pH-induced Conformational Changes of AcrA, the Membrane Fusion Protein of Escherichia coli Multidrug Efflux System*

The multidrug efflux system AcrA-AcrB-TolC of Escherichia coli expels a wide range of drugs directly into the external medium from the bacterial cell. The mechanism of the efflux process is not fully understood. Of an elongated shape, AcrA is thought to span the periplasmic space coordinating the concerted operation of the inner and outer membrane proteins AcrB and TolC. In this study, we used site-directed spin labeling (SDSL) EPR (electron paramagnetic resonance) spectroscopy to investigate the molecular conformations of AcrA in solution. Ten AcrA mutants, each with an alanine to cysteine substitution, were engineered, purified, and labeled with a nitroxide spin label. EPR analysis of spin-labeled AcrA variants indicates that the side chain mobilities are consistent with the predicted secondary structure of AcrA. We further demonstrated that acidic pH induces oligomerization and conformational change of AcrA, and that the structural changes are reversible. These results suggest that the mechanism of action of AcrA in drug efflux is similar to the viral membrane fusion proteins, and that AcrA actively mediates the efflux of substrates.

Emergence of multidrug-resistant bacterial strains not only has hampered the current treatment of bacterial infections but also hindered the development of new therapeutic agents. Resistance mediated by multidrug efflux pumps as a major mechanism has been increasingly recognized. Available clinical data showed that 40–90% of some bacterial pathogens (Streptococcus pneumoniae, Streptococcus pyogenes, and Pseudomonas aeruginosa) bear efflux mechanisms for the major classes of available antibiotics (1–5). Many drug efflux pumps have broad substrate specificity and expel a wide range of completely unrelated chemotherapeutic drugs. The AcrA-AcrB-TolC efflux system of Escherichia coli is such an example and is largely responsible for the intrinsic resistance of E. coli to most lipophilic antibiotics, detergents, and dyes (6, 7). This system consists of a resistance-nodulation-cell division (RND) type efflux pump, AcrB, a periplasmic, membrane fusion protein (MFP), AcrA, and a multifunctional outer membrane channel, TolC. Such organization allows the bacterium expel a wide variety of noxious compounds from the cell directly into the medium, bypassing the periplasm (8). Similar multicompont efflux systems have been found in other Gram-negative bacteria including P. aeruginosa, Enterobacter aerogenes, and Neisseria gonorrhoeae (3, 6).

Major progresses have been made for understanding the efflux mechanism in Gram-negative bacteria, which are highlighted by recent publications of the crystal structures of TolC and AcrB (9–11). The TolC trimer comprises of two barrel-like structures joined together, the outer membrane β-barrel and the periplasmic α-barrel. The long α-barrel (~100 Å) is thought to traverse the periplasm and interact with AcrB or inner membrane (9). Consistently, the AcrB protein, also as a trimer, contains two structural domains, the transmembrane domain (50 Å in thickness) and a headpiece that protrudes about 70 Å in depth into the periplasm (10). It is thought that AcrB and TolC may be directly docked with each other, forming a continuous pathway across the periplasm and the outer membrane. Substrates may gain access to the AcrB central cavity either from the cell interior through the transmembrane region, or from the periplasm through the vestibules of AcrB protein, which are then actively transported through the AcrB pore into the TolC tunnel (10). Indeed, AcrB structures with four structurally diverse substrates demonstrated that they bind to the large central cavity of AcrB (11).

An important question remains to be addressed is the role of AcrB in the efflux process. AcrA is essential for drug efflux, but how AcrA participates in this process is not fully understood. In its mature form, AcrA carries a diacylglycerol group and a palmitic acid chain linked to the N-terminal cysteine residue, which is believed to anchor the protein to the inner membrane. However, the lipid-deficient variant of AcrA carrying a His tag at the C-terminal is functional and has been used for biochemical studies (12). The secondary structure predictions of AcrA suggest that AcrA and its MFP homologs contain two regions of high coiled-coil probability of approximately equal length, flanked by two lipoyl/biotin-binding motifs that are likely β-strands (13). Although the high resolution structure is not available (14), AcrA was found to be a highly asymmetric molecule with an elongated shape of about 200 Å in length (12). Cross-linking experiments showed that AcrA forms a complex with AcrB (15), and may interact with TolC. Therefore, AcrA could provide a seamless link between AcrB and TolC. Alternatively, it may simply bring the outer and inner membranes closer for substrate transfer.

A better knowledge of the structure and function of AcrA is essential for understanding the efflux process. In this study, we
used a sensitive biophysical method, the site-directed spin labeling (SDSL), for studying the structure and dynamics of AcrA. SDSL utilizes site-directed mutagenesis to replace the residue of interest in a protein with a cysteine, which is then modified with a sulphydryl-specific nitroxide to introduce the paramagnetic side chain (Fig. 1). Electron paramagnetic resonance (EPR) spectroscopic analysis of the spin label yields spectral characteristics that are dependent on the local environment, which in turn provide information on the structure and dynamics of the protein (16, 17). Using this method, we demonstrate that AcrA is a dynamic protein that undergoes conformational rearrangements triggered by changes of pH. Such conformational changes may be important for the action of AcrA during the drug efflux process.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmid Construction, and Growth Media—All E. coli strains were grown at 37 °C in LB broth. Antibiotics were added when required, to the following final concentrations: kanamycin, 34 μg/ml; ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; and chloramphenicol, 15 μg/ml.

All plasmids were constructed by standard cloning techniques and propagated in the DH5α strain of E. coli. To construct pBP184, a 3557-bp HindIII-BamII fragment of pBP was ligated into a HindIII- and BamII-treated pACYC184 vector. Plasmid pBP184 expresses AcrB protein under the native acrAB promoter. The pUZ11 (12) was used to construct all plasmids for the expression and purification of single cysteine-containing AcrA-6His variants. The histidine tag was at the C-terminal of AcrA. Unique cysteine residues were introduced into OmpA-AcrA-His, using the QuickChange Site-directed Mutagenesis Kit (Stratagene). All final constructs were verified by DNA sequencing.

Analysis of Function and Expression of AcrACys-6His Variants—The functional fitness of AcrACys-6His variants was evaluated by measuring minimal inhibitory concentrations (MICs) of different antibiotics. For this purpose, AcrACys-6His variants were transformed into AG100A (acrAB-hsa) strain carrying pBP184 plasmid (18). Then exponentially growing cultures (optical density at 600 nm of 1.0) were inoculated at a density of 10⁶ cells per ml into LB medium in the presence of 2-fold-increasing concentrations of the drug under investigation. Cell growth was determined after overnight incubation at 37 °C.

The levels of expression of AcrACys-6His were routinely monitored by Western immunoblotting analysis according to standard protocols using anti-AcrA antibody. E. coli cells were harvested by centrifugation, briefly sonicated, and solubilized by boiling in SDS sample buffer and subjected to SDS-polyacrylamide electrophoresis. In the case of the non-reducing SDS-PAGE, β-mercaptoethanol was omitted from the sample buffer.

Protein Expression and Purification—The E. coli DH5α or AG100A strains containing plasmids with single cysteine substitution were cultured overnight. The overnight culture (5–10 ml) was inoculated into 500 ml of fresh LB medium with appropriate antibiotics and incubated at 37 °C until OD₆₀₀ nm reached 0.5–0.7. Expression of protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside at 1 mM. After 3–4 h, cells were harvested by centrifugation, washed with 20 mM Tris-HCl, 0.2 mM NaCl, pH 8.0, and subsequently disrupted by sonication. AcrA protein was purified from the cell lysates using His-Bind metal chelating resin (Novagen) according to the manufacturer’s protocol with a slight modification (i.e., the concentration of imidazole in the Wash Buffer was 50 mM). To remove imidazole, protein was dialyzed against storage buffer (20 mM Tris-HCl, 0.2 mM NaCl, 1 mM EDTA, 500 mM imidazole) at 4 °C for 24 h.
E. coli cells expressing AcrA and mutants were transformed with plasmids expressing His-tagged AcrA or AcrA mutants, and MICs were determined. The cloning vector pUC18 was included as the negative control.

**TABLE I**

| E. coli cells expressing | MIC | | | | |
|-------------------------|-----|-----|-----|-----|-----|
|                         | Erythromycin | Novobiocin | SDS | Puromycin | Tetracycline |
| AcrA                    | 64  | 32  | >5000 | 32  | 0.625 |
| AcrA A30C               | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A39C               | 64  | 32  | >5000 | 32  | 0.625 |
| AcrA A62C               | 64  | 32  | >5000 | 32  | 0.625 |
| AcrA A103C              | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A146C              | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A172C              | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A204C              | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A242C              | 64  | 16  | >5000 | 32  | 0.625 |
| AcrA A295C              | 64  | 8   | >5000 | 32  | 0.625 |
| AcrA A339C              | 64  | 32  | >5000 | 32  | 0.625 |
| AcrA A390C              | 64  | 8   | >5000 | 32  | 0.625 |
| No AcrA (pUC18)         | 4   | 2   | 40   | 4   | 0.313 |

**FIG. 3.** Representative DSC scans of AcrA variants. Thermal unfolding of AcrACys103 (top) and AcrA103R1 (bottom) are shown. The protein concentrations were 1 mg/ml. The solid line represents the fit of the DSC data to a two-state model.

pH 7.0) overnight. Purified protein is stable for at least 4 weeks at 4 °C. For prolonged storage, 10% glycerol was added, and protein was stored at –20 °C. The resulting protein was >95% pure as judged by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined at 280 nm using the molar extinction coefficient 17,210 M–1 cm–1 or the BCA protein assay (Pierce Chemical Co.).

Spin Labeling and EPR Spectroscopy—Thio-reducing agent DTT (4 mM) was added to the purified protein (~0.5–2 mg/ml) in the storage buffer and incubated at 4 °C for 2–3 h. Samples were then dialyzed against the storage buffer overnight (2–3 buffer changes). Spin label MTS-L, 1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl-methanethiosulfonate (Toronto Research Chemicals Inc., Toronto, Canada), was added at 10-fold molar excess with respect to the AcrA and incubated for 16 h at 4 °C. Excess free spin labels were removed by dialysis. For experiments at pH 5.0, another buffer, 25 mM MES, 0.2 M NaCl, 1 mM EDTA, pH 5.0, was used for dialysis. Proteins were concentrated by ultrafiltration and adjusted to 100–200 μM. For titration experiment, labeled AcrA in appropriate buffer was mixed with excess unlabeled AcrA (1:5 molar ratio) at protein concentration below 50 μM for overnight at 4 °C. The mixture was then concentrated and adjusted to 100–200 μM before acquiring EPR spectra.

EPR spectra were obtained using a Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany). The conventional X-band spectra were obtained using 0.63 milliwatts microwave power and a field modulation of 100 kHz and 1.6 G modulation amplitude. Scan width was 100 G. EPR spectra were analyzed using the software Xport provided by the manufacturer (Bruker).

**Differential Scanning Calorimetry (DSC)—**DSC experiments were performed on a MicroCal VP-DSC calorimeter (MicroCal, Northampton, MA). The protein concentration is at 1 mg/ml and the scan rate is 45 °C/hr. Data analysis was performed using Origin software provided by the manufacturer (MicroCal).

**RESULTS**

**Single Cysteine AcrA-6His Variants Are Functionally Active and Stable in Vivo**—The native AcrA protein has a unique cysteine residue, Cys-25, which is the site for lipid modification (19). However, genetic analysis suggested that neither lipid modification nor Cys-25 residue are essential for AcrA function (12). We have used the previously reported cysteine-less AcrAC25K variant with a hexa-histidine tag on the C terminus (AcrA-6His) as a background to construct 11 single cysteine AcrA (AcrACys-6His) mutants. Mutants were constructed by a PCR-based method and verified by sequencing. Previous sequence analysis predicted that AcrA has a highly hydrophilic central domain with a high propensity to form a trimeric coiled-coil and a C-terminal hydrophobic domain conserved among MFPs (13, 20). In addition, the central α-helical region of AcrA...
is bracketed by sequences homologous to lipoyl/biotin-binding domain (13). We introduced unique cysteine residues in all these putative domains of AcrA (Fig. 2).

All 11 mutants could be expressed in E. coli to the similar level of wild-type AcrA (data not shown). We did not detect any visible degradation products, which could indicate protein instability induced by Cys substitutions. At most positions, cysteine substitutions were well tolerated by the presumed AcrA—AcrB-TolC complex, since plasmids expressing various AcrA variants displayed a single cooperative endotherm centered at a transition temperature of 50°C, as illustrated by the representatives in Table I. In two instances, cells carrying AcrACys295 and AcrACys390 exhibited only partial resistance to novobiocin and puromycin, compared with the wild-type AcrA. However, these mutants fully complemented resistance to other antimicrobials including erythromycin and tetracycline, and detergent SDS (Table I). Although these data do not address if initial rates of drug efflux are affected, they suggest that the mutant proteins are not grossly destabilized.

Spin Labeling and Stability of Labeled AcrACys-6His Variants—To gain insights into the molecular conformation and dynamics of AcrA in solution, ten AcrACys-6His variants were expressed, and purified by the standard methods, and subjected to spin labeling by a nitroxide reagent MTSL. All sites reacted readily with MTSL under the experimental conditions (i.e., without prior unfolding by denaturants) and gave strong EPR signals, suggesting that they are not buried within the structure and are accessible to MTSL, which is comparable in size to a tryptophan.

As mentioned above, most cysteine substitutions did not alter the function of AcrA (Table I). We also examined if the presence of spin label MTSL affects AcrA stability by monitoring its thermal unfolding with DSC. All spin-labeled mutants examined display a single cooperative endotherm centered at a transition temperature of 50°C, as illustrated by the representatives in Fig. 3. The corresponding cysteine mutants (unlabeled) and the parental AcrA protein were also analyzed. The DSC data were analyzed according to a two-state unfolding model, and the derived thermodynamic parameters are summarized in Table II. It is notable that changes of $T_m$ and $\Delta H$ in the presence of spin label fall within the range of values found for substitutions at these sites by cysteine residues with the exception of 62R1, which is slightly more destabilized by the presence of MTSL. These results agree with previous findings from many studies that show MTSL, being relatively small, represents a minimal perturbation of the backbone fold, thermal stability, or function of a protein (21).

Side Chain Mobility of Spin-labeled AcrA—Fig. 4 shows the EPR spectra of individual spin-labeled AcrA at pH 7.0. The 10 spectra are distinctive in terms of mobility, a qualitative descriptor of the dynamic modes of R1 in the protein. Two measurable parameters, the inverse of the peak-to-peak splitting of the central line of EPR spectra ($\Delta H_p^{-1}$) and the spectral breadth that is represented by the separation of magnetic field between the two outermost peaks, have frequently been employed to determine the mobility of spin label, which reflects both the rate and amplitude of motion (17). Such mobility is determined by the immediate environment of a R1

![Fig. 4. EPR spectra of ten spin-labeled AcrA proteins. Numbers indicate the spin-labeled positions (see Fig. 2). All spectra shown were acquired at protein concentration of 100–200 μM (~5–7 mg/ml) at pH 7. The magnetic-field scan width is 100 G. The symbols α and β indicate components of different mobility.](image)
residue that reflects its surrounding protein topography. Correlations between side chain mobility and the details of protein structure and dynamics have been studied extensively in several proteins including T4 lysozyme (T4L) (21), colicin E1 (22), annexin XII (23), and the T domain of diphtheria toxin (24). In general, R1 residues are highly immobilized at buried sites, immobilized or have complex multicomponent spectra at tertiary contact sites, and have relatively high mobility at exposed sites or in loop regions. The side chain mobility of AcrA, measured as $\Delta H^{-1}$, was summarized in Table III. The spectra of 30R1 and 390R1 are dominated by sharp and narrow line-shapes, reflecting a high degree of motion. This suggests that the extremes of N and C termini of AcrA are largely unstructured, making little or no static tertiary contacts with the remainder of the protein. This is consistent with the notion that AcrA is in an extended conformation in solution and the N and C termini do not physically interact with each other but instead are located on two opposite poles of the protein molecule (12). In contrast, other R1 residues yield broadening spectra, which are characteristics of side chains within ordered structures or involving in tertiary interactions. The residues 103R1, 146R1, and 172R1 exhibit anisotropic motions that are typical of solvent-exposed, noninteracting, helix surface sites. The spectrum of 172R1 is complex, reflecting an anisotropic motion about an axis roughly parallel to the nitroxide $2p\pi$ orbital. Nearly identical spectrum was described for residue 72R1 of T4L, which is located on the surface of a helix and experiences no interactions with nearby side chains (21). The

Fig. 5. Spectral changes induced by acidic pH. A, overlay of spectra acquired at pH 7.0 (blue) and pH 5.0 (red). Spectra of spin-labeled AcrA collected under different conditions are normalized to the same spin population. B, EPR spectra of 146R1 at pH 7.0, then pH 5.0 and then by raising the sample at pH 5.0 back to pH 7.0. C, overlay of spectra acquired at pH 7.0 (blue) and pH 6.0 (green).
cause for the constrained motion of 72R1 in T4L has been studied extensively and was recently shown to reflect the backbone motion (25). Consistent with the EPR spectra, residues 103R1, 146R1, and 172R1 are located at the predicted two α-helical regions with high probability of forming coiled-coil (Fig. 2).

Residues 39R1, 62R1, and 204R1 have two spectral components, reflecting two resolved populations of different mobilities (i.e. $H9251$ and $H9252$, Fig. 4). As described previously in many other systems, spin label attached to a single cysteine residues in protein often exhibits spectra composed of at least two motional components. Until recently, the structural basis remained unknown. The crystal structures of spin-labeled T4L proteins indicate that the multiple component spectra reflect bond rotational isomerization, which are modulated by interactions of the R1 side chain with neighbor side chains or backbone (26). Thus, residues 39R1, 62R1, and 204R1 might be involved in tertiary contacts. Similarly, residues 146R1, 204R1, and 339R1 might also have multiple dynamic components reflecting some degree of tertiary interactions (Fig. 4). Residues 62R1 and 204R1 are close to the predicted N- and C-lipoyl/biotin binding motifs, which are mostly β-strands (Fig. 2) (13). Residues 242R1 and 339R1 are close to the conserved C-terminal hydrophobic domain (20). Although no secondary structures were predicted for regions of 39R1, 242R1, and 339R1, the EPR spectra indicate that these residues are located within ordered structures. In general, the EPR spectra are compatible with the predicted structure of AcrA based on sequence analyses.

Conformational Rearrangements of AcrA Triggered by Changes of pH —The AcrAB-ToIC system, like most bacterial efflux pumps, utilizes the proton motive force as the energy source for drug transport (6). In _E. coli_ cells, about half of the proton motive force across the cytoplasmic membrane comes from a proton gradient, with the cytoplasmic pH being higher than the external pH by about 1.7 pH unit (27). The _in vitro_ reconstitution studies showed that AcrA greatly stimulates the transport activity of AcrB transporter by presumably promoting adhesion between two phospholipid bilayers (8). In addition, AcrA alone could mediate hemifusion of lipid bilayer of membrane vesicles without intermixing the vesicle contents when there is a pH gradient across the vesicle (pH of vesicle interior is 7.0 and external 5.0) (8). Triggering of membrane fusion by lowering pH has been well established in the influenza viral membrane fusion protein, hemagglutinin (HA). HA is thought be in a metastable state at neutral pH and refolds into its lowest energy state by exposure to an acidic environment (28). Therefore, we examine whether AcrA undergoes conformational transitions when the environment is acidified.

Lowering the buffer pH from 7.0 to 5.0 caused changes of EPR spectra of residues 62R1, 103R1, 146R1, and 172R1, but little or no change of the others (i.e. 30R1, 39R1, 204R1, 242R1, 339R1, and 390R1, see Fig. 5A and Table III). Addition of MgCl$_2$ (10 mM) did not cause further spectral changes (data not shown). The most pronounced spectral change induced by acidic pH was observed at residues 62R1 and 146R1. At pH 5.0, the spectrum of 62R1 became broadened and a strongly immobilized component appeared (see arrows in Fig. 5A), indicating that the local protein structure in the vicinity of 62R1 underwent a conformational change and consequently, residue 62R1 was in a more restricted environment. Residues 103R1 and 172R1 exhibited a similar conformational change, i.e. they became more restricted in mobility at pH 5.0.

Comparing the EPR spectra of 146R1 at pH 7.0 and pH 5.0, the signal amplitude at pH 5.0 was decreased dramatically as the result of spectral broadening, and the baseline of the spectrum distorted, which are characteristics of spin-spin interaction that occurs when spin labels are at close proximity (<15

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**Fig. 6. Titration of spin-labeled AcrA with excessive unlabeled AcrA protein.** A, overlay of spectra acquired at pH 7.0, prior titration (blue) and after titration (black). B, overlay of spectra acquired at pH 5.0, prior titration (red) and after titration (black). For each spin-labeled AcrA protein, spectra obtained under different conditions are normalized to the same spin population.
Since all AcrA variants studied here are single labeled proteins, this result indicates that acidic pH induces oligomerization of AcrA, and that residue 146R is involved in intermolecular interaction. To further verify this, 146R mutant protein was titrated with unlabeled AcrA protein in five times excess and the spectra compared. Indeed, at pH 5.0, dilution of spin-labeled AcrA 146R mutant leads to spectral sharpening (Fig. 6), confirming that residue 146R is a tertiary contacting site among AcrA monomers. Interestingly, titration of 146R protein at pH 7.0 with unlabeled AcrA also increased the signal amplitude slightly, suggesting even at pH 7.0 AcrA may form an oligomer. Titration of other spin-labeled proteins including 62R and 172R with unlabeled protein did not change their spectra either at pH 5.0 or 7.0 (Fig. 6), consistent with the notion that the pH-induced spectral changes of these sites reflect conformational changes.

The conformational rearrangements of AcrA induced by acidic pH is reversible, e.g. when the pH was changed from 5.0 to 7.0, the EPR spectra of 146R1 (Fig. 5B) and 62R1 (not shown) were reversed to their original lineshapes at pH 7.0. Interestingly, titration of 146R1 protein at pH 7.0 with unlabeled AcrA also increased the signal amplitude slightly, suggesting even at pH 7.0 AcrA may form an oligomer. Titration of other spin-labeled proteins including 62R and 172R with unlabeled protein did not change their spectra either at pH 5.0 or 7.0 (Fig. 6), consistent with the notion that the pH-induced spectral changes of these sites reflect conformational changes.

Oligomerization of AcrA in Vivo—Previous chemical in vivo cross-linking studies suggested that in the periplasm AcrA exists as an oligomer most probably a trimer (15). To investigate if any of cysteine residues can stabilize oligomeric conformation of AcrA through the formation of intermolecular disulfide bonds we also analyzed whole cell extracts from E. coli cells expressing various AcrACys-6His by SDS-PAGE under non-reducing conditions followed by immunoblotting with anti-AcrA antibody. We found that AcrACys-6His mutants spontaneously form intermolecular disulfide bonds in the oxidative environment of the periplasm. The non-reducing SDS-PAGE analysis has shown that all mutants with the exception of AcrACys30 and AcrACys62 form high molecular weight species, which remain stable after purification using Ni/H$_{2}$O$_{4}$ affinity chromatography (Fig. 7A). We analyzed the compositions of these high molecular weight species by running the second dimension of SDS-PAGE in the presence of DTT. AcrA was the only protein present in these high molecular weight species (Fig. 7B).

Intermolecular disulfide bonds are most efficiently formed by Cys residues in positions A39C, A339C, and A390C. These residues are located close to the N and C termini of AcrA. The molecular mass of oligomers stabilized by these residues is about 90 kDa, which is close to the molecular weight of AcrA dimers estimated from the amino acid composition. The centrally located A172C, A204C, and A242C stabilize by disulfide bonds.
Conformational Changes of AcrA

50481

bonds protein species with molecular mass of about 150 kDa. These high molecular weight species could correspond to AcrA trimers (Fig. 7A). On the other hand, the variable mobility in the SDS-PAGE could arise as a result of differences in frictional properties of AcrACys-6His variants cross-linked by disulfide bonds. Indeed, the molecular weight of oligomers stabilized by Cys residues in other positions is of intermediate value. Thus, all high molecular weight species could correspond to either AcrA dimers or trimers.

These results support the notion that in vivo AcrA exists as an oligomer (15). Since protein oligomerization is sensitive to protein conformation, the presence of AcrACys-6His oligomers provides evidence that in vivo the gross conformations of native and AcrACys-6His variants are similar. AcrA molecules are arranged in a parallel manner since the disulfide bonds are formed between cysteine residues located in the same position in the primary sequence of AcrA.

DISCUSSION

The two-membrane envelope of Gram-negative bacteria serves as an effective permeability barrier against noxious compounds in the environment. However, the outer membrane, which does not have direct access to energy, and the periplasmic space, which is about 130–250 Å in depth (29, 30), create problems when molecules have to be exported extracellularly. To overcome this difficulty, a multicomponent export assembly that spans the inner membrane, the periplasm, and the outer membrane is constructed (6). Such organization, such as the AcrA-AcrB-ToIC of E. coli, allows bacterial cells to expel substrates directly into the medium. AcrA is thought to coordinate the concerted operation of AcrB and ToIC such that the substrates are expelled through the multiprotein complex without leaking into the periplasm. How AcrA achieves this goal, however, is not fully understood. In this study, we report the first experimental evidence that AcrA is a dynamic protein that undergoes pH-induced oligomerization and conformational change, and suggest that AcrA plays an active role in the drug efflux process. Although we do not know at present whether the various conformations of AcrA observed under the in vitro conditions reflects its conformational states in vivo, it is conceivable that AcrA could undergo conformational transitions while interacting with AcrB, and/or ToIC, and upon changes of the local environmental conditions within the periplasm, which coordinates the actions of AcrB and ToIC.

Two general models of AcrA action were proposed (31, 32). One model predicts that AcrA acts as an adaptor to dock ToIC and AcrB, which forms a closed channel with ToIC and becomes a route for the expelled substrates to pass through the periplasm (31). The other model proposes that AcrA simply brings the outer and inner membranes into close apposition to allow substrate transfer (32). This model was originally proposed based on the sequence analysis. AcrA belongs to a family of membrane fusion protein (MFP) found in Gram-negative bacteria (20), which share homology with a paramyxovirus membrane fusion protein, SV5 F protein. In addition, structural prediction of MFPs reveals features such as the presence of coiled-coil domains and two hydrophobic regions near the N and C termini, which are reminiscent of viral membrane fusion proteins (13). This model is further supported by several findings including that AcrA is a highly asymmetric molecule with a length of about 200 Å, sufficient to span the periplasm (12), and that the purified AcrA protein alone is able to promote the close association of two membranes, and possibly a hemifusion event (8). In addition, the crystal structure of AcrB shows that like ToIC, which protrudes 100 Å into the periplasm, AcrB contains an extramembrane region that extends into the periplasm by 70 Å (9, 10). The sum of the periplasmic length of AcrB and ToIC is about 170 Å, which could be enough to cross the periplasmic space.

Results presented in this study provide new insight into the mechanism of AcrA action in the efflux process. Our data suggest that AcrA could form oligomers arranged in a parallel manner. Unique cysteine residues introduced in different positions in primary sequence of AcrA readily form intermolecular disulfide bonds confirming previous finding that in vivo AcrA is an oligomer (15). Protomers in the oligomeric AcrA are arranged in parallel manner since the disulfide bonds are formed between cysteines located at the same position in the primary sequence of AcrA. In contrast, purified AcrA exists in solution as a monomer (12). We found that acidic pH induces oligomerization of AcrA in solution. At pH 5.0, spin-spin interaction that only occurs when spin labels come to close proximity was readily detected at residue 146R1, and was titrated out by excessive unlabeled protein, indicating that AcrA forms oligomer under this condition and the residue 146R1 is involved in inter-molecular interaction. The spin-spin interaction was obvious when the concentration of 146R1 protein was >150 μM, but was not detected at concentrations <50 μM (data not shown), suggesting that AcrA exists as a monomer at low concentrations. The K_d for the oligomerization/association was estimated to be ~100 μM by analyzing EPR spectra of 146R1 at several different concentrations (20, 40, 80, 120, 160, 240, and 320 μM). The number of protomers in oligomeric AcrA remains unclear. Low resolution structure of AcrA is consistent with the dimeric conformation of AcrA (14). However, in vivo cross-linking studies showed that DSQ cross-linker with the 7 Å spacer arm stabilizes AcrA trimers (15). Trimeric conformation is also supported by sequence analysis of coiled-coil domains of MFPs (13).

Acidic pH not only facilitates the oligomerization but also causes conformational changes of AcrA, which were detected in local structures of residues 62R1, 103R1, and 172R1. This is illustrated most clearly in the 62R1 mutant. The EPR spectrum of 62R1 at pH 5.0 was significantly broadened, and a strongly immobilized component appeared, suggesting that a significant population of 62R1 side chain moved to a more restricted environment, involving interactions with neighboring side chains or backbone. Unlike residue 146R1, the spectral broadening of 62R1 at pH 5.0 was not affected by titration with excessive unlabeled protein, further supporting the notion that the local structure of residue 62R1 undergoes conformational change, instead of being involved in spin-spin interaction. Similar results were obtained for residues 103R1 and 172R1. Johnson and Church (13) suggested one possible mechanism by which a MFP could bring the outer and inner membranes closer. It was noted that there is a 2-fold symmetry of the coiled-coil and lipoyl motif regions and that a MFP could simply fold back on itself at the gap between the helical regions, forming an intramolecular hairpin (13). Interestingly, the residues of AcrA that exhibit pH-induced conformational change, i.e., 62R1, 103R1, and 172R1, are located in the predicted coiled-coil or lipoyl motifs, suggesting such proposed conformational change of AcrA could occur, although our current data are not sufficient to directly evaluate the proposed model.

Not all residues of AcrA studied herein are involved in intermolecular interaction or undergo pH-induced conformational change. EPR spectra of residues close to the N terminus such as 30R1, and 39R1, as well as residues close to the C terminus including 204R1, 242R1, 339R1, and 390R1 showed little or no change when the pH was lowered from 7.0 to 5.0. The four residues that exhibited pH-induced spectral changes, i.e. 62R1, 103R1, 146R1 and 172R1, are all located in the predicted coiled-coil and the lipoyl/biotin motif regions, indicat-
understanding the dynamics of the AcrA-AcrB-TolC system will elucidate the mechanism of efflux process in Gram-negative bacteria.

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