AcrAB and Related Multidrug Efflux Pumps of Escherichia coli

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Abstract

The AcrAB system of Escherichia coli is a multidrug efflux system composed of an RND-type transporter AcrB and a periplasmic accessory protein AcrA, and pumps out a wide variety of lipophilic and amphiphilic inhibitors directly into the medium, presumably through the TolC outer membrane channel. AcrA, a highly elongated protein, is thought to bring the outer and inner membranes closer. It forms a trimer that interacts with a monomeric AcrB, which was shown by in vitro reconstitution to be a proton antiporter. Details of interaction between the AcrAB complex and TolC remain a major topic for study.

Introduction

Most Gram-negative bacteria are intrinsically less susceptible to many antibiotics, especially amphiphilic and lipophilic ones, than are the average Gram-positive bacteria (with the exception of mycobacteria). It was once thought that this difference can be largely explained by the presence, in the Gram-negative bacteria, of the outer membrane. This additional membrane layer indeed acts as a very effective permeability barrier. Thus the influx of hydrophilic drugs through the porin channels is difficult as they are usually much larger than the typical nutrient molecules. Furthermore, lipophilic drugs cannot penetrate through these channels (owing to the highly ordered nature of water molecules within the channels [Schulz, 1993]) and diffuse only very slowly across the lipid bilayer domain of the outer membrane (Pléniat and Nikaido, 1992), which contains a lipopolysaccharide-containing outer leaflet of unusually low fluidity (Nikaido and Vaara, 1985). However, the discovery of the AcrAB multidrug efflux pump (Ma et al., 1993) destroyed the simplistic and static idea that the resistance of Gram-negative bacteria can be explained entirely by the outer membrane permeability barrier. The acr mutant of E. coli, which is hypersusceptible to many lipophilic inhibitors, was long thought to have defective, “leaky” outer membranes. But cloning and sequencing showed that the genes (acrAB) actually coded for an active multidrug efflux machinery, and the phenotype of acr mutants showed that this pump exported substrates of unprecedented wide range (Nikaido, 1996).

The AcrAB efflux system is composed of a transporter AcrB of the RND family, and a periplasmic accessory protein AcrA. The genes acrAB form an operon. The outer membrane protein TolC, which is encoded by a gene located elsewhere on the chromosome, is likely to work together with AcrAB (Fralick, 1996). Although many multi-component export systems similar in composition to AcrAB-TolC and presumably similar also in the mechanism of action have been known in Gram-negative bacteria, the mode of interaction between components remains largely unknown. The cooperation between AcrB, AcrA, and TolC is hypothesized to allow the direct efflux of drug molecules into the medium, rather than into the periplasm (Nikaido, 1996). Indeed quantitative analysis and modeling of tetracycline efflux in the wild-type and porin-deficient mutant of E. coli suggested that the drug is extruded into the periplasm by the Tet pump, located in the cytoplasmic membrane, but the endogenous efflux machinery, now known to be AcrAB-TolC, produces the direct drug efflux into the medium (Thanassi et al., 1995). Because the drug molecules, once pumped out into the medium, must traverse the low permeability outer membrane in order to come into the bacterial cell again, the AcrAB-TolC and similar systems work synergistically with the outer membrane barrier, and are very effective in creating resistance. The AcrAB system is constitutively expressed in E. coli, and is largely responsible for the characteristic intrinsic resistance of this organism to dyes, detergents, and most lipophilic antibiotics. Since our biochemical studies of this system have recently been summarized (Zgurskaya and Nikaido, 2000a), we will limit our comments to specific areas that were not emphasized earlier.

RND Superfamily

An important advance in our knowledge in this area is the realization (Tseng et al., 1999) that homologs of RND pumps exist also in Gram-positive bacteria as well as eukaryotes and archea. These more distant relatives of AcrB sometimes display interesting functions that contribute to our understanding of not only the origin of RND pumps, but also the way these pumps function. Thus M. tuberculosis, a gram-positive bacterium covered by a thick cell wall of low permeability, contains about a dozen members of RND superfamily (mmpL1 through mmpL12), and indeed transposon mutagenesis led to the discovery that one of them is involved in the export of a very lipophilic cell wall lipid, phthiocerol dimycocerosate (Cox et al., 1999). Also, the human proteins that belong to this superfamily include Nieman-Pick type C protein, thought to be involved...
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in moving cholesterol out of the lysosomal membrane, and even the cholesterol-sensing domain of HMG-CoA reductase (Tseng et al., 1999). These findings suggest that RND proteins evolved mostly as a machinery that senses, or senses and then extrudes, relatively apolar molecules found within the lipid bilayer domains of the membrane. It is also noteworthy that the two very large periplasmic “loops” (each containing about three hundred residues) of the bacterial RND pumps are also found in many eukaryotic homologs. Since the latter do not interact with AcrA homologs, clearly we can rule out the hypothesis that these “periplasmic” loops are needed only for interaction with AcrA and relatives. This conclusion is also consistent with the observation that AcrA homologs interact also with transporters of Major Facilitator superfamily (for example EmrB [Lomovskaya and Lewis, 1992]) and of ABC superfamily (for example HlyB [Blight and Holland, 1994]), neither of which contains these huge periplasmic loops.

**Natural Function of AcrAB in E. coli**

We have purified and reconstituted the AcrB transporter into proteoliposomes, and measured its activity (Zgruskaya and Nikaido, 1999b)(Figure 1). This allowed us not only to conclude that AcrB is a proton antiporter, but also to evaluate the affinity of the pump to various substrates. We found that the substrates with by far the highest affinity to AcrB were bile salts and their derivatives, such as taurocholate and glycocholate. This makes sense because *E. coli* has to survive in the presence of high concentrations of these detergents in its natural habitat; in fact mutant strains lacking the AcrAB pump are hypersensitive to bile salts, and obviously cannot stay alive in the upper intestinal tract (Thanassi et al., 1997). The broad specificity of AcrAB also makes sense because bile acids acquire diverse structures as a consequence of their metabolism by the intestinal flora, including deconjugation and dehydroxylation.

Close homologs of AcrAB are present in the genome sequences of various Gram-negative bacteria, and it is of interest to note that the “environmental” species, presumably living in contact with many potentially harmful agents, contain many putative and demonstrated RND drug efflux genes (4 each in *E. coli* and *Aquifex aeolicus*, between 3-5 in *Synechococcus*), whereas obligate pathogens adapted to only one environment, presumably free of toxic amphiphiles, contain very few (0 for *Chlamydia trachomatis*, 0-1 for *Treponema pallidum*, and 1 for *Haemophilus influenzae*) (Paulsen, 2000).

**Substrate-Binding Sites**

We have proposed that the RND transporters probably prefer to bind their substrates from within the bilayer. So far, it has not been possible to discriminate between the binding from the external leaflet and that from the inner leaflet, as has been done with the Gram-positive transporters (Bolhuis et al., 1996a; 1996b). However, it is easier to envisage that the preferred binding mode involves capture from the external leaflet of the bilayer. (a) Unlike some other “multidrug” ef flux pumps, AcrAB system is unusually broad in its substrate specificity, and extrudes

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**Figure 1. Principle of the AcrB Assay with Reconstituted Proteoliposomes.** Purified AcrB protein was reconstituted into proteoliposomes (donor vesicles), which contained about 0.5% each of fluorescent phosphatidylethanolamine molecules, whose head groups were labeled either with NBD (filled-in ellipses) or with rhodamine (filled-in rectangles). Excitation of NBD group produces only a reduced fluorescence intensity, because of energy transfer to the neighboring rhodamine-lipid. The fluorescence-labeled lipid molecules, however, are exported by AcrB pump, in the presence of ∆pH, to liposomes (acceptor vesicles) nearby, so that the density of fluorescent lipids decreases with time, resulting in less efficient energy transfer and increased fluorescence. The addition of purified AcrA to the external medium enhances the lipid export process, possibly because the acceptor and donor vesicles are brought closer by the AcrA molecules (Zgruskaya and Nikaido, 1999b).
cations, neutral, and anionic substrates (Nikaido 1996; Nikaido et al. 1998). These differently charged substrates will alter membrane potential in different directions. This may require a major expenditure in cellular energy to compensate. In contrast, if substrates enter into the outer leaflet of the inner membrane from the periplasm, becomes captured and then expelled, they will never traverse the cytoplasmic membrane and their export will not generate membrane potential even when they are charged. (b) AcrAB system pumps out some \( \beta \)-lactams with multiple charged groups, which were experimentally shown not to traverse the cytoplasmic membrane. These substrates are likely to be captured from the outer leaflet of inner membrane, whose content should be in equilibrium with the content of the periplasm.

However, this preferred binding mode may not be the only mode in which the substrate binding occurs. If this were the case, then drugs that are uncharged and relatively lipophilic, such as chloramphenicol, would accumulate in the cytoplasm if they succeed in crossing the cytoplasmic membrane by escaping capture by AcrAB. With the AcrB homolog AcrD, aminoglycosides, which are multiply positively charged and highly hydrophilic, are efficiently extruded (Rosenberg et al., 2000); in this case the substrates are unlikely to partition into any leaflet of the cytoplasmic membrane, and possibly they are captured directly from the periplasm or cytoplasm. It is thus likely that the transporter will have multiple substrate-binding mechanisms (and substrate-binding sites) to capture drugs from different locations in the cell. In fact, with a Gram-positive transporter, multiple drug-binding sites were shown to exist (Putman et al., 1999). A similar observation was also made with the QacA efflux transporter in Gram-positive bacteria (Mitchell et al., 1999), as well as with the human P-glycoprotein (Dey et al., 1997).

**Roles of AcrA and TolC in the Direct Efflux into Medium**

AcrA, a member of the MFP family, is anchored to the cytoplasmic membrane through its N-terminal lipid extension. Hydrodynamic properties of AcrA showed that it is an extremely asymmetric protein (Zgurskaya and Nikaido, 1999a). If modeled as a prolate ellipsoid, its axial ratio was estimated as 8 or 9, and its length can be as long as 20 nm. This is certainly enough to cover the depth of the periplasm. This finding led to the hypothesis that AcrA may connect the inner and outer membranes. In fact, our in vitro reconstitution experiment showed that AcrA greatly enhanced the export of fluorescent-labeled lipid from the AcrB-containing proteoliposomes into neighboring acceptor liposomes (Zgurskaya and Nikaido, 1999b). These results were interpreted to mean that AcrA bound to two membrane bilayers, the donor proteoliposome and the acceptor liposome, and brought the two types of vesicles together, to facilitate the lipid transfer (see Figure 1). This hypothesis was supported also by the observation that even the AcrA lacking its normal lipid modification became attached to liposomes. Furthermore, AcrA alone, in the absence of AcrB transporter, produced the slow movement of fluorescent-labeled lipids from the donor to recipient vesicles, at pH 5.0 and in the presence of 5 mM Mg\(^{2+} \), suggesting that AcrA under these conditions catalyzes hemifusion between vesicles. These results are consistent with the conservation of the interrupted coiled-coil domains bracketed by the "lipoyl arm" domains in AcrA and its homologs (Johnson and Church, 1999), a structure that suggests the possibility that these proteins may fold upon themselves to bring the two membranes very close. Even without a hemifusion event, AcrA would at least be able to bring the RND transporter and the outer membrane protein in contact, so that the substrates can be transferred directly from the transporter into the outer membrane channel (see Figure 2).

The recent elucidation of the three-dimensional structure of TolC (Koronakis et al., 2000) influences our model in a major way. Unlike the conventional porin-like proteins, which are nearly completely embedded in the outer membrane, a TolC trimer has, in addition to the 4 nm-thick outer-membrane-spanning domain, a long (10 nm), presumably periplasmic, \( \alpha \)-helical barrel domain,
composed of 12 α-helices. How do these two components, TolC with a 10 nm-long barrel and AcrA with a putative length of 20 nm, interact to produce a direct efflux channel for drugs?

Our recent cross-linking study (Zgurskaya and Nikaido, 2000b) may be relevant to this question. We found that in intact cells, much of AcrA exists as trimers, and these AcrA trimers could be cross-linked to monomeric AcrB. However, we could not cross-link either of these proteins to TolC. With the hemolysin secretion system, composed of the HlyBABC transporter, HlyD periplasmic accessory protein, and TolC (Wandersman, 1992), a HlyD trimer could similarly be cross-linked to HlyB but the cross-linking of this complex to TolC required the presence of the protein substrate secreted (Thanabal et al., 1998). A similar finding was obtained earlier with another type I protein secretion system (Létoffé et al., 1996). This gave rise to the idea that TolC is only transiently recruited for the export of each protein molecule. Perhaps a similar situation exists with AcrAB-TolC complex, but the mechanism cannot be exactly the same because the substrates of AcrAB system are small molecules in contrast to large proteins, which are necessarily exported slowly by the HlyBBD system.

The interaction of a trimeric AcrA with a trimeric TolC is pleasing in terms of symmetry, but the details of this interaction are not known. AcrA could, as was assumed earlier, simply bring the bottom end of TolC in contact with the top end of AcrB. In the model presented in Figure 2A, the long AcrA molecule is assumed to be associated with both the inner and the outer membrane. AcrA is associated with the inner membrane through its lipid extension at the N-terminus. AcrA and its homologs contain a highly conserved, about 100-residue-long domain, predicted to be rich in β-structure, near the C-terminus (Dinh et al., 1994). Because all known outer membrane proteins contain β-barrel structures, it has been suggested that this segment also forms a β-barrel and become inserted into the outer membrane. (Experimental evidence is desperately needed to find out how AcrA and its relatives interact with the outer membrane.) Alternatively, the AcrA molecules may fold upon themselves, using their coiled coil and lipoyl arm domains, and bring the TolC barrel even closer to AcrB pump (Figure 2B). Yet another possibility is that the coiled coil domain of AcrA becomes interwoven with the helices that comprise the helical barrel domain of TolC. Not to be forgotten, also, is that AcrB and other RND transporters contain huge periplasmic “loops.” Thus there is a superabundance of periplasmic domains in the AcrB-AcrA-TolC complex, and the elucidation of the way they interact with each other will be a topic of major interest in the coming years.

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