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GUEST COMMENTARY

Covalently Linked AcrB Giant Offers a New Powerful Tool for Mechanistic Analysis of Multidrug Efflux in Bacteria[▽]

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In gram-negative bacteria, multidrug transporters belonging to the *r*esistance-*n*odulation-cell *d*ivision (RND) superfamily of proteins are responsible for high intrinsic and acquired levels of antibiotic resistance. Since their discovery in 1993 (2), RND transporters have been the focus of intense investigation. Given their low similarity to mammalian RND proteins and high impact on bacterial pathogenesis and infection, the bacterial RND pumps are regarded as very attractive targets for drug development (1). It is now clear that inhibitors of RND pumps potentiate the activity of clinically important antibiotics and could become effective antibacterial agents. At present, there are more than 20 various structures of AcrB transporter from *Escherichia coli*, the best-studied representative of the RND multidrug pumps. This vast structural information yielded a putative biochemical mechanism of how RND transporters expel from cells multiple structurally unrelated compounds. In this issue, Takatsuka and Nikaido provide an elegant functional proof for this mechanism (9).

RND transporters function as homo- or heterotrimers of proteins, or protomers, 1,049 amino acid residues in length (Fig. 1A). Each protomer consists of the 12 transmembrane α-helices (TM1 to TM12) and a large periplasmic domain (4). Takatsuka and Nikaido succeeded in constructing a giant gene which produces the AcrB pump with three protomers covalently linked together into a single ~3,200-amino-acid-residue protein (9). *E. coli* cells have difficulty expressing such large proteins. The choice of an α-helix as a connector between protomers possibly contributed to the successful expression of the tripled AcrB protein. The fused AcrB giant was found to be functionally active, with activities exceeding those of the native protein for some drug substrates.

The clever cloning scheme allows the manipulation of one repeat at a time, making this construct a powerful tool for characterizing the mechanism of AcrB. Protein oligomerization significantly complicates all kinds of analyses, including in vivo mutagenesis. All protomers will contain a desired mutation if a mutated protein is produced in the absence of the native sequence. Expression in the presence of the native gene, however, will yield mixed oligomers of random configurations. In contrast, with the fused AcrB triplet, one can introduce a point mutation in one of the three repeats selectively. The only possible complication in such analysis could be instigated by protein degradation products, which could assemble mutationless oligomers.

The ability to manipulate one subunit at a time is especially critical when protomers interact cooperatively, bringing about isomerization of one or several subunits or, as in the case of AcrB, undergoing sequential conformational transitions. The recently proposed mechanism of transport by AcrB involves sequential ("rotational") conformational changes in each protomer of AcrB (Fig. 1) (3, 5). More importantly, these conformational transitions are asynchronized in such a way that at any given time during transport activity, all three protomers of a functional AcrB trimer adopt three different conformations. These three consecutive conformations were named loose ("access"), tight ("binding"), and open ("extrusion") (Fig. 1C) (3, 5).

Traditional mutagenesis showed that mutations trapping the transporter in one of the three conformations inactivate AcrB. In two independent studies, cysteine residues were introduced into the periplasmic domain of AcrB in such a way that when one of the protomers adopts the extrusion conformation, cysteine residues come at a distance of ~4.4 Å and can form a disulfide bond (6, 8). In contrast, in the other two conformations, these two cysteines are too far away from each other to form a bond. AcrB carrying such cysteine residues was deficient in drug transport but could be revived under reducing conditions, which allow breakage of the disulfide bond and release of the trapped protomer. In these studies, however, all three protomers contained the same mutations, leaving unclear which of the multiple possible events leads to the loss of function. Alternatively, AcrB can be trapped in the extrusion conformation by mutating the critical residues involved in proton translocation (7). Such mutations irreversibly inactivate AcrB. Furthermore, such AcrB mutants have a negative dominant phenotype when overproduced in wild-type *E. coli* cells carrying a chromosomal copy of the native gene (10). This result suggested that AcrB trimers containing both native and mutant subunits are transport deficient. However, alternative explanations for the negative dominant phenotype are possible as well.

Using the fused AcrB giant, Takatsuka and Nikaido now tested unequivocally the idea that all three protomers function as a single unit and that the inactivation of one protomer leads to the complete loss of function of the transporter (9). They introduced double-cysteine substitutions in each of the fused

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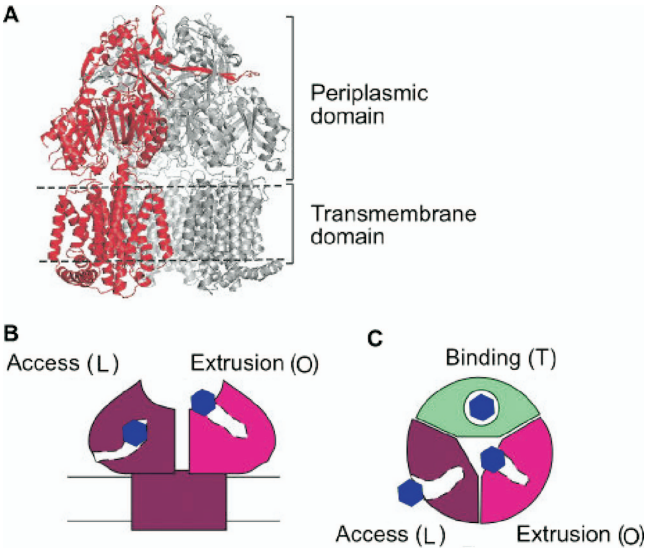

FIG. 1. Structure and mechanism of AcrB. (A) Trimeric AcrB (one protomer is shown in red) is composed of a large periplasmic domain and a transmembrane domain. The periplasmic domain is the site of drug binding and interaction with accessory AcrA and TolC proteins. Takatsuka and Nikaido used a short α-helix located between TM6 and TM7 of AcrB to link the three AcrB protomers together into a single polypeptide (9). (B and C) Schematic representation of the AcrB functional rotation transport mechanism (5). The conformational states loose [Access (L)], tight [Binding (T)], and open [Extrusion (O)] are indicated. A substrate molecule (blue hexagon) enters the binding site of AcrB from the periplasm (L), and after binding (T) and conformational transitions, the substrate is released (O) into the periplasmic funnel of AcrB connected to the TolC channel.

protomers and showed that irrespective of their positions, such mutations inactivate the transporter. Similar results were obtained with mutations in the proton translocation pathways. Taken together, these results provide a functional proof that the functional unit of the AcrB transporter is a trimer and that protomers are likely to alternate their conformations as suggested by structural studies (3, 5).

The fused AcrB protein constructed by Takatsuka and Nikaido opens a new door into mechanistic characterization of RND transporters. One of the important questions is how the conformational transitions in AcrB are coupled to substrate transfer across the outer membrane. Two accessory proteins, the periplasmic membrane fusion protein AcrA and the outer membrane channel TolC, are absolutely required for this activity (1). AcrA and TolC are believed to be integrated into the transport cycle because mutations in any of these accessory proteins results in the complete loss of AcrB function. Using the covalently linked AcrB trimer, it is now possible to determine whether the affinity of AcrA toward AcrB alternates with the conformation of the protomers and how changes in AcrA-AcrB interactions affect TolC. The fused AcrB protein could also offer new insights into the mechanism of AcrB inhibition. A number of broad- and narrow-spectrum inhibitors of RND pumps have been identified (1). Using the linked AcrB trimer, it could now be possible to test whether these inhibitors preferentially bind one of the three AcrB conformations and to design and test new inhibitors. The structural studies could be facilitated as well, because one could produce a homogeneous protein with one of the protomers trapped in the open, tight, or loose conformation. This could also help in cocrystallization of AcrB and substrates/inhibitors. It seems that with more than 20 structures of AcrB available, it is only the beginning of an exciting journey into the mechanism of RND pumps.

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