Published in final edited form as:

FEBS Lett. 2007 October 16; 581(25): 4972–4976.

Ligand-transporter interaction in the AcrB multidrug efflux pump determined by fluorescence polarization assay

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Abstract

The AcrB of *Escherichia coli* pumps out a wide range of compounds, including most of the currently available antibiotics, and contributes significantly to the serious problem of multi-drug resistance of pathogenic bacteria. Quantitative analysis of drug efflux by this pump requires the measurement of the affinity of ligands. Yet there has been no success in determining these values. We introduce here an approach of steady-state fluorescence polarization to study the interactions between four different ligands and the purified AcrB transporter in a detergent environment. Our assays indicate that the transporter binds these drugs with K_D values ranging from 5.5 to 74.1 μ M.

Keywords

Multidrug resistance; AcrB; Multidrug transporter; Membrane protein; Protein-ligand interaction

1. Introduction

The emergence of multidrug-resistant pathogenic bacteria is a major current problem in public health. In gram-negative bacteria, much of the multidrug resistance phenotype is caused by the increased expression of multidrug efflux pumps. These pumps fall into several families, small multidrug resistance (SMR), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), and resistance-nodulation-division (RND). The members of the RND superfamily [1] pump out the widest range of compounds, and are usually the major contributor to both the intrinsic antibiotic resistance, and when overexpressed, the increased resistance levels of multidrug resistant strains [2], as was recognized already in 1993 [3] and 1994 [4]. However, quantitative analysis of the function of RND efflux pumps could not even be attempted during the last 14 years, because all attempts to measure the kinetic constants of the pump resulted in failure.

We here use a relatively simple methodology to quantitatively monitor interaction between a membrane protein, which in this study is the AcrB multidrug efflux pump of *Escherichia coli* [5], and its fluorescent ligands in detergent solution. Binding of a fluorescent ligand to a protein causes a decrease in rotational motion due to an increase in the size of the protein–ligand complex as compared to that of the free ligand, and thus results in increases in the polarization of fluorescence of the bound ligand. Fluorescence polarization is defined as

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$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

where P is the observed polarization, and $I_{\parallel}$ and $I_{\perp}$ represent the intensity of emissions parallel and perpendicular to the incident polarized light.

AcrB [5] is a prototypical member of the RND family of transporters [1]. It recognizes many structurally unrelated compounds, including most of the currently available antibiotics and chemotherapeutic agents, detergents, dyes, and simple solvents, and actively engages to extrude them from cells [6]. This inner membrane efflux pump interacts with a periplasmic membrane fusion protein, AcrA [7], and an outer membrane channel, TolC [8], to mediate the extrusion of toxic compounds directly into the external medium, across both membranes of *E. coli*.

The structure of AcrB has been studied extensively [9–16]. Using the purified AcrB multidrug efflux pump, we succeeded for the first time in determining the affinity of ligands for the pump, and further in demonstrating the competition between ligands for the binding process. This study will be the first step for the quantitative analysis of the kinetics of the most important class of multidrug efflux pumps in gram-negative bacteria.

2. Materials and methods

2.1. Purification of AcrB

The AcrB protein that contains a 4×His tag at the C-terminus was overproduced in *E. coli* BL21-Gold (DE3) cells (Stratagene) using the plasmid derived from pSPORT1 (Invitrogen) [17]. Cells were grown in 6 L of LB medium with 100 µg/ml ampicillin. Cells were disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10% glycerol, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF), and once with 20 mM HEPES–NaOH buffer (pH 7.5) containing 1 mM PMSF. The membrane proteins were then solubilized in 1% (w/v) n-dodecyl-β-D-maltoside (DDM). Insoluble material was removed by ultracentrifugation at 370000 × g. The extracted protein was purified with hydroxyapatite, Cu²⁺-affinity and G-200 sizing columns [10,18].

2.2. Fluorescence polarization assays

Fluorescence polarization assays [19,20] were used to determine the drug binding affinities of AcrB. The experiments were done using a ligand binding solution containing 20 mM Tris (pH 7.5), 0.05% DDM, and 1 µM ligand [rhodamine 6G (R6G), ethidium (Et), proflavin (Pf), or ciprofloxacin (Cip)]. The AcrB protein solution in 20 mM Tris (pH 7.5), 0.05% DDM, and 1 µM ligand was titrated into the ligand binding solution until the polarization (P) became unchanged. In this assay, the protein–drug interaction would reach equilibrium within 1 min. As this is a steady-state approach, fluorescence polarization measurement was taken after incubation for 5 min for each corresponding concentrations of the protein and drug to ensure that the binding has reached equilibrium. It should be noted that the detergent concentration was kept constant at all times to eliminate the change in polarization generated by drug–DDM micelle interaction. All measurements were performed at 25 °C using a PerkinElmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelengths were 527, 483, 447, and 330 nm, respectively, for R6G, Et, Pf, and Cip. Fluorescence polarization signals (in ΔP) were measured at emission wavelengths of 550, 620, 508, and 415 nm, respectively, for these ligands. Each titration point recorded was an average of 15 measurements. Data were analyzed using the equation, $P = \{ (P_{\text{bound}} - P_{\text{free}})[\text{protein}] / (K_D + [\text{protein}]) \} + P_{\text{free}}$, where P is the polarization measured at a given total protein concentration, P_{free} is the initial polarization of free ligand,

P_{bound} is the maximum polarization of specifically bound ligand, and [protein] is the protein concentration. The titration experiments were repeated for three times to obtain the average K_D value. Curve fitting was accomplished using the program ORIGIN [21].

3. Results

3.1. Binding affinities of various AcrB ligands

The goal of this research was to determine the binding affinities of a variety of ligands to AcrB. Fluorescence polarization based-assay was initially carried out to study the interaction between AcrB and R6G. Fig. 1a illustrates the binding isotherm of AcrB in the presence of 1 μM R6G. As presented in the figure, a simple hyperbolic curve was observed with the K_D of 5.5 ± 0.9 μM . The Hill plot of the data (Fig. 1b) yields a Hill coefficient of 1, suggesting a simple drug binding process with no cooperativity.

Fluorescence polarization was also used to observe the binding of Et, Pf and Cip to the transporter. In comparison with R6G, other ligands tested show somewhat lower affinity. Thus, Et bound with a K_D of 8.7 ± 1.9 μM (Fig. 2a), Pf with that of 14.5 ± 1.1 μM (Fig. 2b), and Cip with that of 74.1 ± 2.6 μM (Fig. 2c).

3.2. Binding of two drugs by AcrB

We carried out several titration experiments that involved the initial saturation of AcrB with 100 μM of Et. To ensure that AcrB and Et formed a complex, the mixture was incubated for at least 2 h before titrating with 1.5 μM Pf. Fluorescence polarization experiments indicated that AcrB-Et binds Pf with a K_D of 61.0 ± 0.9 μM (Fig. 3). This value is about four times of the K_D of Pf (14.5 ± 1.1 μM) in the absence of Et, indicating that Et interferes with the binding of Pf to AcrB, possibly because the two compounds compete with each other for the same binding site.

We carried out similar titration experiments with the pre-formed AcrB-Et complex using 1.5 μM Cip. The results gave a K_D value of 70.4 ± 15.4 μM for Cip (data not shown), which is not significantly different from the K_D of Cip (74.1 ± 2.6 μM) determined in the absence of Et. Although it is tempting to conclude that Et and Cip bind to non-overlapping sites, the numerical precision of K_D values is not high in this case because of the quite low affinity of this ligand, and we believe that further experiments utilizing different approaches are needed in this case.

3.3. pH dependence of drug binding to AcrB

We performed fluorescence polarization experiments to determine the binding of R6G to AcrB at different pH values. Fig. 4 illustrates the decrease in K_D values for rhodamine 6G as the pH increases from 5.5 to 8.4.

3.4. Detergent concentration does not affect drug binding to AcrB

To analyze if the detergent concentration affects the fluorescence polarization results, we measured the dissociation constant of the AcrB-R6G complex in different concentrations of detergent, DDM. We found that at moderate detergent concentrations, the concentration of detergent does not have much influence on the results. At 0.5%, 0.05%, and 0.005% DDM, the dissociation constants of the AcrB-R6G complex were 7.2 ± 0.1 , 5.5 ± 0.9 , and 4.4 ± 0.8 μM , respectively.

4. Discussion

We demonstrated that the technique of fluorescence polarization is sensitive and precise enough to detect ligand binding of *E. coli* AcrB, an intrinsic membrane protein in a detergent

environment. As this is a steady-state approach, it is important to ensure that the binding equilibrium has reached before data collection. A stop-flow study of an oligopeptide OppA transporter, belonging to the ABC superfamily, indicated that peptide binding to OppA would reach equilibrium within 20 ms when the concentrations of the protein and peptide are in the micromolar range [22]. Thus, a 5 min period for incubation in our experiments should be more than enough to allow the binding to reach equilibrium. We obtained results showing that the ligands bind to AcrB with their K_D values between 5.5 (R6G) and 74.1 μ M (Cip) (Figs. 1 and 2). These values are similar to the K_D for most substrates for the MdfA (a MFS transporter) [23] and EmrE (a SMR transporter) [24] multidrug pumps, determined by competition with tetraphenylphosphonium binding. Although our data suggested the binding stoichiometry of 1:1 monomeric AcrB-to-drug molar ratio, there is a formal possibility that any compound may bind to more than one site on the protein with similar affinity that could not be discriminated using this technique. Thus, further experiments utilizing different approaches are needed to confirm this drug binding stoichiometry.

One of the main goals of our work is to introduce a relatively simple approach to study membrane protein–ligand interaction in a detergent environment. It should be noted that detergent solubilized protein in detergent micelles may behave differently from that reconstituted in a lipid environment, including altering the binding affinity of drugs to AcrB in this case. However, a study of the EmrE transporter suggested that EmrE binds drugs with similar strengths in different membrane mimetic environments, including those in DDM micelles and in reconstituted lipid vesicles [25]. The observed dissociation constants were in the micromolar range for the EmrE drugs in any of the membrane mimetic environments [25]. Detailed study of AcrB–ligand interaction in the lipid environment would be necessary to determine whether the reconstituted AcrB behaves differently in terms of drug binding affinity.

As our fluorescence polarization assay was performed in a detergent environment, we are aware of the fact that detergent micelles in solution may have interaction with the drug molecules. To eliminate this “non-specific” binding component that may cause an error for the measurement of drug binding affinity, it is important to keep the detergent concentration constant during titrations. It has been reported that the dissociation constants of DDM micelle with Et and Pf are 5.2 and 17.1 mM, respectively [25]. We measured the binding affinities of DDM micelle with R6G and Cip using fluorescence polarization assay (data not shown). We found that the interactions between the micelles and drugs are quite weak. Thus, we could only estimate these K_D values, which were 2.9 mM for R6G–DDM micelle and 899.7 mM for Cip–DDM micelle, respectively. Apparently, the drug–DDM micelle interactions are in the millimolar range, which are about three orders of magnitude weaker than those of the drug–transporter interactions.

We also examined the question of simultaneous binding of two different drugs to AcrB, by using fluorescence polarization assays. Et was a convenient ligand because it absorbs and emits light at very long wavelengths, far away from the wavelengths used by other dyes. Titrations of Pf into Et-saturated AcrB yielded a much increased dissociation constant compared with that in the absence of Et, suggesting strongly that both drugs bind in a competitive manner to an overlapping binding site in AcrB.

To confirm that two drugs compete with one another for a specific binding site in the protein, we performed competition experiments in which tetraphenylphosphonium chloride (TPP) was titrated into a solution containing the preformed AcrB–R6G complex. In this case, TPP was chosen as a second ligand to knock off the bound R6G from AcrB. The absorption spectra of TPP (from 200 to 600 nm) showed that this molecule absorbs light at the wavelengths of 224.9, 268.0 and 275.9 nm. At $\lambda = 527$ nm, which is the excitation wavelength for R6G, the energy

is too low to excite TPP. Thus, TPP was treated as a non-fluorescent ligand in the “knock off” experiments. The data revealed that TPP was able to bind AcrB and replace the bound R6G molecule from the protein as demonstrated by the release of R6G that resulted in the reduction of polarization (Fig. 5). This binding assay provides direct evidence that TPP interferes with the binding of R6G. However, it is not known that this heterologous displacement is due to a truly competitive binding or via negative heterotropic allostery between distinct binding sites of the ligands. We were unable to determine the binding affinity of TPP through these competition experiments due to precipitation of the protein at higher TPP concentrations. Regardless, the titrations demonstrate that R6G is bound specifically in the AcrB transporter.

We also tried these competition experiments using erythromycin, which is another AcrB drug, as a second ligand to replace the bound R6G from the transporter (not shown). The data revealed that erythromycin is not capable of knocking off the bound R6G, as the polarization of R6G does not change during titrations. Thus, there is a chance that different class of drugs may bind at different sites in AcrB.

The binding of R6G to AcrB was strongly pH-dependent. A trivial explanation of this result was the pH-dependent alteration of charge state in the ligand. However, we found that there is essentially no change in the emission spectra of free R6G in the pH range examined in Fig. 4, suggesting that the electronic arrangement of the protonated and de-protonated states of R6G are very similar. The pK_a of R6G is nearly neutral, with the value equals 7.5 [26] (the pK_a s of Pf and Cip are 8.1 and 6.1, respectively [27]). At $pH < pK_a$, R6G should exist in its protonated form. When $pH > pK_a$, this dye should be predominantly unionized. It is expected that R6G should bind tighter to the transporter at acidic pH, as it is predominantly in the positively charged form. However, the reverse case is seen from the pH dependent data. One possible explanation is that R6G possesses a large degree of delocalization. It is this large extent of electron delocalization that makes the chemical properties of the protonated and de-protonated forms of R6G very similar. Indeed, it has been reported that the observed octane/water partition coefficient of R6G remains the same from pH 4 to 11 because of this charge delocalization phenomenon [26].

Judging from the binding isotherms of AcrB in the presence of 1 μM R6G, these curves maintained the simple hyperbolic shape and reached saturation at the same AcrB concentration at different pH values (as shown in Fig. 1a). Thus, it is not likely that altering the pH affects the capacity of binding. Perhaps the pH effect is related to the protonation state of acidic residues in the binding pocket: there are several acidic residues in the ligand binding site of the asymmetric AcrB crystal [12], with Asp276 particularly close to the ligand. The strong pH dependence for drug binding has been observed as the result of binding of cationic drugs with the glutamate residue in the EmrE multidrug transporter [24]. Alternatively, it could involve global conformational changes of the transporter that utilizes the transmembrane electrochemical gradient of protons for activity.

Fluorescence polarization assay has been widely used for studying protein–DNA interactions [19,20,28,29], and lately for determining affinities of drug binding in transcriptional regulators [29–31]. To our knowledge, this is the first attempt using this methodology to investigate interaction between membrane protein and bound drug. The availability of fluorescence polarization to measure affinities of a variety of AcrB substrates will provide the means for studying the interactions of other purified transporters with their substrates in detergent solutions.

Acknowledgements

This study was supported in part by US Public Health Service Grants AI-09644 (to H.N.) and GM074027 (to E.W.Y.).

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Fig. 1.

Representative fluorescence polarization of AcrB in 0.05% DDM with R6G. (a) Binding isotherm of AcrB with R6G, showing a K_D of $5.5 \pm 0.9 \mu M$, in buffer containing 20 mM Tris (pH 7.5) and 0.05% DDM. (b) Hill plot of the data obtained for R6G binding to AcrB. α corresponds to the fraction of bound R6G. The plot gives a slope of 1.12 ± 0.02 , indicating a simple binding process with a stoichiometry of one AcrB protomer per one drug molecule. The interception of the plot provides a K_D of $5.4 \pm 1.0 \mu M$ for the R6G binding.

Fig. 2. Fluorescence polarization of AcrB with Et, Pf and Cip. Binding isotherms of AcrB with (a) Et, showing a K_D of $8.7 \pm 1.9 \mu M$, (b) Pf, showing a K_D of $14.5 \pm 1.1 \mu M$, and (c) Cip, showing a K_D of $74.1 \pm 2.6 \mu M$.

Fig. 3. Binding of ligands by AcrB in the presence of 100 μM of Et as determined by fluorescence polarization assay. The change in fluorescence polarization signals (ΔFP) of Pf was measured at an emission wavelength of 508 nm. The binding curve suggests a K_D of $61.0 \pm 0.9 \mu\text{M}$ for Pf.

Fig. 4. Effect of pH on the K_D of R6G binding to AcrB. The resulting K_D s were plotted against pH.

Fig. 5.

AcrB binding competition experiment between R6G and TPP. AcrB (10 μM) was pre-incubated with R6G (1 μM) for 2 h before titration. The change in fluorescence polarization signals (ΔFP) of R6G was measured at an emission wavelength of 550 nm. TPP was non-fluorescent in the experimental conditions. The decrease in ΔFP showed that the bound R6G was knocked off by TPP.