STRUCTURE AND MECHANISM OF RND-TYPE MULTIDRUG EFFLUX PUMPS

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I. Introduction

It has long been known that gram-negative bacteria are usually much more resistant than gram-positive bacteria to the actions of antibiotics and
chemotherapeutic agents. The cells of the former group are surrounded by an extra membrane layer, the outer membrane (OM), and it was suspected that this additional membrane layer acted as a general barrier for the influx of agents. Indeed, the nonspecific diffusion channels of OM, the porins, limit the influx of small hydrophilic agents, because their channels are quite narrow (7 by 11 Å in the *Escherichia coli* OmpF porin) (1, 2). In addition, the lipid bilayer domain of the OM is unusual in its extreme asymmetry by having the outer leaflet composed nearly exclusively of lipopolysaccharides containing only saturated fatty acid chains (3), and the very low fluidity of this outer leaflet decreases the spontaneous permeation rates of hydrophobic probe molecules by nearly two orders of magnitude compared with conventional phospholipid bilayers containing many unsaturated fatty acid residues (4). Nevertheless, when the permeability coefficients of β-lactams across *E. coli* OM were measured, even the slowest-penetrating compounds were found to equilibrate across the outer membrane usually within a minute, that is, in a time span that is much shorter than the generation time of *E. coli* (5), in part because the surface-to-volume ratio is very high in these small bacterial cells. It was thus clear that the OM barrier alone cannot generate significant levels of resistance, and another major process must work synergistically with this barrier (5). With β-lactams, the ubiquitous periplasmic β-lactamases can fulfill this role. However, with other classes of antibiotics, the nature of this second process remained obscure until the constitutive multidrug efflux pumps were found to be widespread in gram-negative bacteria and to act synergistically with the OM barrier (6, 7).

Gram-negative bacteria usually contain multidrug efflux pumps belonging to several families, such as ABC (ATP-binding cassette), SMR (small multidrug resistance), MFS (major facilitator superfamily), MATE (multiple antibiotic and toxin extrusion), and RND (resistance–nodulation–division) (8–10). Among these, most are pumps located in the cytoplasmic membrane and must pump out drugs rapidly into the periplasm because the drugs can penetrate back into cytosol frequently by spontaneous diffusion. Only the RND pumps (and a few exceptional pumps belonging to other families) exist in a tripartite form traversing both the OM and the inner membrane, in a manner first suggested by Wandersman and co-workers for a protein-secreting apparatus of gram-negative bacteria (11). This complex involves, in addition to the RND pump protein located in the inner membrane, an outer membrane channel such as TolC of *E. coli* (belonging to OMF [outer membrane factor] family (12)), and a periplasmic adaptor.
protein (belonging to the MFP [membrane fusion protein] family (13)). As shown in Figure 1, this construction allows the bacteria to pump out drug molecules directly into the external medium. This is a huge advantage for bacteria, because the drug in the medium has to cross the low-permeability OM in order to reenter the cells, in contrast to the drug molecules in the periplasm, which can easily penetrate the high-permeability inner membrane. Thus, the tripartite RND pumps are expected to produce drug resistance very effectively by working synergistically with the outer membrane barrier (6, 7).

Wild-type strains of most gram-negative bacteria are resistant to most lipophilic antibiotics (for *E. coli* they include penicillin G, oxacillin, cloxacillin, nafcillin, macrolides, novobiocin, linezolid, and fusidic acid), and this “intrinsic resistance” was often thought to be caused by the exclusion of drugs by the outer membrane barrier. Indeed, breaching the outer membrane barrier does sensitize *E. coli* cells to the drugs just mentioned (14). However, inactivation of the major and constitutively expressed RND pump AcrB also

![Diagram of the tripartite pump complex](image-url)

Figure 1. Early schematic view of the tripartite pump complex. Note that amphiphilic drugs (empty and solid rectangles represent hydrophobic and hydrophilic parts of the molecule) are hypothesized to be captured from the periplasm–plasma membrane interface or possibly from the cytosol (or the cytosol–membrane interface). For the latter process, two possible pathways are envisaged (dashed arrows): Either the substrate is flipped over to the outer surface of the membrane first and then follows the regular periplasmic capture pathway, or it follows a different capture pathway from the cytosol. For the AcrAB–TolC system, the efflux transporter corresponds to AcrB, the accessory protein to AcrA, and the OM channel to TolC. [Modified from (7).]
makes the bacteria nearly completely susceptible to these agents [the minimal inhibitory concentration (MIC) of a lipophilic penicillin, cloxacillin, goes down from 512 µg/mL in the wild type to only 2 µg/mL (15)] even in the presence of the intact outer membrane barrier. Thus, the characteristic intrinsic resistance of gram-negative bacteria owes as much to the RND pumps as to the outer membrane barrier.

Since some tripartite pumps give resistance to drugs that cannot penetrate the inner membrane, such as the dianionic compound carbenicillin, they were proposed to be able to capture drug molecules from the periplasm (6, 7). Finally, in order to produce significant levels of drug resistance, the drugs that were pumped out into the periplasm by the simple pumps located in the inner membrane need to be captured and then extruded across the OM into the medium by tripartite RND pumps (16, 17). For example, in Pseudomonas aeruginosa, the expression of a simple tetracycline pump TetA produces only a very modest degree of resistance (MIC = 32 µg/mL) in the absence of the main tripartite pump MexB–MexA–OprM, but MIC is raised up to 512 µg/mL if the tripartite pump is, in addition, expressed at the normal, constitutive level. That the high resistance is not due to the pumping of tetracycline by the tripartite pump alone is seen from the fact that the tripartite pump, in the absence of TetA, produces an MIC of only 4 µg/mL (16). Thus, these two types of pumps appear to act in a truly synergistic manner.

The first example of an RND pump that functions as a multidrug exporter was identified in 1993 (18); this was AcrB (called AcrE in that paper) in E. coli. The immediately upstream gene, acrA, was known to be involved in acriflavine resistance, but the mechanism was thought to be the decreased permeability of the envelope through the global chemical modification of its components. We showed that acriflavine penetrates rapidly across OM in both the wild-type and acrAB-inactivated strain, yet accumulates at a much higher level in the cells of the latter. We also noted the similarity of the AcrB sequence to CzcA, a divalent cation efflux pump, and proposed that AcrB functions by actively pumping out acriflavine, not by strengthening the passive permeability barrier of OM. The extremely wide substrate specificity of this pump was indicated by the fact that inactivation of the acrAB genes made E. coli hypersusceptible not only to several dyes but also to detergents (such as SDS, Triton X-100, and bile salts) and to a wide range of antibiotics, including macrolides, β-lactams, tetracycline, chloramphenicol, fusidic acid, and novobiocin (but not aminoglycosides) (Figure 2).
Figure 2. Examples of substrates for the AcrB pump.
At about the same time, Poole and co-workers (19) found that *P. aeruginosa* mutants capable of growth in the presence of an iron chelator, α,α′-dipyridyl, overproduced an outer membrane protein (OprM, called OprK in the paper), and that the *oprM* gene was a part of an operon with upstream genes *mexAB* that are homologous to *acrAB*. Indeed, the inactivation of these genes made the organism hypersusceptible to chloramphenicol, tetracycline, ciprofloxacin, and streptonigrin. Meanwhile, our studies on clinical isolates of *P. aeruginosa* whose β-lactam resistance could not be explained fully by the combination of OM barrier and the periplasmic β-lactamase showed that unidentified multidrug efflux pump(s) are overexpressed in these strains, leading to simultaneous resistance to a large number of agents (20, 21). The most likely candidate for this pump was found to be the MexAB–OprM, the major constitutive RND multidrug efflux transporter of *P. aeruginosa*, through collaboration with the Poole laboratory (22). MexAB–OprM extrudes not only fluoroquinolones, chloramphenicol, and tetracycline, but also novobiocin and a number of β-lactam antibiotics. These studies also gave the first indication that the pump functions as a tripartite complex, as *mexA*, *mexB*, and *oprM* genes comprised a single operon; in *E. coli*, the outer membrane channel TolC is coded elsewhere on the chromosome (23). Importantly, each of these three component proteins is essential for drug efflux, and the absence of even one component makes the entire complex totally nonfunctional (18, 19, 24). To this day, AcrB and MexB have been the most intensively studied RND drug transporters in bacteria and have served as the prototype for biochemical and structural studies of such pumps.

Some of the observations made in this early period were important in formulating our concepts on the function of RND drug efflux transporters.

1. Some β-lactam compounds, such as carbenicillin and ceftriaxone, which contain multiple charged groups and therefore cannot diffuse across the inner membrane, as verified experimentally, are nevertheless very good substrates of MexAB–OprM (21) and AcrAB–TolC (15). This observation indicates that the RND pumps are capable of capturing substrates from the periplasm (6, 7, 21). The periplasmic capture also fits with the observation that the same pump can catalyze the efflux of substrates with diverse charges: uncharged, anionic,
cationic, or zwitterionic (7). Export of these substrates from cytosol will generate different effects on membrane potential, which may be difficult to deal with.

2. Although the pumps often handle a very wide range of substrates, they all seem to have a significant lipophilic portion (7, 15). This observation led to a hypothesis that many substrates become partially partitioned, from the periplasm into the outer leaflet of the inner membrane (see Figure 1), before being captured by the pump (7). This association with the membrane surface would involve an energetic cost in terms of decreased entropy, but a similar phenomenon is well known to occur with the interaction of amphiphilic anesthetics with lipid bilayers (25). An alternative interpretation of the requirement of lipophilic domain in the ligands, however, might be that hydrophobic interaction plays a predominant role in the binding of substrates at the binding pocket of the pump (see Section IV).

Finally, it should be mentioned that the tripartite architecture is not completely limited to RND pumps. In *E. coli*, an MFS efflux pump EmrB is known to form a tripartite structure together with the periplasmic adaptor EmrA and the outer membrane channel TolC (26), and to pump out weakly acidic or largely uncharged substrates, such as the proton uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), nalidixic acid, and thiolactomycin (27). An ABC efflux pump, MacB, pumps out various macrolides with the periplasmic adaptor MacA, as well as the OM channel TolC (28). Although MacA has been crystallized (29) and an elegant proteoliposome reconstitution of MacB has been achieved (30), the totality of our knowledge on these non-RND systems cannot be compared with that on the RND systems, and we concentrate on RND systems in this chapter.

Recent reviews deal with various aspects of bacterial multidrug efflux; in addition to the comprehensive reviews cited earlier (9, 10), there are reviews emphasizing different aspects, such as multidrug resistance (31, 32), structure (33–35), and mechanisms (36). We do not discuss the regulation of pump production [which was reviewed recently (37)] or the clinical microbiology of the pumps, as they are outside the scope in this chapter. We also do not discuss in detail the outer membrane channels nor the periplasmic adaptor proteins [reviewed recently by Zgurskaya and associates (38)].
II. Biochemical and Genetic Studies

A. RANGE OF SUBSTRATES

Comparison of MIC values between the wild-type and ΔacrAB strains (7, 18, 24) showed that AcrB of *E. coli* can handle a very wide range of compounds. These include cationic dyes such as acriflavine, crystal violet, ethidium bromide, and rhodamine 6G; and antibiotics such as penicillins, cephalosporins, fluoroquinolones, macrolides, chloramphenicol, tetracyclines, novobiocin, fusidic acid, oxazolidinones, and rifampicin; and detergents such as Triton X-100, sodium dodecyl sulfate, and bile acids. The range even extends to simple solvents such as pentane and cyclohexane (39, 40) (Figure 2). It was obvious that there is no structural similarity between most of these compounds. Furthermore, the charge states of the ligands are diverse, some nonionic, some anionic, some cationic, and others containing multiple ionizable groups (see Figure 2). However, there was one common feature: that the substrates were either relatively lipophilic or at least contained lipophilic domains (7). This led to the hypothesis that the substrates first interact with the membrane lipid bilayer (7), and become captured either from within the bilayer or from the bilayer–aqueous interface (7) (Figure 1). However, alternative interpretations are possible, as mentioned earlier.

The capture of substrates from the periplasm or a location that is in rapid equilibrium with periplasm was described in Section I. In this connection it is interesting that the general inhibitor of *P. aeruginosa* and *E. coli* RND pumps, MC-207,110 (phenylalanyl-arginyl-β-naphthylamide (41), which apparently is a favored substrate of the pumps, affected the carbenicillin MIC only to a small extent (41). Thus, the addition of this inhibitor decreased the MICs of drugs such as erythromycin and levofloxacin to almost exactly the same degree as the genetic inactivation of the MexB–MexA–OprM pump. In contrast, the inhibitor produced only a fourfold decrease in carbenicillin MIC, although the genetic inactivation decreased it 512-fold. A similar discrepancy, a strong decrease in MIC by genetic inactivation vs. little decrease in the presence of the inhibitor, was also found for tetracycline and ethidium bromide (41). Since carbenicillin must be captured exclusively from periplasm, these results were sometimes interpreted to mean that this particular inhibitor acts more effectively for drugs that are predominantly captured in the cytosol. Currently, we are more inclined to assume that AcrB captures all drugs exclusively from the periplasm (see Section IV). If so, these results are perhaps caused by the
binding of various substrates to different subdomains of the large binding pocket of the transporter.

B. TRIPARTITE ASSEMBLY

As described in Section I, the drug efflux function of AcrB requires the simultaneous presence of the periplasmic adaptor protein AcrA and the outer membrane channel TolC. Chemical cross-linking showed that the adaptor protein AcrA can be cross-linked to the RND transporter AcrB in intact cells (42), and more recently, the entire trimeric complex was isolated from gently lysed *E. coli* cells without cross-linking (43). Interestingly, the association of the component proteins was stabilized by the presence of substrates (43).

When the crystallographic structures of the transporters AcrB (44) and, most recently, MexB (45), the adaptor proteins MexA (46, 47) and AcrA (48), and the outer membrane channel TolC (49) and OprM (50) became available (Figure 3), it became possible to speculate on the details of interaction among these components. Space does not permit a discussion of the details of these structures, but some of the prominent features will be listed.

1. From the amino acid sequences, AcrB (18) and other RND pumps (51) were expected to contain 12 transmembrane helices, with two extremely large periplasmic domains between helices 1 and 2 and between helices 7 and 8. Indeed, one can estimate that the size of the periplasmic domain would almost surpass that of the transmembrane domain, a prediction borne out with the x-ray crystallographic structures (44, 45). What the amino acid sequence did not tell us was that these pumps existed as trimers and that the tip of the large periplasmic domain, often called the TolC-docking domain, would fit the internal end of the TolC channel (44).

2. The adaptor proteins had a very elongated shape, as predicted from physicochemical studies (52). This shape was created by a very long α-helical hairpin structure on top of another elongated domain, the “lipoyl” domain, which usually carries bound lipoic acid in various enzymes. Finally, this structure was followed by a β-barrel domain. [The structure of the fourth domain at the opposite end of the molecule from the α-hairpin, called the membrane-proximal domain, was solved recently (53) and is discussed below.] All these
separate domains create a flexible structure, again as predicted by site-directed spin labeling studies (54).

3. The structure of TolC (49) was perhaps the most spectacular. It is a trimer containing the OM-traversing channel of a β-barrel, with each protomer contributing four transmembrane β-strands of the
12-stranded barrel. Even more interestingly, the channel in the β-barrel is continued by a long helical tunnel, approximately 130 Å long, made of long α-helical hairpins and extending deep into the periplasm.

These structures obviously suggest that the tip of the periplasmic domain of AcrB, or the TolC-docking domain, contacts the end of the TolC periplasmic tunnel. This contact was also confirmed by introducing cysteine residues to the “top” of AcrB and the “bottom” of TolC channel, and observing the formation of a disulfide linkage (55). Although this end of the TolC tunnel is closed in the first structure published, there are now structures in partially open states (56).

Modeling efforts for the trimeric complex were also helped by studies that involved alteration of the structure of component proteins. For example, we found that the adaptor protein of another E. coli tripartite system, YhiU (also called MdtE), could not replace AcrA in the formation of tripartite system containing AcrB, and tested chimeric AcrA molecules containing different parts of YhiU (57). Within the 398-residue sequence of AcrA, residues 1–290 could be substituted by the YhiU sequence, but substitution of residues 290–357 produced a protein inactive in forming a functional tripartite complex with AcrB, suggesting that the C-terminal portion of the adaptor proteins was important in interaction with the RND pump proteins. Study of component interaction in vitro by the use of titration calorimetry also gave results (58) consistent with the chimeric AcrA data. Thus, both the full-length AcrA and its C-terminal half (residues 172–397) are associated with AcrB, but the N-terminal half (residues 24–172) or the central fragment (residues 45–315) showed no evidence of interaction with AcrB. In this connection, it must be mentioned again that until very recently the published x-ray crystallographic structures of the adaptor proteins were incomplete, corresponding roughly to the central fragment just mentioned, thus lacking the most important C-terminal part that interacts with the RND transporter. In another approach it was noted that AcrA can function with MexB and TolC, but the full spectrum of drug resistance found in the MexAB–OprM system was not attained (59). Random mutagenesis of AcrA resulted in the isolation of mutants that work better with MexB, and some of them were located in the β-barrel domain.

As regards the interaction between AcrA and TolC, Lobedanz and co-workers (60) were able to carry out a fine resolution analysis by
cross-linking Cys residues of AcrA, introduced by site-directed mutagenesis, with amino groups on the surface of TolC, establishing how only one side of the \(\alpha\)-helical-coiled coil hairpin in AcrA interacts tightly with the intramolecular groove in the TolC helical bundle.

Hypothetical models of the tripartite assembly were proposed in the past. In one model (46), trimers of MexB and OprM are surrounded by either 12 or six MexA proteins. In another (61), nine AcrA protomers circle the trimers of AcrB and TolC. However, there were some major problems in building these models.

1. The oligomeric state of the adaptor proteins is uncertain. Cross-linking studies showed that overexpressed AcrA existed as dimers and trimers in the cell (42), whereas AcrA lacking the N-terminal lipid modification behaved as monomers when isolated (52). Adaptor proteins have been crystallized as giant complexes containing more than 10 protomers (46–48). These are obviously crystallographic artifacts that do not tell us anything about the physiological state of their self-association.

2. As mentioned above, the structure of the adaptor proteins used in modeling lacked the extreme N-terminus and a large C-terminal domain, the portion known to be essential for the interaction between the adaptor and the RND pump protein.

3. The stoichiometric ratio between the adaptor and the pump protein (or TolC–OprM) within the tripartite complex was not known.

A giant stride in this area was recently made by the Koronakis group (53). They have refined the data obtained previously for the MexA crystal (47) and succeeded in obtaining the structure of the hitherto missing domain containing the N-terminus and the large C-terminal segment. This is now known to form the fourth domain or membrane-proximal domain of the adaptor, containing an \(\alpha/\beta\) structure with a characteristic concave surface (Figure 3). By using this MexA structure as a template, they built the complete structure of AcrA. Finally, by applying the method used earlier to study the interaction between TolC and AcrA (60) [i.e., site-directed insertion of Cys followed by cross-linking], they were able to determine precisely where defined portions of AcrA fit into which surface of AcrB. Interestingly, in this cross-linking study in intact cells, AcrA seems to interact with only one contiguous stretch of AcrB within the periplasmic domain, thus suggesting a 1:1 stoichiometric ratio between
these two proteins. Based on these observations, a data-driven model of the AcrAB complex could be built, which is shown as Figure 4.

Another significant advance in the study of component interaction was the in vitro examination of association between MexA and OprM (62).
Such studies are difficult because both are membrane proteins [although some adaptor proteins appear to work in intact cells without the lipid modification at the N-terminus (52)], and the adaptor inserted into one membrane must interact with the OM channel inserted into another membrane (see Figure 1). Reffay and co-workers (62) used an ingenious system of L3 phase (sometimes called “sponge phase”) surfactants, where the distance between neighboring bilayers can be adjusted. In this system, the lateral diffusion rates of MexA become greatly diminished when it forms a complex with the OprM trimer, a much larger protein, in another bilayer. The MexA immobilization as a function of OprM concentration showed that the MexA monomer to OprM trimer stoichiometry was strongly dependent on pH [a finding reminiscent of an earlier study of AcrA conformation (54)] and ranged from 2 at pH 7.5 to 6 around pH 6. The former value is more or less consistent with the model of Symmons et al. (53), but it is not yet clear how the second adaptor protein can be accommodated in the final tripartite complex. We note that these ratios are consistent with the abundance of MexA, MexB, and OprM protomers in the cell reported by the Nakae group (63): namely, 2 : 1 : 1. However, in *E. coli*, AcrA appears much more abundant, Tikhonova and Zgurskaya (43) reporting an abundance of 10 : 1: 3 for AcrA, AcrB, and TolC protomers; this ratio, however, may reflect the fact that AcrA and TolC also function for other systems: AcrA, for example, for AcrD (64) and TolC not only for all other RND systems but also for some non-RND systems such as EmrAB and MacAB.

One observation that may be pertinent here is that some RND systems apparently contain a pair of adaptor proteins. For example, the TriABC–OpmH system of *P. aeruginosa*, which pumps out triclosan, contains two adaptor protein genes (triA and triB) within the operon, and studies clearly showed that both genes are needed for triclosan resistance (65). The ZrpADBC system of *Serratia* sp. appears to be yet another example of such a system containing a pair of adaptor proteins (66). If we assume that the pump proteins in these systems function as trimers according to the functionally rotating mechanism (described in Section III.C), each protomer of the pump is likely to contact a pair of the adaptor proteins, giving rise to a 2 : 1 : 1 ratio between the adaptor, pump, and OM channel proteins. However, an alternative arrangement of 1 : 1 : 1 stoichiometry is still possible, producing a situation in which different adaptor–pump complexes would behave similarly to the different pump protomers in the MdtBC-type complexes (see Section IV.E).
C. KINETIC BEHAVIOR OF THE AcrB PUMP

To understand the behavior of the pump in intact cells, it is obviously essential to know its kinetic constants. Hints on the binding affinity of various substrates were obtained by using them as competitive inhibitors in the reconstitution assay of AcrB (67) (see below). In this assay, which depended on the export of fluorescent phospholipids by the purified AcrB protein, taurocholate, a conjugated bile salt, inhibited the reaction most strongly (presumably by competing as a substrate), the 50% inhibition occurring at 15 μM. In contrast, antibiotics were less efficient inhibitors, and cloxacillin and erythromycin caused 50% inhibition only around 100 μM. These results are important in showing that the conjugated bile salts are probably the preferred, and most likely the natural, substrates of the AcrB pump of *E. coli*, an organism that must live in an environment full of these membrane-disrupting detergents. In the same study (67), proton movement accompanying the pumping of substrates was also measured. Although a single concentration of substrates (0.2 mM) was used, again the conjugated bile salts were most effective in increasing the proton flux. However, these assays do not measure substrate binding per se, and we do not know the true affinity of each substrate to the pump. (We should also mention that running similar assays in intact cells is difficult because both the substrate and the competitive inhibitor must diffuse across the outer membrane before binding to the pump.)

There was a recent attempt to measure the binding of substrates to purified AcrB protein by the use of fluorescence polarization (68). Because binding of fluorescent substrates to a large AcrB trimer is expected to slow the tumbling of these molecules, this method offers advantages. The apparent dissociation constants reported ranged from 5.5 μM for rhodamine 6G to 74 μM for ciprofloxacin. However, the method does not give reliable information on binding stoichiometry, and because the authors did not use, as controls, mutant AcrB proteins that are altered in substrate binding, it is unclear whether the binding occurred to the true binding sites or to some extraneous pockets that bind lipophilic molecules in a nonspecific manner.

The efflux of fluorescent dyes from cells after their energization was followed in several studies. Typically, cells are preloaded with dyes that become fluorescent only within the membrane or the cytosol. The preloading requires deenergization of the RND pump by a proton conductor such as CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), and reener-
gization of the cells is done by adding compounds such as glucose or formate. If everything goes perfectly, the time-dependent decrease in fluorescence intensity should follow the integrated form of the Michaelis–Menten equation, which should give us the kinetic constants $K_m$ and $V_{max}$. In the first paper reporting the use of one of these probes (69), the results were not convincing because the efflux rates from *P. aeruginosa* strains either producing or not producing the MexB–MexA–OrpM efflux complex showed little difference, presumably because deenergization with cyanide could not be reversed rapidly. However, efflux assays were performed successfully since then as a qualitative assay, for example with NPN (*N*-phenylnapthylamine) and *E. coli* (41) and with DASPEI (2-[[4-dimethylamino]styryl-*N*-ethylpyridinium iodide) and *E. coli* (70). No effort to use the efflux curve for quantitative analysis of the transport process was reported. We have tried to do this quantitative analysis with a fluorescent dye, Nile Red, using *E. coli* (70a). However, the system is extremely complex, because CCCP used in the dye-loading period may still persist in the cells, at least in the beginning, and the full membrane energization with carbon sources will take a few seconds, thus making analysis of the early phase of the efflux curve quite difficult.

An early study that attempted to determine the kinetic constants of efflux was that of Narita et al. (63). Here the authors calculated the rate of ethidium efflux from the difference in the ethidium influx rates between efflux-incompetent and efflux-competent cells of *P. aeruginosa*, and reported a $V_{max}$ value of 100 nmol/s per $3 \times 10^8$ cells, said to correspond to a turnover number of 500 s$^{-1}$. However, the authors did not show how these numbers were arrived at. In fact, inspection of the data shows that the efflux rate plotted against intracellular ethidium concentration was essentially linear, and little sign of saturation can be observed. Thus, these conclusions are not convincing.

Recently, we had the first success with the estimation of kinetic constants of the AcrB pump (71). With attempts using intact cells, the difficulty is always with estimation of the substrate concentration within the periplasm, where it is presumably captured by the pump. We solved this problem by using $\beta$-lactams as substrates. Since $\beta$-lactams are hydrolyzed by the periplasmic $\beta$-lactamase, and since we know the $K_m$ and $V_{max}$ values of the enzyme, we can calculate the periplasmic concentration of $\beta$-lactams, $C_p$, from the Michaelis–Menten equation if we determine spectrophotometrically the $\beta$-lactam hydrolysis rate by intact cells. Once the periplasmic concentration $C_p$ is known, the rate at which the $\beta$-lactams
cross the outer membrane by simple diffusion through porin channels \( (V_{\text{in}}) \) can be calculated from the permeability coefficient of the OM, because it is proportional to the concentration difference of the \( \beta \)-lactam between the external medium and the periplasm. The efflux rate \( V_e \) is then the difference between the influx rate \( V_{\text{in}} \) and the hydrolysis rate \( V_h \), or \( V_{\text{in}} - V_h \). Initially, this approach was applied successfully to the influx, efflux, and hydrolysis of a cephalosporin, nitrocefin, and we found that the efflux occurs with the Michaelis–Menten saturation kinetics, with an apparent \( K_m \) value of about 5 \( \mu \)M (Figure 5A). There was little sign of positive cooperativity, a result that is interesting in view of the functional rotating mechanism of the pump discussed later in this chapter. A turnover number of about 10 \( s^{-1} \) was calculated. The low turnover number was as predicted in early studies, on the ground that the tripartite pumps may have to deal only with the small number of drug molecules that have trickled through the effective outer membrane barrier (7).

Interestingly, when the assay was extended to cephalosporins that were clinically useful, such as cephalothin, cefamandole, and cephaloridine, kinetics with strong positive cooperativity was observed (Figure 5B). Although it is not clear why such kinetics were not observed with nitrocefin, we note that nitrocefin has a much lower \( K_m \) value than that of these compounds. Interestingly, compounds showing strong cooperativity also have high \( V_{\text{max}} \) values, so the ratio of \( V_{\text{max}} \) to \( K_{0.5} \) (the substrate concentration giving a half-maximal rate, \( K_m \) in the case of noncooperative kinetics) did not change much for the four compounds tested (range: 0.5 to 1.9 \( \times 10^{-2} \) cm\(^3\) mg\(^{-1}\) s\(^{-1}\)), although the \( V_{\text{max}} \) for cephaloridine was 80 times higher than that for nitrocefin. The results on cephaloridine merit close attention for several reasons.

1. We have shown that cephaloridine cannot diffuse across the cytoplasmic membrane (15, 21). Thus, these results represent the kinetics of drug export initiated by the periplasmic capture of the drug.

2. The criterion for the evaluation of the range of substrates for an efflux pump was usually the comparison of MICs of the drug in wild-type cells and pump gene deletion mutants. By this criterion, the presence or absence of AcrAB made little difference to cephaloridine MICs (15, 70), so this compound was thought to be either a nonsubstrate or at best a marginal substrate for the pump. However, detailed kinetic analysis showed that cephaloridine may be pumped out very rapidly, with the turnover number approaching 1000 \( s^{-1} \) (71), although the
A. Nitrocefin

B. Cephaloridine

C. Cefazolin
$K_{0.5}$ value was very high (288 μM). Thus, the MIC comparison can be a poor and sometimes a misleading indicator of the pump function.

In this case, the AcrAB function made little difference to the MIC values, because the bacteria are killed at β-lactam concentrations usually below 5 μM (72, 73), and in this concentration range, the efflux is insignificant because of the high $K_{0.5}$ value and especially because of the strongly sigmoidal kinetics. The high turnover number for some substrates explains the observation that AcrAB can pump out simple solvents (39, 40), as these compounds must traverse the OM bilayer rapidly because they are small and uncharged and must be pumped out very rapidly by the efflux pump in order to produce resistance.

Interestingly, one cephalosporin, cefazolin, showed no sign of getting pumped out by the AcrAB pump (Figure 5C). This compound has very hydrophilic substituents (a tetrazole and a thiazole) at the 7- and 3-positions, respectively, and presumably the binding of the substrates to the AcrB binding site requires the presence of lipophilic moiety, preferably an aromatic ring, in at least one of these positions [see the article by Nikaido et al. (15)]. Kinetic constants for the AcrAB-mediated efflux of penicillins were also determined by a similar approach (73a).

Recently, binding of 24-, 25-, or 27-hydroxysterols to Niemann–Pick C1 protein (NPC1), a member of the RND family involved in cholesterol trafficking in vertebrate cells (74), was reported (75). The binding took place despite the presence of a high concentration (1%) of the detergent Nonidet P40, and the half-saturation was reached with about 0.1 μM of 25-hydroxysterol. 7-, 19-, or 20-Hydroxysterol did not bind to this protein. In comparison with bacterial RND pumps, the NPC1 protein has an N-terminal extension consisting of an extramembranous domain of about 240 residues and an additional transmembrane segment. This extramembranous domain is secreted from cultured cells as a soluble dimer, which bound 25-hydroxysterol with a strong affinity ($K_d = 10$ nM) (76). Since this domain is uniquely present in NPC1, it is unclear whether these beautiful

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Figure 5. Kinetics of AcrAB–TolC-catalyzed efflux of nitrocefin (A), cephaloridine (B), and cefazolin (C). The rates of efflux from the periplasm ($V_e$) are plotted against the periplasmic drug concentration ($C_p$) for each drug. Nitrocefin efflux follows a classical Michaelis–Menten kinetics, but cephaloridine efflux shows a sigmoidal kinetics. The rates of β-lactamase-catalyzed hydrolysis ($V_h$) for cephaloridine and cefazolin are shown for comparison. [From (71).]
results would help us in our effort to understand the ligand-binding process in efflux transporters (see Section IV.B).

D. GENETIC STUDIES OF AcrB AND ITS RELATIVES

To identify domains responsible for pump functions, large segments of genes were replaced with sequences coming from another homolog, producing chimeric genes. For example, Elkins and Nikaido (77) used *E. coli* AcrB, which shows strong resistance to ciprofloxacin, novobiocin, fusidic acid, erythromycin, and taurocholate, and AcrD, which generates no resistance to these agents except a modest one against taurocholate. Replacing both of the large periplasmic loops of AcrD with those from AcrB created a transporter pumping out all of the tested AcrB substrates efficiently. Replacing loops of AcrB with those from AcrD created a transporter that behaved like AcrD in terms of its substrate specificity. Finally, replacing the transmembrane regions of AcrD with corresponding sequences from AcrB did not alter the substrate specificity of the transporter. These results show that the substrate specificity is determined nearly entirely by the periplasmic domain (and that the critical binding of substrates presumably occurs here), and the transmembrane domain probably plays a role only in energy coupling. That the exchange of transmembrane domain does not alter the substrate specificity was also confirmed later by using *P. aeruginosa* MexB and MexY (78).

Tikhonova and co-workers (79) used AcrB and *P. aeruginosa* MexB. The results of chimeric exchange suggested that the substrate specificity was determined largely by the second external loop (called PC2 in AcrB) of these transporters, containing residues 612–849, a conclusion consistent with the location of the substrate-binding pocket identified in the asymmetric trimer crystal of AcrB (see Section IV.A). [This study was recently refined by the use of site-directed mutagenesis (80), which pinpointed the difference in macrolide extrusion activity to residue 616, which is Gly in AcrB and Asn in MexB]. They also showed that replacing the region coding for the transmembrane helices 8 to 12 of AcrB with the MexB sequence produced a somewhat MexB-like pattern for a few drugs (a lower efflux of ethidium bromide and lincomycin, and a higher efflux of cinnoxacin); currently, it is difficult to explain these results in terms of structure, although the elongation of helix 8 into the periplasmic domain appears crucial in the conformational change that leads to drug extrusion (Section III.C). Finally, this group showed that the N-terminal periplasmic loop
(PN1 and PN2) and the first part of the C-terminal loop (PC1) essentially
determine the interaction of the RND pumps with their cognate periplasmic
adaptor proteins, a conclusion that is in complete agreement with the recent
study based on the crystal structures (see Figure 4).

Use of point mutations could in principle allow us to produce more
detailed knowledge on the binding and export of substrates. In an approach
pioneered by Lomovskaya’s laboratory, random mutants of *P. aeruginosa*
MexD that acquired the ability to extrude carbenicillin, which is not
handled by the wild-type MexD, were isolated (81). All mutants mapped
to the periplasmic domain, none to the transmembrane domain, further
supporting the conclusion mentioned above. Among the residues identified,
Phe608 (corresponding to Phe610 of AcrB) is a part of the substrate-
binding pocket more recently identified (see below). Some others [Gln34,
Gln89 (corresponding to Glu 89 of AcrB), Asn673(Thr676)] are on the
walls of the large lateral cleft of the periplasmic domain, which is discussed
below (Section IV.B) as a potential site of periplasmic entry for substrates.
These residues are shown as space-filling models in the AcrB structure
(Figure 6).

Bohnert et al. (82) used an *E. coli* strain that lacked both AcrB and AcrF
and instead overproduced another RND pump, YhiV. A spontaneous
mutant obtained after repeated exposure to levofloxacin was shown to
owe its increased resistance to the change of an aliphatic Val610 residue of
the YhiV into an aromatic Phe residue. This mutant pump has an altered
specificity. It produces a stronger resistance to relatively small aromatic
compounds such as levofloxacin, linezolid, and tetracycline, but the resis-
tance to nonaromatic, bulky macrolides becomes weaker than in the parent
protein. This residue, which corresponds to Val612 of AcrB, is seen to be a
part of the substrate-binding pocket in the asymmetric AcrB trimer
structure (discussed in Section IV.A).

Middlemiss and Poole (83) used a different approach and carried out an
in vitro random mutagenesis of the *mexB* gene from *P. aeruginosa*. This is
a more comprehensive approach, but is expected to generate more “noise.”
Indeed, the group of mutants that decreased the level of resistance to most
drugs included the presumed proton relay mutants in Asp407 and Asp408
of the transmembrane domain (see Section III.B), or Gly220 mutant in the
“peg” that is inserted into the periplasmic domain of the neighboring
protomer. The mutants that are significantly altered in the substrate
specificity, in contrast, often contained alterations in the periplasmic
domain, as expected. Among these, alterations of Ala618 and Arg716
Point mutations can also be introduced into the transmembrane domain by site-directed mutagenesis. In 1999, inspection of amino acid sequence of an RND-family toxic cation efflux pump CzcA of *Ralstonia* sp. and other RND pumps showed that there are several conserved charged residues in (corresponding to Ala618 and Arg717 of AcrB) occur on the opposing walls of the large lateral cleft. However, there were several mutants in the transmembrane domain, and their phenotypes remain to be explained.

**Figure 6.** Large cleft of the AcrB periplasmic domain, seen from the outside. The opening of the cleft is indicated approximately by the large red ellipse. AcrB residues corresponding to those MexD residues affecting the substrate specificity (81) (Phe610, Gln34, Glu89, and Thr676) are shown in space-filling models, as well as those that were shown to affect drug pumping by site-directed mutagenesis (88) (Phe664, Phe666, and Glu673). The binding of drugs to the Asn109Ala mutant AcrB (88), discussed in Section III.A, is shown by stick models (ciprofloxacin, yellow; nafcillin, green; ethidium, magenta; rhodamine 6G, salmon; and Phe-Arg-β-naphthylamide, orange). (See insert for color representation.)
the transmembrane domain, including Asp402, Asp408, and Glu415 (84). Changing these residues into nonacidic residues abolished the cation pumping activity. This was followed up a few years later by the site-directed mutagenesis study of MexB (85). In MexB, the Asp402 and Asp408 of CzcA are replaced by a consecutive pair of aspartate residues, Asp407 and Asp408 (corresponding to Asp407 and Asp408 in AcrB) on transmembrane segment (TMS) 4, and both of them were found to be essential for the pump function. This study also identified an essential Lys939 (corresponding to Lys940 of AcrB) located in TMS 10 and suggested that these three residues produce a proton-relay network. Finally, we have identified Thr978 of AcrB on TMS 11 as another, functionally essential component of this network (86) (see Figure 10 below). (This study also identified Arg971, located close to the cytosolic end of TMS11, as an essential residue (86), although it is far away from the network of other charged residues.) These results emphasize the role of the transmembrane domain in proton translocation, which presumably provide energy for drug pumping by the periplasmic domain.

Site-directed mutagenesis was also applied to residues assumed to be involved in the binding or movement of substrates. When we reported on the crystal structures of AcrB with ligands in the central cavity of the transmembrane domain (87), we were aware of the potential problem that the ligand might be binding to any hydrophobic pocket of the protein, which has nothing to do with the normal pathway of the exported ligand. Thus, we changed residues that appeared to be involved in the binding through site-directed mutagenesis, and we obtained an apparently assuring result that conversion of Phe386 to alanine nearly totally abolished the resistance to ethidium, rhodamine 6G, and dequalinium (87). However, this result was obtained by using a very high copy number vector of the pUC series, and we are now aware that this approach may possibly produce misleading results by making the cell membrane leaky through strong overproduction of intrinsic membrane proteins. Indeed, Phe386Ala mutation hardly affected susceptibility of E. coli to most drugs when tested with expression by the vector pSPORT1 (88). Nevertheless, Hearn and co-workers (89), using a Pseudomonas fluorescens homolog of AcrB, EmhB, expressed from a medium copy number plasmid, found that decreased efflux of dequalinium was produced by the same change (Phe386Ala) as well as the change of Asn99 to alanine. Furthermore, changing Asp101, which is located at the “ceiling” of the central cavity, into alanine decreased the efflux of most drugs as well as of polycyclic hydrocarbons.
With AcrB mutant Asn101Ala, described later in Section III.A, the efflux activity was decreased by changing into alanine the residues Glu673, Phe664, or Phe666, all located in the right (looking into the center of molecule with the periplasmic domain up) wall or the bottom of the large periplasmic cleft, close to its entrance (89) (Figure 6). With the Cu\(^+\) and Ag\(^+\) RND-type efflux pump CusA of *E. coli*, changing into leucine the methionine residues 573, 623, and 672, all close to the entrance of the periplasmic large cleft in the AcrB model, strongly reduced its activity (90). Together with the binding of substrates to this cleft observed in the Asn101Ala mutant AcrB (89), described below, these results favor the assumption that the periplasmic uptake of substrates by these pumps involves the transient binding to, and then entry from, the large periplasmic cleft.

Dastidar et al. (91) introduced cysteine residues to various positions of *Haemophilus influenzae* AcrB, chosen on the basis of their effects on substrate specificity in MexD (81) (described above), and measured, in intact cells, their accessibility to a relatively hydrophilic probe, fluorescein maleimide in the presence and absence of efflux substrates such as erythromycin, novobiocin, cloxacillin, ethidium bromide, Triton X-100, and the inhibitor Phe-Arg-\(\beta\)-naphthylamide. Most substrates, except ethidium, inhibited strongly the modification of cysteine at position 288, which corresponds to Gly290 of AcrB that is close to the bottom of the substrate-binding pocket in the asymmetric trimer structure (described in Section IV.A). Interestingly, the modification of cysteine at position 601, corresponding to Phe617 of AcrB, one of the residues lining the substrate-binding pocket, is inhibited by Triton X-100, but not by other substrates. These data suggest the large size (and/or the flexibility) of the binding pocket that would accommodate different ligands in different ways (see also Section IV.B).

Murakami et al. (70) used cysteine scanning mutagenesis to examine the role of the narrow central “pore” of the trimer, which appears to be closed in the crystal structure of AcrB. Although alterations of the residues facing the center of the pore, including Asp101 just mentioned, were found to decrease the extrusion of drugs, the interpretation is complicated by the fact that the pore is made by a three-stranded coiled-coil structure composed of all three protomers of AcrB, so that intersubunit disulfide bonds are formed in some cases. Now that the drug-binding site and the likely drug extrusion pathway were discovered within the AcrB protomer (Section III.C), it seems unlikely that the interprotomeric central pore plays a direct role in substrate movement.
E. MEMBRANE VESICLES AND PROTEOLIPOSOME RECONSTITUTIONS

In the biochemical studies of most bacterial transporters, the most useful approaches were often the use of membrane vesicles, either right-side-out or inverted. However, with RND family multidrug efflux pumps, this approach was not successful, although it was pursued vigorously in several laboratories, including our own. The likely reasons for this failure include the hydrophobic nature of most substrates, which allows a spontaneous equilibration of transported substrates across the membrane bilayer. Furthermore, “transport” by RND transporters may not change the transmembrane location of the substrates since the major pathway of substrate capture is from the periplasm. Another possibility may be that the periplasmic adaptor protein, which seems to be necessary to activate the transporter (see below), becomes stripped off during the preparation of the membranes.

The first successful functional reconstitution of an RND pump was achieved by Zgurskaya and Nikaido (67) in 1999. Cells containing *acrAB* genes on a high-copy-number pUC plasmid were grown without induction, and the membrane proteins were solubilized with 5% Triton X-100 overnight in the cold. The native AcrB protein contains four histidine residues among the eight residues at its C-terminus, and thus the protein could be purified by adsorption to a Cu$^{2+}$-chelate matrix followed by elution with imidazole, always in the presence of Triton X-100. Just before the reconstitution, AcrB was again adsorbed to the Cu$^{2+}$-chelate matrix, and Triton X-100 was exchanged with octyl-$\beta$-D-glucoside by elution with a buffer containing the latter detergent as well as imidazole. Since AcrB formed aggregates in octylglucoside within a few hours, it was necessary to carry out reconstitution into proteoliposomes immediately by the octylglucoside dilution method.

Innovative approaches were required for assays of the transport activity of AcrB, because most of the substrates of AcrB are quite hydrophobic (7) and are expected to diffuse spontaneously across the membrane bilayer, as mentioned above. Thus, it was impossible to rely on the quantitation of ligands accumulated within the intravesicular space. Inspired by the report that mammalian P-glycoprotein, which also transports hydrophobic compounds, has activity as a phospholipid flippase (92), we added 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled fluorescent phospholipid to the phospholipid mixture used in proteoliposome reconstitution. Phospholipids, however, are likely to become reinserted into the original bilayer even when
they are expelled from it by the AcrB pump. To minimize the effect of this process, an excess of “acceptor” liposomes, which did not contain AcrB, were used to “trap” the fluorescent phospholipids extruded. Finally, the amount of the NBD-labeled phospholipids remaining in the AcrB-containing “donor” proteoliposomes was estimated by initially quenching the NBD fluorescence through the fluorescence energy transfer to rhodamine-labeled phospholipids added initially (together with NBD-labeled phospholipids) to the proteoliposome reconstitution mixture.

Phospholipid extrusion, as indicated by time-dependent increase in NBD fluorescence, required the addition of Mg$^{2+}$ (Figure 7), which is consistent with the observation that in intact cells, another RND-family multidrug efflux pump MexXY requires the presence of Mg$^{2+}$ in the

![Figure 7. Phospholipid extrusion assay of reconstituted AcrB. When a transmembrane pH gradient was generated by diluting the vesicles (internal pH, 7.0) into a buffer of pH 6.0, donor vesicles not containing any AcrB (bottom trace) do not show much change in fluorescence, nor do AcrB-containing vesicles in the absence of pH gradient (middle trace). However, when AcrB-containing vesicles are exposed to pH gradient, there is an expected gradual increase in NBD fluorescence in the presence of Mg$^{2+}$. Note that the rate of fluorescence increase is strongly accelerated if AcrA (15 µg/mL) is also added. [From (67).]
medium for its function (93). Remarkably, strong pumping also required the addition of lipid-free AcrA. This result was interpreted as the result of AcrA protein connecting the donor and acceptor vesicles (67). However, knowledge of the structure of AcrA (see Figures 3 and 4) makes this hypothesis unlikely, because AcrA can connect to the second membrane only through TolC, which was not present in our system. Rather, AcrA could have activated the pumping activity of AcrB directly, in view of the fact that AcrD, a close homolog of AcrB, is activated in a reconstituted system by AcrA (64) (see below).

To show more directly that AcrB functions as a proton/drug antiporter, the system was energized by using the valinomycin-induced efflux of $K^+$, which was converted into an interior-acidic proton gradient in the presence of KCl. By measuring the intravesicular pH with a fluorescent, membrane-impermeable pH indicator pyranine, we confirmed that proton efflux occurred accompanying the pH-gradient-driven operation of the pump in the presence of substrates (67) (Figure 8). When the number of protons moved per vesicle is calculated, it appears that the AcrB pump is functioning extremely slowly, with a turnover rate of less than one per minute. We note that the assay was carried out without the addition of Mg$^{2+}$ and AcrA, which were needed for the activation of AcrB according to the fluorescent phospholipid extrusion assay. Possibly an even more important factor could have been the location of the substrates, which were added to the extravesicular space that corresponded to cytosol as the acidified vesicle interior corresponded to the periplasm. Thus, it seems likely that we were measuring the slow spontaneous influx of substrates into the intravesicular space, followed by their capture by the periplasmic domain of AcrB.

The affinity of substrates for AcrB was estimated in two ways. First, the substrates were added as potential competitors in the fluorescent phospholipid efflux assay. This showed that conjugated bile acids, such as taurocholate, were the most effective inhibitors of phospholipid efflux. Second, the potential substrates were added in the proton efflux assay, and again taurocholate was the most effective in producing proton efflux among the compounds tested (Figure 8). These results fit the notion that the natural substrate for the AcrB pump are the bile salts, most of which exist in the conjugated form in the intestinal tract of higher animals.

Soon afterward, the successful reconstitution of another RND pump, the CzcA protein of *Ralstonia*, was reported (83). CzcA is not a multidrug pump, but catalyzes the export of toxic divalent cations such as $Zn^{2+}$, $Co^{2+}$, and $Cd^{2+}$. The PCR-amplified gene was inserted into a commercial
vector pASK-IBA3, which supplies a C-terminal, eight-residue tag. The protein was purified by affinity chromatography with a modified streptavidin matrix that binds the C-terminal tag (94). Solubilization and purification were performed in the presence of phospholipids (95). The purified CzcA was mixed with Triton X-100-treated liposomes, and the proteoliposomes were formed by removal of the detergent with Bio-Beads. A pH gradient was imposed across the membrane by diluting proteoliposomes containing 0.1 M KCl into 0.1 M NaCl and then by adding valinomycin (arrow). The acidic internal pH was maintained for several minutes in the absence of drug (curve 1). When erythromycin (curve 2), chloramphenicol (curve 3), cloxacillin (curve 4), glycocholate (curve 5), or taurocholate (curve 6) were present at 0.2 mM, the internal pH returned to its initial value more rapidly, presumably due to the proton efflux accompanying the influx of drugs by the AcrB antiporter. [From (67).]

Figure 8. Proton flux assay of reconstituted AcrB. AcrB-containing vesicles were made in the presence of 1 mM pyranine, a fluorescent pH indicator, and the extravesicular pyranine was removed by gel filtration. The interior-acidic pH gradient was generated by diluting the vesicles containing 0.1 M KCl into 0.1 M NaCl and then by adding valinomycin (arrow). The acidic internal pH was maintained for several minutes in the absence of drug (curve 1). When erythromycin (curve 2), chloramphenicol (curve 3), cloxacillin (curve 4), glycocholate (curve 5), or taurocholate (curve 6) were present at 0.2 mM, the internal pH returned to its initial value more rapidly, presumably due to the proton efflux accompanying the influx of drugs by the AcrB antiporter. [From (67).]
with Zn\(^{2+}\) were reported to show a sigmoidal kinetics with a Hill coefficient of 2, although only four concentrations of the substrate were used. These data, unfortunately, may not have much physiological relevance because the half-maximal rate was achieved at an absurdly high concentration of 6.6 mM, where micromolar (or even lower) values of \(K_{0.5}\) were expected. Furthermore, the experiment measured the movement of cations from outside (corresponding to cytosol) to intravesicular space (corresponding to periplasm), whereas the real pump is likely to export substrates from periplasm to the external medium. Indeed, we now know that a similar cation pump, CusAB system captures ions such as Cu\(^+\) from the periplasmic binding protein CusF before their efflux (96, 97).

Reconstitution of another multidrug efflux RND pump AcrD was achieved in 2005 (64). AcrD was of special interest to us, as some of its substrates, aminoglycosides, are very hydrophilic and are not expected to cross membrane bilayers spontaneously. AcrD, in a form with a C-terminal hexahistidine tag, was expressed from a high-copy-number plasmid with isopropyl-\(\beta\)-D-thiogalactoside (IPTG) induction, and was purified in dodecylmaltoside. The AcrD preparations in dodecylmaltoside were used directly for proteoliposome reconstitution by octylglucoside dilution method, without the prior removal of dodecylmaltoside. Routine assays relied on the proton efflux determined with the intravesicular pH indicator pyranine, as described above. It was soon discovered that no acceleration of proton flux occurred, with aminoglycosides (70 \(\mu\)M) either outside or inside vesicles, unless AcrA (with the lipidation site removed) was added at the time of reconstitution, and thus to the interior of the vesicles. It was known that AcrA was required for the activity of AcrD in intact cells (77), but this was expected because AcrA (or one of its homologs) would be needed for the construction of a tripartite efflux machinery. In the proteoliposomes containing AcrD, a tripartite structure is not needed for efflux, and the result strongly suggested that AcrA and its homologs of the MFP family had another important function in directly activating the pumping function of the cognate RND efflux transporter. A similar situation was found more recently with the MFP MacA for the activation of an ABC efflux transporter MacB (30).

Results obtained with streptomycin are shown in Figure 9. When streptomycin was added to the external medium, the drug is occupying a more alkaline compartment that is similar to the cytosol in intact cells. Under such a setup, we could not detect stimulation of proton efflux (curve 2); however, a strong stimulation was observed when streptomycin
was added to the more acidic intravesicular space, corresponding to the periplasm (curve 3). These results are important, as they were the first results showing that at least one drug was captured exclusively from the periplasm. Adding kanamycin, tobramycin, gentamicin, or amikacin to the interior of the vesicles similarly produced a strong increase in the efflux of protons (64). However, these aminoglycosides seemed to produce some stimulation even when they were added to the outside medium, and we then concluded that these drugs may be captured both from the periplasm and from the cytosol. However, examination of the data (64) shows that the stimulation tended to be marginal or small when the drugs were added to the outside medium, and I now believe that data were overinterpreted at that
time and that the capture occurs only from the periplasm with all aminoglycosides. We tested the intravesicular accumulation of $[^3$H]gentamicin by adding it to the external medium (64). Although the vesicles seemed to accumulate the drug slowly, the background accumulation was also significant, and the calculated turnover number of AcrD was in the range of 0.01 s$^{-1}$. This is insignificant in comparison to the now determined turnover number of AcrB for cephalosporins, between 10 and 1000 s$^{-1}$ (Section II.C), and it fits with the notion that there is little evidence for the capture of aminoglycosides from inside the cytosol.

III. Structure of AcrB

A. SYMMETRIC STRUCTURES OF AcrB

Crystallography supplied essential pieces of knowledge on the mechanisms of the RND pump complex. The crystallographic structures of the outer membrane channels and of the periplasmic adaptor proteins were described briefly in Section II.B. Again, as mentioned earlier, Murakami et al. solved the structure of AcrB as a homotrimer with a threefold rotational symmetry in 2002 (44) (Figure 3), the first crystallographic structure for a proton-motive-force-driven transporter solved.

We mentioned earlier that AcrB probably captures its substrates from the periplasm, and the prediction was made in the early days that drugs may first partition into the membrane–periplasm interface (Figure 1). Indeed, the AcrB trimer structure showed that there was a small opening (vestibule) between subunits at the bottom of the periplasmic domain, close to the external surface of the membrane bilayer (44), and this led to the top of the large central cavity in the transmembrane domain (Figure 3). It was thus hypothesized that the drugs diffused through the vestibule into the central cavity, where it was captured. Indeed, co-crystallization of AcrB with various drugs (rhodamine 6G, ethidium, dequalinium, and ciprofloxacin) produced crystals with drug molecules within this central cavity (98). However, our conclusions were less than watertight. First, the resolution was poor (3.5 to 3.8 Å), and thus the identification of the drug molecules could not be made with absolute confidence, although drugs with polyaromatic structures were used precisely for the ease of detection in the crystal structure. Second, the wall of the central cavity, where these drugs were thought to be bound, appeared quite open and totally unlike the traditional ligand-binding sites. To explain drug binding to such sites, we
had to propose the existence of “composite” binding sites with the involvement of head groups of phospholipids that were assumed to be present in the central cavity (87). To confirm that the bound drugs were on their correct path to export, we applied site-directed mutagenesis to change Phe386, which seemed to be close to most substrates, into Ala, and found that the efflux activity was nearly abolished (87). However, as described already (Section II.D), this result is likely to be an artifact resulting from the use of high-copy-number vectors. Thus, our earlier conclusion that the drugs bind to the walls of the central cavity is not totally convincing. Nevertheless, the AcrB–YajC co-crystal obtained accidentally from E. coli grown in ampicillin-containing medium (99) was reported to contain two ampicillin molecules per AcrB protomer at this position near the ceiling of the central cavity, although the resolution was again poor (3.5 Å) and it is difficult to imagine that these ampicillin molecules survived attack by the high levels of plasmid-coded β-lactamase during production and purification of the protein.

In one symmetric structure of AcrB, the revealed N-terminal structure was shown to narrow the cytosolic opening of the central cavity somewhat (100). However, it is unclear whether this has any bearing on the function of the pump, as the role of the central cavity in substrate binding is uncertain, as described above.

We obtained another set of drug co-crystallization data with a site-directed mutant of AcrB, Asn109Ala (88). In the simple hypothesis we had at that time for drug extrusion, the drug molecules are captured near the ceiling of the central cavity, enter the central pore bordered by three helices (corresponding roughly to Asp99 through Leu117) from the three protomers, and pass through this pore to reach the top of the periplasmic domain (see Figure 3). However, the pore in the crystal structure appears closed. Since the side chain of Asn109 appears to extend deeply into the center of the pore, we naively thought that changing it to alanine might weaken the interaction of helices from the three subunits and “loosen up” the pore. The mutant AcrB functions almost normally as judged from the resistance levels to several antimicrobial agents. Interestingly, when the mutant protein was crystallized in the presence of several ligands [ethidium, rhodamine 6G, ciprofloxacin, nafcillin, and the inhibitor Phe-Arg-β-naphthylamide (41)], ligands were found to be bound not only to the ceiling of the central cavity [as seen earlier (98)] but also to the periplasmic domain, near the entrance of the large, deep, external cleft (Figure 6). Again, there was the problem that the ligands could have become bound to
any lipophilic pocket, which may not be on the proper pathway for export. Changing residues that appeared to be interacting with the ligands into alanine through site-directed mutagenesis produced a drastic decrease in resistance to most drugs in the case of Glu673, and more modest, yet significant, decreases with Phe666 and Phe664. Although we cannot exclude the possibility that they may inactivate the transporter by a mechanism unrelated to ligand binding, the more recent asymmetric AcrB structures (discussed in Section III.C) are not inconsistent with the hypothesis that this is the portal for the periplasmic entry of substrates. The putative pathway for the drugs to the binding site is closed in the “access protomer” (see below), which resembles the protomers in the symmetric trimer in conformation; thus, the drug molecules cannot go further and may be forced to interact with the entrance of this pathway in the symmetric structure. Furthermore, there are random and site-directed mutation data that suggest the importance of residues within the cleft (Section II.D) as well as the recent data using Cys mutants (see below).

Finally, it should be noted that the symmetric AcrB crystals soaked in deoxycholate contained deoxycholate molecules within the external cleft of the periplasmic domain (101), in a position essentially identical to the various substrates mentioned above.

B. ATTEMPTS TO DETERMINE THE STRUCTURE OF AcrB DURING THE DRUG EXPORT CYCLE

Because of the uncertainties involved in the modeling of ligand electron density and in the possibility of nonspecific interaction between the ligand and the protein surface, we tried a different approach. We crystallized AcrB mutants in which one of the transmembrane domain residues putatively involved in proton translocation, Asp407, Asp408, Lys940 (corresponding to Lys939 of MexB), or Thr978 (see Section II.D), was changed to Ala (86, 102). In the crystal structure (44), the side chains of these residues are very close and appear to interact with each other either by salt bridge or hydrogen bonding (Figure 10). We assumed that during the normal function cycle of AcrB, a proton antiporter, one of these residues will be altered in its protonation state, most probably by the protonation of Asp, and this may disrupt the tight interactions among these residues and eventually, cause conformational changes of the entire protein, which would be coupled to ligand export. If so, our mutant proteins would mimic this transient, protonated intermediate state during the process of drug extrusion, and
allow us to see this elusive structure that cannot be deduced from the ground-state structures of the wild-type protein. When the crystal structures (without ligands) of these mutant proteins were examined (102), we found the following.

1. The tight interaction between Asp407, Asp408, Lys940, and Thr978 indeed became disrupted, and the Lys940 side chain, which interacted strongly with both of the carboxyl groups of Asp407 and Asp408 in the wild-type AcrB, went through a strong flipping motion and now faced away from these residues (Figure 10). Remarkably, the alterations were quite similar regardless of which residue was changed to Ala (102).

2. An extensive conformational alterations were found in the transmembrane domain (102). Again, it was reassuring that the conformational alterations were similar in all four mutants, confirming that they were
due to the disruption of the salt bridge/hydrogen-bonding network among these four residues rather than to specific effects of individual mutations. Most of the changes involved the shortening or melting of helices, except that helix 8, one of the helices connecting the transmembrane domain to the periplasmic domain, became extended into the periplasmic domain (102).

3. When the structure of the periplasmic domain was examined, however, there were only very small changes (102), a disappointing result because we expected major conformational alterations in the periplasmic domain during substrate binding and extrusion (see above). The reason for this failure was suggested from the studies describing the asymmetric trimer structure of AcrB, described below.

C. SOLUTION OF THE ASYMMETRIC AcrB TRIMER STRUCTURE

At about the same time as our study of the proton-relay network mutants, several papers appeared describing the asymmetric trimer structure of AcrB (103–105) (Figure 11). In contrast to the symmetric structures seen before, in these structures each protomer, especially its periplasmic domain, takes a unique conformation (Figure 11). All groups identified one protomer in which a pocket is opened up in the periplasmic domain, a likely substrate-binding site, and in the study by the Murakami group (103), this pocket was indeed found with a bound substrate molecule (minocycline or doxorubicin). The interpretation of electron density as the bound substrate was strengthened by using minocycline that was labeled with a heavy atom, bromine. This protomer is called the binding protomer by the Murakami group or T (for “tight”) by others. Other protomers are called the access (or L for “loose”) and extrusion (or O for “open”) protomers. In the extrusion protomer, which shows the largest conformational alteration, the binding pocket becomes collapsed, and there appears to be an opening of the pathway from the binding pocket to the top (central funnel) of the periplasmic domain. Inspection of the structures of the transmembrane domains shows that the tight association of Asp407–Asp408–Lys940–Thr978 becomes disrupted in the extrusion protomer, probably as a consequence of the protonation of Asp407 and/or Asp408; indeed, the arrangement of these side chains here is nearly identical to that found in our mutant proteins (102) (Figure 10, green sticks). This is consistent with our hypothesis that the disruption of the
Figure 11. Periplasmic domain of the asymmetric crystal structure of AcrB (A) and the functionally rotating mechanism proposed (B), from studies of Murakami et al. (103), drawn using Pymol from PDB file 2DRD. The view is from the top (periplasm), and the transmembrane domains were removed for clarity. The three protomers, each with a distinctive conformation, are shown in different colors. In part A, the binding protomer (green) contains the bound ligand minocycline (in a stick model). When the protonation of the Asp407, Asp408, or both changes the alignment of proton relay residues shown in Figure 10, the conformational alteration is transmitted to the periplasmic domain, which is changed into an extrusion protomer, which extrudes the drug molecule outward. After reprotonation of Asp residue(s) in the transmembrane domain, the initial conformation of the Access protomer is restored. Thus, each protomer was proposed to go through cyclic changes of conformation (part B, where each protomer retains its initial color scheme) in a functionally rotating manner (103–105). Note that the large external cleft of the periplasmic domain becomes closed in the extrusion protomer. (See insert for color representation.)
network among the four residues mentioned alters conformation of the periplasmic domain. However, the asymmetric structure also shows that extensive conformational changes in the periplasmic domain require complementary accommodation by the neighboring protomers (103–105). This was not possible in our construct, in which all protomers corresponded to the mutant protein.

As mentioned already, the extrusion protomer is the subunit where the conformation of the periplasmic domain is altered most strongly from the conformation seen in the symmetric trimer. Presumably, this conformational alteration is driven by the extension of helix 8 toward the periplasm. This change, in turn, is caused by disruption of the salt bridge/hydrogen-bonding network among Asp407, Asp408, Lys940, and Thr978, as seen in the structures of both the wild-type protein in the asymmetric model (103–105) and the structure of the proton-relay network mutants (102).

The asymmetric structure was important because the structure of one single trimer suggests three stages in the drug extrusion process. Thus, the protomers appear to go through a cyclic conformational change, from the open, access conformation (which would allow the access of substrates), through the ligand-bound, binding confirmation, and finally, the extrusion conformer, which propels the substrates out and whose transmembrane domain shows signs of disrupted network among proton-translocating residues (103–105) (as shown in Figure 11B). Thus, this mechanism is called the functionally rotating mechanism; it is reminiscent of the mechanism of F$_1$-ATPase, although there is nothing that corresponds to the physical rotation of its $\gamma$-subunit. The “power stroke” of this entire process occurs during the change of binding into extrusion conformation, which involves the largest movement of various sections in the periplasmic domain. This large conformational alteration is initiated apparently by the protonation of one or both of the Asp residues (Asp407 and Asp408) in the midst of the transmembrane domain, which leads to a significant elongation of TM helix 8 into the periplasmic domain and all the subsequent conformational changes in the periplasmic domain.

Recently, the reactivity of the carboxyl groups in AcrB was assessed by labeling with a hydrophobic reagent, dicyclohexylcarbodiimide, followed by CNBr cleavage of the protein and analysis of the fragments by mass spectrometry (106). It was found that Asp408 was especially reactive to carbodiimide, with a high $pK_a$ value of 7.4, implicating the protonation/deprotonation of at least this Asp residue as a key step in the proton
translocation pathway, and eventually, in generating conformational alterations in AcrB that leads to drug extrusion.

IV. Mechanism of Drug Efflux

A. MAIN SUBSTRATE-BINDING SITE

The possible route of substrate binding and extrusion is suggested by the structure of the asymmetric trimer (103–105). In the binding protomer, the periplasmic domain contains an expanded binding pocket containing several aromatic and hydrophobic residues [Phe136, 178, 610, 615, 617, and 628; Val139 and 612; Ile277 and 626; and Tyr327 (104)]. This pocket was indeed found to contain the drugs minocycline and doxorubicin (103), and its role is also supported by the mutation data with the AcrB homolog YhiV (82) previously described as well as the more recent data on AcrB (107). In the latter study, each of the Phe residues in the binding site was changed to Ala by site-directed mutagenesis. Among them, Phe610Ala mutant became strongly more susceptible to almost all drugs, and Phe178A and Phe615Ala had a somewhat less extensive effect. Changing Phe628 and Phe136, and especially Phe617, decreased the AcrB activity only to a limited range of substrates, perhaps understandably, as these side chains occupy a peripheral position in the binding pocket.

The significance of this binding site is also suggested by the observation that the pocket becomes collapsed in the extrusion protomer. Finally, its physiological significance is supported further by a comparison of its surface in AcrB and in the homology-modeled AcrD (36); although the surface is entirely hydrophobic in AcrB, in the aminoglycoside pump AcrD it contains many oxygen atoms that may function in the binding of the hydrophilic basic substrates.

B. PATHWAYS FOR DRUGS: A CURRENT HYPOTHESIS

How do the substrates reach the binding pocket? All three groups that studied the asymmetric trimer structure agree that there is an open pathway in the binding protomer between the binding pocket and the external large cleft of the periplasmic domain of the binding protomer (102–104) (see Figure 12). Although this cleft was earlier thought by some investigators to be the site of interaction between AcrA and AcrB, in the most up-to-date model of AcrA–AcrB interaction this cleft remains fully open (53) (Figure 4). [The entry of the substrate from the cleft was advocated by Lomovskaya and...
We have recently examined experimentally the path(s) of the drugs within the periplasmic domain of AcrB (108a). We first changed each of the 48 residues that may line the drug path into Cys, and tested if the Cys residue was covalently modified by fluorescein maleimide. This reagent did not label sites obviously outside the path, even when they were surrounded by hydrophobic residues; thus the labeling appears to occur only when the reagent behaves as the AcrB substrate, and becomes concentrated in the drug pathway. Some of the residues thus identified were also shown to

Figure 12. Cutout view of the binding protomer with the bound minocycline in a green stick representation (A) and the extruding protomer (B), both from PDB file 2DRD, drawn using the UCSF Chimera package (133). The wide passageway from the external cleft (dashed green arrow) seen in A becomes closed in B. The passageway in A was also enlarged, so that the extremely hydrophobic surface in this area can be seen more easily. [Modified from (36), with permission from Elsevier.] (See insert for color representation.)
block the efflux of Nile Red when convalently modified by bulky substituents, further confirming their location in the substrate path. In this manner, we showed that, in addition to the residues lining the binding pocket identified in the asymmetric crystal (103), the residues at the entrance of the large lateral cleft (Figure 6) were also in the substrate path, in support of the idea that the substrates mainly enter the AcrB periplasmic domain from the lateral cleft and then reach the binding pocket.

The Murakami group found, in addition, the second pathway, which connects the binding site with the vestibule, the passageway between protomers, located close to the external surface of the inner membrane. A similar pathway between the binding site and the vestibule area could also be seen in the Darpin-containing crystal, which so far has shown the highest resolution (105). Thus, there is a possibility that the substrate may go through the initial part of the vestibule and then go into the binding pocket.

In the extrusion protomer, because of the conformational changes probably initiated by the protonation of Asp408 in the transmembrane domain, the binding pocket becomes narrower and the entrance pathway(s) become closed, and at the same time a new pathway from the binding pocket to the funnel-like structure at the top becomes open (Figure 12). This would favor the extrusion of drug molecules into the TolC channel.

Thus, the basic principles of the drug pumping mechanism, based on the functionally rotating model, are now reasonably well understood. However, some details are still at the stage of speculation. For example, for the drug extrusion to occur with a reasonable efficiency, the drug molecule must remain in the binding pocket for some time. How would this be achieved when both the drug entry pathway and the pocket are surrounded primarily by hydrophobic side chains (Figure 12)? Furthermore, how does the AcrB pump accommodate such a wide range of substrates, some of which are shown in Figure 2? The answer to these questions is not immediately apparent from the publications describing asymmetric crystal structures (103–105), because the only drug molecules found in co-crystals are minocycline and doxorubicin, despite trials with many dozens of drugs by the Murakami group. Because of this situation, we have tried a computer-simulated docking approach (108b). With the binding protomer of minocycline-bound AcrB (103), the binding of about two dozen known AcrB substrates was estimated. Minocycline was shown to bind to the apoprotein in a location and conformation found in the crystal structure,
although this was not the complex predicted to be the most stable. This result validates the use of the computational approach, at the same time suggesting that the energy scoring function of the program may not yet be perfect. Currently, there is no way to include the movement of the backbone of this rather flexible protein in simulation. Thus, the details of the binding interactions of drugs predicted give us only broad hints. Nevertheless, examination of the possible binding modes of many substrates was instructive, in a way somewhat similar to the usefulness of comparison of many homologous sequences. Many substrates (including minocycline, tetracycline, and nitrocefin) prefer to bind, at least in this modeling exercise, with their hydrophobic domain bound to the narrow groove of the binding site, with their hydrophilic group(s) exposed either at the “top” and/or “bottom” minipockets within the site. These subdomains are much more hydrophilic than the central groove. This is very similar to the binding of minocycline (103) (for the structure, see Figure 2), which is bound to the groove mainly by using its hydrophobic middle of the molecule and has its one polar domain (containing the acidic OH, an amino group, and an amide group) on top and its other polar group (an amino group) at the bottom. Murakami et al. (103) argue that the polar groups on top interact with an Asn274 side chain, and the amino group at the bottom interacts with Gln176. However, none of these groups are at hydrogen-bonding distance, and are at best 4 to 5 Å away. Thus, it seems best not to think of these interactions as being specific. Rather, the top and bottom of a minocycline seem to fit into more hydrophilic, larger minipockets which may even be filled with water.

Interestingly, some ligands are predicted to bind nearly exclusively to the bottom pocket and its neighboring hydrophobic patch, completely avoiding the central groove where the bulk of minocycline binds. This group includes the inhibitor NMP (naphthylmethylpiperazine (109)) as well as chloramphenicol. These observations are consistent with the finding that the efflux of nitrocefin, a groove-binder, is inhibited by the simultaneous presence of another groove-binder, minocycline, but not at all by the presence of a cave-binder, chloramphenicol (102b).

In this light, we can imagine the movement of substrates as follows. Initially, substrates are attracted to the hydrophobic surfaces of the pathways, either from the periplasm through the large external cleft of AcrB periplasmic domain or possibly from near the external surface of the plasma membrane through the vestibule. However, the protein–substrate complex is not in its most stable conformation here, because the hydrophilic part of the substrate is not stabilized optimally. The final stabilization
is achieved at the binding site, because of the presence of two features here: one, interface(s) for hydrophobic interactions, and the other, rather large hydrophilic pockets next to it. The substrate range of AcrB is likely to be determined essentially by this structure of the binding site. The substrates bound here is then ready for extrusion through the conformational change of the binding protomer into the extrusion protomer.

C. BIOCHEMICAL STUDIES OF CONFORMATIONAL ALTERATIONS

Although the structure of the asymmetric AcrB trimer strongly suggests the mechanism of ligand extrusion coupled to proton influx, this mechanism remains a hypothesis. We set out to prove this hypothesis by carrying out biochemical studies of the pump. The proposed cyclic change involves the opening and closing of the large external cleft in the periplasmic domain (Figure 11). Thus, if the opening of the cleft is prevented, we can predict that the cyclic conformational changes will be prevented and that the pump will cease to function. We introduced pairs of cysteine residues at various positions on the opposing walls of the cleft (110). Although single cysteine mutations produced little inhibition of transport, proteins containing double mutations (Asp566Cys and Thr678Cys; Phe666Cys and Thr678Cys; Phe666Cys and Gln830Cys) on the opposing walls of the cleft were strongly compromised in function. This is likely to be due to the forced closing of the cleft by disulfide bonds formed between these residues, but we cannot rule out the possibility that the trimeric assembly failed because of the premature formation of disulfide bonds between protomers. Indeed, with cysteine pairs at other positions, there were data suggesting this interpretation. To avoid this problem, we expressed mutated AcrB in a host strain with a defective DsbA, a periplasmic disulfide oxidoreductase that plays a major role in the formation of disulfide bonds in this compartment (111, 112). Indeed, in this strain, the AcrB protein containing Phe666Cys and Thr678Cys (or Gln830Cys) largely retained its transport activity, presumably because disulfide bond formation did not occur in the absence of DsbA enzyme activity. We tried to see, by using these mutants, if the cross-linking and inactivation of AcrB can be observed in real time by using a fast-acting disulfide cross-linking agent based on the methanethiosulfonate groups (113). Indeed, addition of such cross-linkers to cells that are pumping out a fluorescent dye, ethidium, was shown to stop the function of the pump instantaneously (110), thus providing biochemical support for the rotating mechanism of drug transport.
A similar approach was also pursued by the group of K. M. Pos (114). They introduced pairs of cysteines at a wide range of positions, importantly including those that were expected to become closer in the access or the binding protomers, in addition to those in the extrusion protomer. The double cysteine mutant of Ser132 and Ala294, which are expected to become closer only in the access protomer, inactivated the pump as estimated by the decreased resistance to a number of drugs, and the pumping activity was increased to a detectable extent upon the addition of disulfide-reducing agent dithiothreitol to intact cells (114).

D. USE OF COVALENTLY LINKED TRIMERS

The functional rotation mechanism of AcrB drug pumping process (103–105) predicts that if one of the three protomers is defective in the proton relay network, the pumping action by the entire trimer should come to a halt. This hypothesis could not be tested by the disulfide cross-linking experiment described above, because all protomers had the same Cys mutations. We devised a way to test this hypothesis by creating a giant gene in which three acrB sequences were connected together through a linker sequence (115). When the sequence of the cytosolic, horizontal helix (Met496 through Arg540) connecting the two halves of the transmembrane domain was used as the linker, the linked trimer was expressed well as a single giant protein and produced drug resistance levels sometimes even higher than that produced by the monomeric acrB gene. When only one of the three-component acrB sequences in the giant gene was changed to include mutations in the proton-relay network, the entire trimer became inactive. When only one of the three acrB sequences was made to contain the Phe666Cys and Gln830Cys, cross-linking of these two cysteine residues by a fast-acting methanethiosulfonate cross-linker instantaneously inactivated the entire trimer, regardless of the position of the altered protomer in the giant sequence (Figure 13). These results strongly support the notion that the trimer acts by a functionally rotating mechanism.

E. RND SYSTEMS APPARENTLY REQUIRING PAIRED TRANSPORTERS

The RND transporters we have discussed so far, AcrB, MexB, or AcrD, exist as homotrimers and are now assumed to work by a functionally rotating mechanism. There are, however, RND transporter genes that occur in tandem, such as MdtBC, discovered by our group (116) as well as by
Both groups showed that both of the RND transporters, MdtB and MdtC, had to be present for the full pumping activity. Interestingly, Baranova and Nikaido (116), who relied on the expression of these genes from the chromosome, found that MdtB or MdtC alone (in the presence of MFP MdtA) was completely inactive, whereas Nagakubo et al. (117), who expressed the genes from plasmids, reported that MdtC alone gives some marginal activity.

When the C-terminus of either the B or C protein was labeled with hexahistidine tag, metal affinity purification produces a complex containing...
both MdtB and C, confirming that these proteins produce a heteromultimeric complex (115a). The size of the complex showed that it was a trimer, and the average composition was B\textsubscript{2}C. To establish the precise composition of the active trimer, the \textit{mdtB} and \textit{mdtC} genes were connected by linker sequences to produce covalently linked trimers of all possible combinations. The results showed that the highest activity was obtained only when the linked trimer contained two copies of MdtB and one copy of MdtC.

Since the heterotrimer contains two different protomers, the functionally rotating mechanism seems unlikely for this pump, at least in its unmodified form. Furthermore, when the proton-translocation pathway residues such as Asp\textsubscript{410} (corresponding to Asp\textsubscript{407} of AcrB) were changed to Ala in one of the MdtB sequences, the complex lost its activity, although similar changes in the MdtC sequence (e.g., in Asp\textsubscript{401}, corresponding to Asp\textsubscript{407} of AcrB) resulted only in a small change in activity (H. S. Kim and H. Nikaido, manuscript in preparation). Since we know from AcrB that protonation in this network of amino acids is needed for the extrusion of substrate drugs (see above), we assume that MdtB performs this function by changing from the binding conformation to the extrusion conformation. We do not know at present the role of MdtC. It could simply play a role in mechanically stabilizing the trimeric complex, in facilitating the capture of substrates in the neighboring MdtB unit, or in actually capturing the substrates by itself. Regardless of the precise roles of protomers, it is clear that the operating principle here is somewhat different from that of the homotrimers. Interestingly, MdtC is a basic protein with the calculated pI of 8.4, in stark contrast to other RND transporters that are acidic (AcrB, 5.4; AcrD, 5.6; MdtB, 5.2). We also do not know if this heterotrimeric structure gives advantages, if any, in terms of pumping activity. Such RND pumps requiring a paired set of transporters are also found in related bacteria, such as SdeAB of \textit{Serratia marcescens} (118), whose substrate range is limited as in MdtBC (bile acids and novobiocin). However, SmeJK of \textit{Stenotrophomonas maltophilia} is reported to have a wider range of substrates (119).

V. AcrAB–TolC and Drug Resistance

We have so far outlined our current knowledge on the mechanism of the AcrAB–TolC complex, beginning with the capture of substrates either from the periplasm or from the periplasm–inner membrane interface, followed by the conformational change in the AcrB periplasmic domain initiated by the flow of proton(s) through the AcrB transmembrane domain, finally resulting
in the extrusion of drugs into the external medium through the long channel of TolC. However, gram-negative RND-type efflux pumps have attracted attention because they are thought to play a large role in drug resistance, a major public health problem, which is becoming more and more acute because this type of multidrug efflux greatly diminishes the activity of most of the antimicrobial agents currently available (31). Thus, this review would not be complete without discussing the effect of AcrAB–TolC on the drug resistance. Furthermore, the mechanisms and kinetics of the pump we have discussed so far cannot be confirmed unless they explain, quantitatively, the level of resistance finally achieved in the living bacterial cell.

The analysis is the simplest with β-lactams, as their target is in the periplasm and as most of them are relatively hydrophilic, so that we can neglect their diffusion across the inner membrane. We have attempted this analysis at the time when multidrug efflux pumps were not yet known (73). In that study we considered the influx across the outer membrane, which follows Fick’s first law of diffusion, and the drug degradation by the periplasmic β-lactamase, which follows Michaelis–Menten kinetics. By solving these two simultaneous equations for the external drug concentration ($C_O$) that would just produce the periplasmic concentration that would kill the cell ($C_{inh}$), we could predict MIC values for β-lactams. Thus,

$$\text{MIC} = C_O = C_{inh} + \frac{V_{\text{max}} C_{inh}}{P A (C_{inh} + K_m)}$$  

where $V_{\text{max}}$ and $K_m$ are the usual kinetic constants of the β-lactamase, $P$ is the permeability coefficient of the β-lactam across the outer membrane and $A$ is the area of the outer membrane per unit weight of bacteria.

This procedure was applied to four strains of $E. coli$, producing widely different levels of the chromosomally coded AmpC β-lactamase ($V_{\text{max}}$ of 0.013 to 16.6 nmol mg$^{-1}$ min$^{-1}$ with cephaloridine), and the MIC values predicted were very close to the values observed for all of the cephalosporins tested. However, with some penicillins, there was a significant difference, which was most pronounced with carbenicillin, whose MIC values were usually eight times higher than the values predicted. We can now understand these results at least qualitatively. With most cephalosporins, at very low periplasmic concentrations where the cells become killed [$C_{inh}$ is usually less than 5 μM (73)], the efflux is insignificant, especially because of its sigmoidal kinetics (71); thus, exclusion of efflux from consideration produces only small errors. However, with carbenicillin, efflux is important, as seen from the strongly decreased MIC values in
acrAB-inactivated strain (15, 72), whereas the chromosomal β-lactamase of *E. coli* works poorly with this compound (72); hence, the prediction without consideration of efflux underestimates MIC values greatly.

We can also carry out a more quantitative analysis. The strain used by Nagano and Nikaido (71) for the analysis of nitrocefin was a rather artificial construct, containing a mutant porin with a wider channel, to facilitate the analysis. Nevertheless, with this strain, when both the AcrAB efflux pump and the AmpC β-lactamase were deleted, the nitrocefin MIC dropped from 38 µM to 2.2 µM. We can calculate the MIC value expected in the parent strain as follows.

When the periplasmic concentration reaches $C_{\text{inh}}$, the rate of efflux $V_e$ is $V_{e}^{\text{max}} C_{\text{inh}}/(K_m^e + C_{\text{inh}})$, and the rate of hydrolysis is $V_{h}^{\text{max}} C_{\text{inh}}/(K_m^h + C_{\text{inh}})$, both from the Michaelis–Menten relationship, where the superscripts $e$ and $h$ indicate constants related to efflux and hydrolysis, respectively. Then, since $V_{\text{in}} = PA(C_O - C_{\text{inh}})$, $C_O$ or MIC can be calculated as $C_{\text{inh}} + (V_e + V_h)/P/A$. As was noted earlier in the analysis without the consideration of efflux (73), the constant that is most difficult to estimate is $C_{\text{inh}}$. It has to be smaller than 2.2 µM, the MIC of the ΔacrAB ΔampC mutant, but how much smaller? As there may be other efflux pumps that slowly pump out nitrocefin, we assume arbitrarily that $C_{\text{inh}}$ is one-half of this concentration. Then the calculations give us the predicted MIC of 72 µM, or 36 µg/mL. Although this is twice the observed MIC of 18 µg/mL, the discrepancy is not too large, considering that MIC was determined under somewhat different conditions in comparison with the kinetic constants (e.g., temperature and medium).

An interesting exercise is to estimate the effect of outer membrane barrier on MIC values. We can see that the more permeable outer membrane would decrease the second term of the formula used above, by increasing $P$, and would make the MIC closer to $C_{\text{inh}}$. Thus, the effect of efflux (or periplasmic hydrolysis) will become minimized. Conversely, in an organism with a more efficient outer membrane barrier, the term $(V_e + V_h)/P/A$ will become larger, and thus efflux (and hydrolysis) will contribute more effectively in raising the MIC values, the situation we find, for example, in *P. aeruginosa* (20–22).

When different strains were compared, it was evident that the MIC values did not have a linear relationship with the amount of efflux pumps produced. Here a relevant study is the rigorous analysis from the group of Frère (120) on the effect of the β-lactamase level and of the target sensitivity to MIC. It was shown that if a high-affinity β-lactamase is overproduced, a 5000-fold difference in the sensitivity of the target (penicillin-binding proteins) will
produce only a twofold difference in MIC. We can see in equation (1) that under certain conditions the $C_{\text{inh}}/(K_m + C_{\text{inh}})$ term approaches 1, so that the MIC will be affected solely by the balance between permeability and the enzyme concentration (i.e., by $V_{\text{max}}/P/A$), and not much by the sensitivity of the target, or $C_{\text{inh}}$. These conditions are attained, for example, when the $K_m$ of the enzyme is very low, as with cefotaxime, in comparison with $C_{\text{inh}}$. Thus, the presence of $\beta$-lactamase will “mask” the difference in target sensitivity. Of course, if the $\beta$-lactamase and efflux were totally absent, there would be a 5000-fold difference in MICs. Although at the time of the work by Frère’s group, the kinetic behavior of the AcrB pump was not known, the conclusions may be relevant, as the efflux follows saturation kinetics such as enzymatic hydrolysis.

It must be remembered, however, that the $K_m$ of the AcrB pump is usually much higher than $C_{\text{inh}}$ (see Section II.C), and under such conditions the sensitivity of the target will continue to affect the magnitude of MIC. If we neglect the sigmoidal nature of the efflux kinetics (Section II.C), we can calculate the situation with cephalothin as follows. Here the $K_m$ (actually, $K_{0.5}$) of efflux is about 100 $\mu$M (71), much larger than $C_{\text{inh}}$, which earlier we estimated to be around 1 $\mu$M for cephalosporins. Thus, the $C_{\text{inh}}/(K_m + C_{\text{inh}})$ term of equation (1) may be approximated as $C_{\text{inh}}/K_m$ (i.e., 0.01 in this case). The $V_{\text{max}}$ value for cephalothin efflux was about 1 nmol s$^{-1}$ mg$^{-1}$ cells (71). As $P$ for cephalothin is around $10^{-5}$ cm/s (73) and $A$ is 132 cm$^2$/mg cells, the $V_{\text{max}}/P/A$ term in (1) will be around 1000 $\mu$M and the second term of (1) will be close to 10 $\mu$M, yielding a predicted MIC of 11 $\mu$M (neglecting the periplasmic hydrolysis). If the target becomes twice as resistant, with a $C_{\text{inh}}$ value of 2 $\mu$M, the MIC predicted will be 22 $\mu$M, following the changes in target sensitivity in a nearly proportional manner. If the efflux rate is decreased by one-half, the MIC predicted will be 6 $\mu$M, an almost but not completely proportional change. In reality, the sigmoidal nature of the efflux kinetics is likely to produce significant deviations from proportionality in these situations.

Carrying out similar analysis for antibiotics that have targets in the cytoplasm is much more complicated, because we have to consider the additional step of fluxes across the cytoplasmic membrane. In addition, the efflux across the cytoplasmic membrane may be catalyzed by “singleton” pumps located in this membrane, rather than by AcrB, as shown recently by Tal and Schuldiner (121) for ethidium bromide (pumped out into the periplasm by an MFS pump MdfA and an SMR pump EmrE). In this case, the drug efflux from cytosol to external medium would require successive action by at least two pumps, the export into periplasm catalyzed by a singleton pump and then the export from periplasm to external
medium catalyzed by the AcrB complex. The kinetic behavior of some of the singleton pumps has been elucidated in great detail: for example, that of EmrE (122–124). However, the kinetic constants of AcrB for non-β-lactam substrates are currently unknown, and building mathematical models of transport of these compounds would be very difficult at present.

There are some interesting observations on the effect of the presence of AcrB on MICs of antibiotics with intracytoplasmic targets. Thus, a mutation in the ribosomal protein L22 lowers the affinity of ribosomes to erythromycin (about a fivefold increase in $K_d$), and makes the *E. coli* cells resistant to this drug, raising the growth-inhibitory concentration (at 50% growth) from 100 μg/mL to 800 μg/mL (125). In a strain with a deletion of the *tolC* gene (therefore, in the virtual absence of efflux), this difference largely disappears, the 50% growth-inhibitory concentration increasing from 3.5 μg/mL to only 5 μg/mL by the introduction of the same L22 mutation (125). A similar observation was made in another laboratory (126), which found that the difference in the 50% growth-inhibitory concentrations between the wild type and an L22 mutant strain decreased from tenfold in the efflux-competent background to only 1.5-fold in the ΔacrAB background. These authors considered the possibility that erythromycin might increase the translation of AcrAB proteins, but could not find any evidence for such a phenomenon. Synergy between the efflux and the target mutations was also found in *Campylobacter* spp. (127). Thus, these observations are the opposite of masking of the target sensitivity by β-lactamase mentioned above.

An attempt has been made to explain this phenomenon of *resistance masking* in efflux-deficient strains by mathematical modeling (128, 129). Since nothing is known about the kinetics of efflux, the efflux pump is assumed to operate at a substrate concentration far below $K_m$, and the efflux is assumed proportional to intracellular drug concentration. Most important, because the increase in cell volume due to growth was assumed to be the only mechanism that decreases the drug concentration inside, the rate of net influx of drugs through a loosely defined “cell envelope” needed to be set extremely low, on an order of magnitude similar to the growth rate. I remain quite skeptical about the validity of such a modeling approach carried out in the almost total absence of experimental constraints, as nothing is known about the kinetics of OM diffusion of macrolides or about that of active efflux of these compounds. Possibly the exaggeration of the difference in target sensitivity by efflux could be brought about by the sigmoidal kinetics of the pump (71), but we need to know much more before we get into more realistic simulations.
VI. Coda

As a participant who was there at the discovery of *E. coli* AcrB as a multidrug efflux pump (18) 17 years ago, I am impressed and gratified by the amount of progress that scientists have made in this area. At that time, the mere notion that a single transporter is capable of exporting such a wide variety of compounds (see Figure 2) and that it may capture substrates in the periplasm was met with a great deal of skepticism, and the publication of our reports on the *P. aeruginosa* multidrug efflux process (20, 21) was delayed for at least several months because the reviewers simply refused to believe in the existence of such a process.

In contrast, we now know the detailed three-dimensional structures of all of the components of the tripartite pumping complex (Figures 3 and 4), and we understand the mechanism of drug extrusion, at least in its basic outline. It seems likely that drugs are captured from the periplasm perhaps exclusively, because so far, structures do not give any hint as to how the drugs captured in the cytosol may be brought out to the periplasmic binding site. We understand the pathway(s) through which drugs reach the binding site and how the protonation of one or two Asp residues in the transmembrane domain causes conformational changes that are transmitted to the periplasmic domain to produce drug extrusion. We understand that the AcrB protomers within the trimeric complex act by a functionally rotating mechanism, so that all of them have to be functional for any drug molecule to be extruded, a situation that produces sigmoidal kinetics with some substrates. We are even approaching the stage where we can understand how various substrates bind to the various subdomains of the large binding sites (Section IV.B).

However, many questions remain. I hope that I emphasized the need to correlate our biochemical knowledge of pump action to the final effect of the pump in raising MIC (Section V), an area still in its infancy. Indeed, the kinetics of AcrB has been examined only for cephalosporins and penicillins, and we totally lack comparable data for all other classes of antimicrobial compounds. We need to know more precisely how various drugs bind to the binding site. The computer docking studies (Section IV.B) can give us only hints, and they remain to be proven by real biochemistry. We also do not have any idea how AcrA stimulates the function of AcrB.

The functions of the adaptor (or MFP), AcrA and its homologs, have been reviewed (38). However, it should be mentioned that the MFP CusB of the Cus system, a toxic cation exporter of *E. coli*, is known to bind the substrate Cu⁺ (96), and most excitingly, direct transfer of metal ion from the
periplasmic binding protein CusF to CusB was recently demonstrated (97). Thus, it seems quite possible that in this system the substrate ion flows from the periplasmic binding protein to the periplasmic adaptor protein, and then, finally, to the periplasmic domain of an RND transporter, CusA.

There are also unresolved questions on the function of tripartite RND multidrug pumps in intact cells. For example, *P. aeruginosa* strains over-expressing a tripartite pump MexCD–OprJ were often found to have reduced functions of other tripartite pumps, MexAB–OprM and MexXY–OprM, although the latter pumps are expressed at the normal level (130). Another curious observation is that TonB, a protein that functions in energizing the outer membrane siderophore receptors, is needed in *P. aeruginosa* for functioning of the MexAB–OprM pump (131, 132). As far as the authors are aware, there is no explanation of this effect at the molecular level, but this reminds us that a small protein YajC was accidentally co-crystallized with AcrB (99). Although the deletion of gene *yajC* had only a small effect on drug susceptibility, this observation certainly requires further follow-up study, especially because this co-crystal was obtained from cells expressing both YajC and AcrB at normal constitutive levels, without the lopsided, plasmid-driven overexpression of only AcrB.

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