,” “binding,” and “extrusion.” The conformation of the proton-binding structure in the TM domain, involving the four amino acids mentioned above, was very similar in the extrusion protomer described by Murakami et al. (18) and our site-directed mutant proteins (27). However, the latter did not show the remarkable conformational alteration seen in the periplasmic domain of the extrusion protomer, presumably because this alteration requires cooperative conformational alterations of the neighboring protomers, which were not possible in our homotrimers, which contained the same mutant protomers at all three positions.

The model for the drug extrusion mechanism proposed by Murakami et al. (18) is thus very attractive. At present, however, it remains a model and proposal. We set out to test this model by taking advantage of the observation that the external

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Strains		
DH5 α	Standard host strain for cloning	24
RI90	MC1000 <i>phoR</i> Δ <i>ara714</i> <i>leu</i> ⁺ <i>dsbA1::kan</i>	23
AG100	K-12 <i>argE3</i> <i>thi-1</i> <i>rpsL</i> <i>xyl</i> <i>mlt</i> <i>supE44</i> Δ (<i>gal-uvrB</i>)	9
HNCE1a	AG100 <i>marR1</i> <i>acrB::kan</i> Δ <i>acrD</i>	7
AG100YB	AG100 Δ <i>acrB::Spc</i> ^r	This study
AG100YBD	AG100 Δ <i>acrB::Spc</i> ^r <i>dsbA1::kan</i>	This study
Plasmids		
pKD46	λ <i>red</i> recombinase (γ , β , <i>exo</i>) expression plasmid; <i>amp</i> , <i>ara</i> -inducible expression, temperature-sensitive replication	6
pGB2	Low-copy-number cloning vector; <i>Spc</i> ^r	5
pSPORT1	High-copy-number cloning and expression vector; <i>amp</i> , <i>lac</i> -inducible expression	Gibco BRL
pUCK151A	6.5-kb BglII fragment containing the entire <i>acrAB</i> operon cloned into pUC19 vector	14
pU Δ <i>acrB::Spc</i> ^r	3.0-kb BsrGI-NsiI fragment of pUCK151A replaced by 1.4-kb <i>Spc</i> ^r gene from pGB2	This study
pSacrB ^{His}	<i>acrB</i> gene with His ₄ tag sequence at the 3' end cloned into pSPORT1	29
pSCLBH	Derived from pSacrB ^{His} ; codons for two intrinsic cysteines (Cys493 and Cys887) of the <i>acrB</i> gene converted to codons for serines, producing cysteineless and His ₄ -tagged AcrB	This study

cleft in the periplasmic domain becomes much narrower in the extrusion protomer and also the observation that there are significant changes in the interface between the periplasmic domains of protomers. We introduced cysteine residues by site-directed mutagenesis and examined if disulfide bonds were generated between certain residues and if the formation of such disulfide bonds inhibited the function of AcrB, presumably by fixing the conformation of one of the protomers within the trimeric structure.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* DH5 α was used for construction and propagation of various plasmid constructs. Cells were grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 μ g/ml), kanamycin (35 μ g/ml), and/or spectinomycin (50 μ g/ml) when necessary.

Construction of plasmids. pSCLBH, which was used to express cysteineless and hexahistidine-tagged AcrB, was constructed from pSacrB^{His} (29); in this pSPORT1-based plasmid, the AcrB protein containing a hexahistidine C-terminal sequence (two intrinsic and four additional histidine residues) is expressed under the control of the *lac* promoter. The codons for two intrinsic cysteines (Cys493 and Cys887) of *acrB* in pSacrB^{His} were converted to codons for serines by site-directed mutagenesis as described previously (29), using primers C493SFw (5'-CTCCAGCTCTTCTGCCACCATGC-3'), C493SRv (5'-GCATGTGGCAGAAAGAGCTGGAG-3'), C887SFw (5'-GTCGTGTTCTGTCTGTGGCGCGC-3'), and C887SRv (5'-CGCCGCGAGAGACAGAACACGA C-3') (underlined sequences encode serine residues).

To construct pU Δ *acrB::Spc*^r, which was used to construct the Δ *acrB::Spc*^r strain, pUCK151A (14) was digested with BsrGI and NsiI and blunt ended by the Klenow fragment of DNA polymerase I (New England Biolabs). A 1.4-kb NciI fragment containing the spectinomycin resistance gene (*Spc*^r), *aadA*, from pGB2 (5) was end filled and inserted into the BsrGI-NsiI-restricted pUCK151A described above. Since the 3-kb BsrGI-NsiI fragment contained the *acrB* gene sequence from 231 bp downstream of the start codon to 46 bp downstream of the stop codon, in the resulting plasmid, pU Δ *acrB::Spc*^r, 90% of the *acrB* gene was deleted and replaced by the *aadA* gene.

Site-specific mutagenesis. Point mutations were introduced into plasmid pSCLBH by PCR as described previously (29). Sequences of all primers used are available upon request.

Construction of strains. An *E. coli* mutant with an *acrB* deletion, AG100YB (Table 1), was generated by a method involving the introduction of a linear homologous DNA fragment, often called "recombineering" (25), but the λ *red* genes expressed from the pKD46 plasmid of Datsenko and Wanner was used (6). In this manner, no extraneous FLP recognition target sequences were introduced into the genome of the resulting strain. AG100 cultures containing the heat-labile plasmid pKD46 (6) were grown at 30°C, induced with 1 mM L-arabinose to

express the *red* genes on the plasmid, and made competent for electroporation. A 5.0-kb portion of linear DNA containing Δ *acrB::Spc*^r and the flanking regions was amplified by PCR from pU Δ *acrB::Spc*^r, using primers K151AFw (5'-AGA TCTCACTGAACAAATCC-3') and K151ARv (5'-CTGGATTACGCCCCAC G-3'). Transformants were selected on LB agar containing spectinomycin (50 μ g/ml) at 37°C and screened for the expected recombination by PCR.

For construction of AG100YBD (Table 1), the *dsbA1::kan* mutation of strain RI90 was transduced into AG100YB, using P1cml, *chr100* phage according to the protocol described by Miller (16).

Drug susceptibility assays. *E. coli* HNCE1a (7), AG100YB, or AG100YBD cells harboring pSPORT1-derived plasmids were tested, without isopropyl- β -D-thiogalactopyranoside (IPTG) induction, for drug susceptibility by the gradient plate method as described previously (4, 29). A linear concentration gradient of cholic acid (Sigma) was prepared in square LB agar plates containing 8,000, 10,000, and 16,000 or 18,000 μ g/ml of cholic acid in the lower layer for HNCE1a, AG100YB, and AG100YBD, respectively. Mid-exponential-phase cultures were diluted to an optical density at 660 nm (OD₆₆₀) of 0.1 with LB broth supplemented with ampicillin (100 μ g/ml) and streaked as a line across the plate, parallel to the drug gradient. Bacterial growth across the plates, from low to high drug concentrations, was measured after 24 h at 37°C. All mutants were assayed at least four times, using strains harboring wild-type cysteineless pSCLBH and the vector pSPORT1 as controls. The relative activity of each mutated AcrB protein was calculated as described previously (29), and the activities of controls with full efflux activity (pSCLBH) and no efflux activity (pSPORT1) were defined as 100 and 0%, respectively.

Ethidium accumulation assay and effect of methanethiosulfonate (MTS) cross-linking reagents. AG100YBD cells harboring pSPORT1-derived plasmids were grown in LB medium with ampicillin without IPTG induction to an OD₆₆₀ of 0.7 to 0.9 at 37°C. Cells were then harvested at room temperature, washed once, and resuspended in buffer A (50 mM sodium phosphate buffer [pH 7.0] containing 100 mM NaCl and 0.1% [vol/vol] glycerol). The accumulation of ethidium by cells was monitored by determining the fluorescence of the cells with a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) at room temperature as described previously (13). The final concentration of ethidium bromide used was 5 μ M, and the concentration of bacterial cells corresponded to an OD₆₆₀ of 0.2. The excitation and emission wavelengths were 520 and 590 nm, respectively, with slit widths of 5 nm for excitation and 10 nm for emission.

To investigate the effect of MTS reagents on the ethidium accumulation, after 2 min of preincubation with ethidium bromide, 5 mM 1,2-ethanedithiol bismethanethiosulfonate (MTS-2-MTS) and 10 mM pentyl MTS (5-MTS) (Toronto Research Chemicals, Toronto, Ontario, Canada), freshly dissolved in dimethyl sulfoxide-ethyl acetate (3:1, vol/vol), were added to the cells to final concentrations of 20 and 40 μ M, respectively, and the accumulation of ethidium was followed. A similar cross-linking experiment was also carried out by using 1,5-pentanedithiol bismethanesulfonate (MTS-5-MTS) (Toronto Research Chemicals) or dibromobimane (Molecular Probes).

To examine the effect of substrates on MTS-2-MTS cross-linking, cells were

harvested at an OD_{660} of 0.7 to 0.9, washed once, and resuspended in buffer B (50 mM sodium phosphate [pH 7.0] containing 100 mM NaCl). Cells (1 ml; OD_{660} , 0.4) were incubated for 5 min with 2 to 20 μ M MTS-2-MTS with or without substrate (chloramphenicol) at final concentrations of 4.5 to 100 μ M in buffer B at room temperature. The cells were then rapidly pelleted, washed once with buffer B, and resuspended in 2 ml of buffer A for measurement of the ethidium accumulation rate.

Analysis of AcrB expression levels and disulfide bond formation in whole cells and in the inner membrane fraction. Exponential-phase cells grown in LB broth supplemented with ampicillin were harvested, resuspended in 10 mM HEPES-KOH (pH 7.5) buffer at an OD_{660} of 10, and broken by sonication. In order to prevent the formation of new disulfide bonds during the manipulation, the buffer contained 20 mM iodoacetamide. In the analysis of whole cells, the sonicated suspensions were treated with the sample buffer (with 2-mercaptoethanol omitted) with or without 20 mM dithiothreitol (DTT), and proteins were resolved by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis. The method used for preparation of the inner membrane fraction was the method described previously (29), using 10 mM HEPES-KOH (pH 7.5) buffer containing 1.5% (wt/vol) *N*-lauroylsarcosine for solubilization. The levels of AcrB expression were monitored by Western blot analysis, using polyclonal rabbit antibody anti-AcrB (35) and an alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma), and protein-antibody conjugates were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Labeling and detection of free cysteine residues in Cys mutant AcrB with biotin-maleimide. HNCE1a or AG100YBD cells harboring pSPORT1-derived plasmids were grown at 37°C in LB medium with ampicillin without IPTG induction to an OD_{660} of 0.8 to 1.0. Cells from a 15-ml culture were collected, washed twice, and resuspended in 0.5 ml of 10 mM HEPES-KOH (pH 7.5). A freshly prepared 10 mM biotin-maleimide [*N*-biotinoyl-*N'*-(6-maleimidohexanoyl)hydrazide] (Sigma) solution in dimethylformamide was added to a final concentration of 0.2 mM, and then cells were left at room temperature for 30 min with gentle mixing. The reaction was stopped by addition of 2-mercaptoethanol to a final concentration of 20 mM, and then the cells were washed once and stored at -80°C. To purify AcrB proteins, the cells were resuspended in 300 μ l of solubilization buffer (20 mM HEPES-KOH, 0.3 M NaCl, 20 mM imidazole, 10% [vol/vol] glycerol, 2% [wt/vol] dodecyl maltoside (DDM); pH 7.5), and membrane proteins were extracted with sonication, followed by a 30-min incubation on ice. After removal of cellular debris by ultracentrifugation at $150,000 \times g$ for 30 min at 4°C, supernatants were incubated with 30 μ l of BD TALON cobalt chelating resin (BD Bioscience) in Micro Bio-Spin chromatography columns (Bio-Rad) at 4°C for 1 h. The resin was washed with buffer C (20 mM HEPES-KOH, 0.3 M NaCl, 10% glycerol, 0.05% DDM; pH 7.5) containing 20 mM imidazole and then eluted with 50 μ l of buffer C containing 200 mM imidazole. The purified AcrB fractions were obtained after buffer exchange with 20 mM HEPES-KOH (pH 7.5) containing 50 mM NaCl, 0.02% DDM, and 10% glycerol, using Bio-Gel P-6DG gel (Bio-Rad) with Micro Bio-Spin columns. Identical amounts of the purified AcrB mutant proteins, as determined by normalization after Western blotting with a polyclonal anti-AcrB antibody (35), were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred to a nitrocellulose membrane, which was exposed to horseradish peroxidase-conjugated streptavidin (1:5,000; Pierce), and then visualized using Western Lightning chemiluminescence reagent plus (PerkinElmer LAS, Inc). The intensity of the bands was measured by use of the NIH ImageJ program. No band was detected for the cysteineless AcrB (CL-AcrB) protein, which was used as a control.

RESULTS

Functional characterization of single- or double-Cys mutants with mutations in the cleft or interface between subunits. As we stated in the Introduction, the model proposed by Murakami and coworkers (18) suggests that the activity of the AcrB multidrug efflux pump involves a cycle of conformational changes that include the opening and closing of both the external clefts of and the interface between the periplasmic domains of AcrB protomers. To examine the closure of the clefts and the alteration of intersubunit distances at the subunit interface by disulfide cross-linking, we chose several amino acid residues for introduction of Cys residues. The choice of these residues was based on the asymmetrical crystal structure of

FIG. 1. External cleft in the periplasmic domain of AcrB. The image shows the surface of two of the protomers (the binding protomer on the left and the extrusion protomer on the right) of the asymmetric AcrB structure (18) from PDB file 2DRD, viewed from the outside. The three domains within each protomer are indicated on the left as follows: T, TolC-binding domain; P, periplasmic domain; and M, TM domain. It is evident that the large external cleft in the periplasmic domain, wide open in the binding protomer (left), is completely closed in the extrusion protomer (right). Asp566, on the left wall of the cleft in the binding protomer, and Thr678, on the right wall, are shown as stick models with CPK colors. The Asp566 residue moves strongly to the right in the extrusion protomer, so that it becomes located very close to Thr678, just above it in the image. The figure was generated from PDB file 2DRD by using UCSF Chimera package (22) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

AcrB (18, 26), and we chose positions where the introduced Cys residues might produce a disulfide bond only in a particular protomer or protomer pair within the asymmetric trimer of AcrB.

In the extrusion protomer, the distances between residues on the two sides of the large external cleft of the periplasmic domain are 3.8 Å between D566 and T678, 4.2 Å between F666 and T678, and 4.4 Å between F666 and Q830, as measured between the ends of the side chains. (D566 and T678 are shown with the clefts in the binding and extrusion protomers, as examples, in Fig. 1.) In contrast, the same distances are over 9 Å in the access and binding protomers and also in the crystal structure of the symmetric trimer (19). In the subunit interface, the distances between F316 in the extrusion protomer and Q687, A688, and G854 in the neighboring access protomer are 3.7, 5.2, and 4.3 Å, respectively, although the distances are >6 and >5 Å between the binding and extrusion protomers and between the access and binding protomers, respectively. On the basis of these observations, we substituted Cys residue for the amino acids just mentioned. For some of the residues, Ser was also used as a control.

We first examined if conversion of any of these residues into Cys affected the function of the AcrB transporter. The transport activity of each single-Cys mutant was evaluated by examining the drug susceptibility of plasmid-containing HNCE1a cells on gradient plates containing 8,000 μ g/ml cholic acid in the lower layer, as described in Materials and Methods. To avoid the nonreproducible drug susceptibility patterns caused by strong overexpression of AcrB alone (29, 33), *acrB* was

FIG. 2. Activities of AcrB single- and double-Cys mutants. Single- and double-Cys mutations were introduced into the cleft or interface of subunits of AcrB. The mutant AcrB proteins were expressed in the *acrB acrD* host strain HNCE1a, and their efflux activities were estimated from their levels of resistance to cholate. The positions of the mutations in the AcrB protein are indicated at the bottom. For details, see Materials and Methods. In the mutation designations "x" corresponds to Cys for black bars and to Ser for gray bars.

expressed without IPTG induction. We also observed that the levels of resistance of HNCE1a cells expressing AcrB mutants from pSPORT1 derivatives often varied after storage of the transformed strains for 3 to 4 days at 4°C. Therefore, for these experiments we used only freshly transformed cells. The lengths of growth across the gradient were reproducibly 65 to 75 and 25 to 30 mm for the *acrB::kan ΔacrD* host strain HNCE1a expressing wild-type CL-AcrB^{His} and the strain containing only the vector, respectively, indicating that the CL-AcrB^{His} protein was fully functional in cholate efflux, as noted previously (27).

As shown in Fig. 2, single-Cys substitutions, such as CL-D566C, CL-F666C, CL-T678C, and CL-Q830C in the cleft or CL-F316C, CL-Q687C, CL-A688C, and CL-G854C at the interface of subunits, did not severely diminish the transport function, although CL-F666C (73% ± 3%) and CL-T678C (77% ± 5%) showed slightly decreased activity (Fig. 2) as assayed by resistance to cholate. The same was true for single-Ser substitutions when such substitutions were examined (Fig. 2). Similar results were obtained for the resistance to ethidium bromide, chloramphenicol, tetracycline, sodium dodecyl sulfate, and tetraphenylphosphonium (results not shown).

In contrast, the transport activity was essentially abolished in all of the double-Cys mutants, which were expected to form a disulfide bond in the periplasmic domain either in the cleft or in the interface between subunits, except the combination F316C/Q687C, which still showed 28% of the activity of the parent CL-AcrB^{His} (Fig. 2). When one of the amino acids of a Cys pair was replaced by serine, as in CL-D566S/T678C, CL-F666S/T678C, and CL-F666S/Q830C in the cleft and CL-F316S/Q687C, CL-F316S/A688C, and F316S/G854C at the subunit interface, most of the molecules retained over 76% of the activity; the only exception was CL-F666S/T678C, whose activity was 63% ± 4% of the parent activity (Fig. 2). These data suggest that the introduced double-Cys substitutions pro-

duced disulfide bonds which caused the loss of activity, even though in the symmetric trimer model (19) they are far from each other (>9 Å in the cleft).

Expression levels and detection of disulfide linkages in AcrB. We noted that all of our mutant proteins were present in the inner membrane at levels similar to that of CL-AcrB^{His} (not shown).

We tried to detect disulfide-cross-linked AcrB by Western blot analysis of whole-cell extracts solubilized without the use of reducing agents. With the mutant AcrB proteins in which disulfide cross-links were expected in the cleft region, we could not detect a strong difference in migration, although some portions of the CL-D566C/T678C protein migrated slightly faster than others (not shown). With the proteins in which cross-linking was expected in the subunit interface, portions of double-Cys mutant proteins (CL-F316C/Q687C, CL-F316C/A688C, and CL-F316C/G854C) often occurred as very-high-molecular-weight bands, which failed to enter the gel (not shown). These results showed that there was formation of disulfide bonds, but they prevented us from carrying out more detailed analysis of the cross-links generated.

We tried to estimate the number of free Cys residues left in the Cys mutant AcrB by carrying out sulfhydryl-specific labeling with biotin-maleimide. The extent of labeling of the CL-D566C/T678C double-Cys mutant AcrB was 52% of the sum of the labeling of two single-Cys mutants, D566C and T678C, and the extent of labeling of CL-F666C/Q830C was 57% of the sum of the labeling of the two single mutants. The CL-F666C/T678C double-mutant protein was labeled even more poorly (7% of the sum of the component single-mutant proteins). As controls, the mutant AcrB proteins mentioned above were labeled after expression in a *dsbA1::kan* host strain that prevented the formation of disulfide bonds in the periplasm (see below); the double-mutant proteins were labeled at a level that was 101% ± 16% of the sum of the levels of the single mutants.

These results suggest that in the *dsbA*⁺ wild-type host strain, some of the Cys residues in the double-mutant AcrB indeed become unavailable for maleimide modification, confirming the presence of disulfide bonds. It is also of interest that not all of the Cys residues become linked in disulfide bonds; in principle, this is consistent with the “functionally rotating model” of Murakami et al. (18) that predicted that only one of three protomers would have a conformation favoring the disulfide bond formation.

Effect of *dsbA* mutation on activity of double-Cys mutants. The absence of activity in the double-Cys mutants (Fig. 2) does not unequivocally prove that the disulfide bond formation inactivates the transporter, because it is still possible that the combination of two mutations, which singly do not inactivate AcrB (Fig. 2), may inactivate AcrB by mechanisms that do not involve disulfide bonds. In order to obtain stronger evidence for the contribution of disulfide bonds to AcrB inactivation, we used *dsbA* mutants. In *E. coli*, the main enzyme catalyzing disulfide bond formation in the periplasm is DsbA, a 23-kDa protein with a thioredoxin-like fold (3, 10). Mutations in *dsbA* cause a pleiotropic defect in disulfide bond formation in the periplasm (3, 17). We constructed for our assay a pair of isogenic strains, AG100YB (Δ *acrB*::*Spc*^r) and AG100YBD (Δ *acrB*::*Spc*^r *dsbA1*::*kan*). Since the levels of resistance of the AG100YB and AG100YBD strains to cholera were different from that of HNCE1a, to evaluate the transport activity of each mutant on gradient plates, the concentration of cholic acid in the lower layer for the strains was adjusted to 10,000 μ g/ml for AG100YB and to 16,000 or 18,000 μ g/ml for AG100YBD. Under these conditions, the lengths of growth across the gradient were reproducibly ~75 mm for both strain AG100YB and strain AG100YBD expressing CL-AcrB^{His} and ~30 mm for the strain containing only the vector.

In the *dsbA*⁺ host strain AG100YB (Fig. 3A), double mutants with mutations in the cleft showed resistance patterns similar to those of HNCE1a, as expected. However, in *dsbA* strain AG100YBD, the activity of the AcrB efflux pump was restored in the CL-F666C/T678C double mutant; this activity was 1% $\pm$ 1% in AG100YB and 57% $\pm$ 6% in the *dsbA* strain. Similarly, the activity of CL-F666C/Q830C double-mutant AcrB increased from 19% $\pm$ 3% in AG100YB to 70% $\pm$ 4% in the isogenic *dsbA* host. These results strongly support the hypothesis that the inactivation of the double-Cys mutant AcrB in the *dsbA*⁺ host strains is indeed due to the formation of disulfide bonds. The activity of CL-D566C/T678C mutant AcrB, however, was not restored in the *dsbA* background (Fig. 3A). This may have been because the distance between the two Cys residues was too small (about 3.8 Å) in the extrusion protomer, and the remaining mechanisms for disulfide bond formation were sufficient for this pair.

Addition of 3 mM DTT to both layers of a gradient plate also resulted in some restoration of the activities of AcrB double mutants in AG100YB. Thus, the activity of the CL-F666C/T678C mutant increased from 1% $\pm$ 1% without DTT to 8% $\pm$ 5% with DTT, and the activity of the CL-F666C/Q830C mutant increased from 19% $\pm$ 3% without DTT to 39% $\pm$ 3% with DTT.

In contrast to these results with the cleft mutants, the *dsbA* mutation in the host did not restore AcrB efflux activity in any of the three double-Cys mutants at the subunit interface (Fig.

FIG. 3. Effect of *dsbA* mutation on the activities of AcrB double-Cys mutants. (A) Cleft mutants. (B) Cys mutants with mutations in the subunit interface. Open bars, host strain HNCE1a; gray bars, AG100YB; black bars, AG100YBD.

3B). Western blot analysis showed that portions of these proteins sometimes formed very-high-molecular-weight aggregates, which failed to enter the gel, as mentioned above. It is possible that the sulfhydryl forms of these proteins eventually formed improper disulfide bonds, leading to the formation of inactive large aggregates.

Cross-linking by use of fast-acting, SH-specific reagents. In order to confirm further that the disulfide cross-linking between the two Cys residues is responsible for the inactivation of mutant AcrB proteins, we used MTS cross-linkers, which are known to act much more rapidly than the usual cross-linkers and which therefore can be used in “real-time” inactivation experiments with transporters (2, 11). MTS-2-MTS (~5.2-Å spacer) was added to AG100YBD cells expressing CL-F666C/Q830C in the presence of ethidium bromide. The double-Cys mutant AcrB was active in the efflux of ethidium, so that only very slow entry of ethidium, which causes fluorescence following dye binding to nucleic acids, was seen initially (Fig. 4, bottom right panel). However, upon addition of MTS-2-MTS, cross-linking apparently occurred, so that AcrB became inactivated, inducing rapid ethidium accumulation and increased fluorescence (Fig. 4, bottom right panel). In contrast, MTS-2-MTS had little effect on the ethidium entry rate in cells expressing no-Cys (CL-AcrB^{His}) or a single-Cys (CL-F666C and

FIG. 4. Effect of cross-linker addition on ethidium accumulation in AG100YBD cells expressing AcrB Cys mutants. Cellular accumulation of ethidium was monitored continuously by measuring the fluorescence of the ethidium-nucleic acid complex at excitation and emission wavelengths of 520 and 590 nm, respectively. After 2 min of incubation with 5 μ M ethidium bromide (arrow), MTS reagents or solvent alone (dimethyl sulfoxide-ethyl acetate [3:1, vol/vol]) was added to 2 ml of cell suspension. Additions were as follows: curve 1, 8 μ l of solvent; curve 2, 40 μ M 5-MTS (8 μ l of a 10 mM stock solution); curve 3, 20 μ M MTS-2-MTS (8 μ l of a 5 mM stock solution). Ordinates show fluorescence intensity in arbitrary units.

CL-Q830C) AcrB (Fig. 4). (Although there was a very slow, gradual increase in ethidium accumulation with the two single-Cys mutants, this could have been the result of cross-linking of Cys to amino groups, as all the electrophilic reagents do react with amino groups, albeit slowly.) As a control reagent, a non-cross-linker, 5-MTS (whose length is similar to that of MTS-2-MTS) was used; this produced no AcrB inactivation in any of the mutants (Fig. 4). As another positive control, 40 μ M (final concentration) carbonyl cyanide *m*-chlorophenylhydrazone, a proton conductor, was added to the cells expressing CL-AcrB^{His}, resulting in a rapid influx of ethidium because of inactivation of AcrB due to the loss of the proton motive force (Fig. 4, top left panel).

We also examined 1,5-pentanedithiol bismethanesulfonate (~ 9.1 -Å spacer) and dibromobimane (~ 5 Å long; not an MTS reagent) as cross-linkers and obtained similar results (not shown). However, inactivation of the double-Cys AcrB transporter occurred more slowly with dibromobimane, as expected.

Attempts to detect the substrate-induced movement of the cleft. According to the asymmetric AcrB model of Murakami

et al. (18), the two cysteine residues that we introduced on both sides of the cleft approach the cross-linking distance only in the extrusion protomer. Thus, we reasoned that the rapid cycling through of the three conformations of AcrB actively extruding the substrates may enhance the cross-linking. We incubated *dsbA* cells expressing double-cysteine mutants of AcrB in the presence of the MTS cross-linker MTS-2-MTS with and without substrates of AcrB, such as chloramphenicol and, after washing the cells, determined the degree of inactivation of AcrB with the ethidium influx assay. We have not been able to detect reproducible effects of the presence of substrate on the extent of inactivation in repeated trials under different conditions (not shown).

DISCUSSION

The molecular mechanism of drug translocation by the proton motive force-dependent drug transporter AcrB is not known, and information is needed to determine how H⁺ transport and substrate transport are coupled and how the protein

changes its conformation during this process. The recent crystallographic elucidation of the structure of the asymmetric AcrB trimer (18, 26) showed that each protomer has a conformation significantly different from that of its neighbors, and crystallization of AcrB with a drug ligand allowed Murakami et al. (18) to propose that the three conformations represent the successive stages of drug transport (i.e., drug access, drug binding, and drug extrusion).

In this work, we utilized the observation that the external large cleft in the periplasmic domain of AcrB appears to become closed in one of the protomers (extrusion protomer) in the asymmetric trimer model (18) (Fig. 1), and we demonstrated that the cleft indeed becomes closed *in vivo*, resulting in inactivation of AcrB. Double-Cys mutants with mutations at the cleft almost completely lost its function, even though a mutant protein was properly expressed and localized to the inner membrane of the cells. The fact that this was due to the formation of disulfide bonds and not the result of the presence of two cysteines per se was shown by the observation that CL-F666C/T678C and CL-F666C/Q830C showed restored activity in the *dsbA* background, which diminished the rate of disulfide bond formation in the periplasm. However, assays dependent on the formation of disulfide bonds had to rely on the slow and indirect assay of growth inhibition, during which assembly of newly made AcrB complex must take place (see below). We therefore also used MTS cross-linking reagents, which allowed us to observe inactivation of the pumps *in situ* in real time (that is, within a few seconds after the addition of the reagent). Our conclusion was supported by these experiments (Fig. 4). All these results provide proof that the external cleft in the periplasmic domain of AcrB, which is wide open in two of the protomers of the asymmetric trimer model, as well as in the symmetric trimer model, becomes sufficiently closed to allow the formation of a disulfide bridge or cross-linking, which results in a loss of flexibility in the conformation, inactivating the efflux pump. These results strongly support the model of Murakami et al. (18), requiring a cyclic conformational alteration in each of the protomers for drug export, a mechanism that becomes impossible when even one of the protomers becomes inflexible by cross-linking. In this context, it is interesting that we observed that a large fraction of the Cys sulfhydryl groups are still free for modification with biotin-maleimide after the nearly complete inactivation of the pump by disulfide cross-linking.

The attempt to inactivate AcrB by creating intersubunit disulfide bonds was not as successful as the intrasubunit disulfide bond experiments discussed above. We observed inactivation with double-Cys mutants and little inactivation with controls (Fig. 2). However, examination of cross-linked products by Western blotting showed that in some experiments a portion of the proteins formed very large aggregates, and the inactivation still occurred in the *dsbA* host strain (Fig. 3B). It is difficult to interpret these data in a conclusive way. One likely possibility is that the cross-linking occurs during the assembly of these multisubunit pumps. If this is true, it suggests possible pitfalls with *in vivo* disulfide cross-linking approaches that utilize a slow, growth-dependent assay and adds emphasis to the real-time assays performed with MTS cross-linkers with the cleft mutants, as discussed above.

Finally, we attempted to determine if the cross-linkers inac-

tivate the functioning AcrB proteins more effectively, but we could not obtain convincing results (see Results). There are several explanations for this. First, even the "resting" AcrB transporters may exist as asymmetric trimers, and they may contain protomers whose conformation is either similar or identical to that of the extrusion protomer, which can be easily cross-linked. Second, even cells without the added substrate drugs may be pumping out endogenous substrates or, for that matter, the cross-linkers themselves. AcrB can use even (modified) phospholipids as substrates at least *in vitro* (35), and it may be difficult to keep a transporter with such wide specificity in a truly resting state. Lastly, the cross-linking reagent could have been too efficient, so that nearly complete inactivation could have resulted even under suboptimal conditions.

It is tempting to interpret the inactivation of cleft-cross-linked AcrB in terms of the model of Murakami et al. (18). However, it should be noted that alternative interpretations are also possible. The external cleft in question has been thought to be the site where AcrA molecules fit in (19). If this is the case, then AcrA access may become difficult for cross-linked AcrB. In this connection, we emphasize that AcrA is not simply a linker of two membranes; with the AcrB homolog AcrD, the presence of AcrA was necessary for the activity of the pump *in vitro* (1). Another possibility is that the cleft might be on the pathway of drugs for exclusion. We have indeed shown that the wall of the cleft can bind various substrates of AcrB (33), and substitution of Phe666 with other amino acid residues does decrease the efflux activity (33) (Fig. 2); thus, at present we cannot completely exclude the hypothesis that the cross-linking and closing of the cleft inactivate AcrB through prevention of substrate access. However, these alternative hypotheses appear to be rather unlikely in view of the fact that complete inactivation depends on the cross-linking and that the single-cysteine substitutions have either no or only minimal effect on activity (Fig. 2 and 4).

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