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SMR proteins SugE and EmrE bind ligand with similar affinity and stoichiometry ☆

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Abstract

Suppressor of a *groEL* mutation protein E (SugE) is a small multidrug resistance (SMR) homologue. In comparison with other SMR proteins, SugE promotes bacterial resistance to a narrow range of quaternary ammonium compounds (QACs). Isothermal titration calorimetry was used to study the binding of QACs to *Escherichia coli* SugE in different membrane mimetic environments. In this study, the binding stoichiometry of SugE to drug was found to be 1:1, and the binding of SugE to drug was observed with the dissociation constant (K_D) in the micromolar range for each of the drugs in the membrane mimetic environments explored. This interaction appears to be enthalpy-driven with enthalpies of 8–12 kcal/mol for each of the drugs. These results are similar to those found with drug binding to the SMR protein EmrE in an earlier study.

Keywords: SugE; Membrane proteins; Detergents; Drug binding; Membrane mimetics; ITC; Calorimetry

Multidrug resistance is the ability of cells to grow in the presence of and survive lethal doses of a variety of drugs. Several mechanisms of multidrug resistance exist such as the efflux of drug out of the cytoplasm by membrane transporters. These transporters have been divided into four major families: the major facilitator superfamily, the ATP-binding cassette family, resistance/nodulation/cell division family, and the small multidrug resistance (SMR) family [1,2]. Members of the SMR family are the smallest known functional unit of multidrug resistance composed of approximately 110 residues and 4 transmembrane helices [3,4]. The SMR family can be divided into two categories: small multidrug efflux proteins (SMR) and suppressor of a *groEL* mutation (Sug) proteins [1,3].

SugE was initially identified as a suppressor of a *groEL* mutation, since the presence of *sugE* on a multicopy plasmid would restore nitrogenase activity when GroEL was mutated and non-functional [5]. It would later be classified as an SMR protein as its sequence was similar to other SMR proteins such as the ethidium multidrug resistance protein (EmrE) protein (Fig. 1). *E. coli* SugE and EmrE share 27% sequence identity and 52% sequence similarity (Fig. 1). However, unlike EmrE, SugE does not transport the drugs commonly transported by other multidrug resistance proteins that are illustrated in Fig. 2 [6]. In fact, when key residues of SugE were mutated to those conserved amongst Smp proteins (highlighted in blue in Fig. 1), SugE would promote sensitivity to drug rather than resistance [6]. It was concluded that SugE was functioning as a drug importer as a result of these mutations.

With the activities of SugE poorly defined and with conflicting phenotypes, we asked the question here if SugE can bind to drug substrates similar to EmrE. In this study, isothermal titration calorimetry was used to

☆ Abbreviations: C:M, chloroform/methanol; DM, *N*-dodecyl-β-D-maltoside; ITC, isothermal titration calorimetry; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles.

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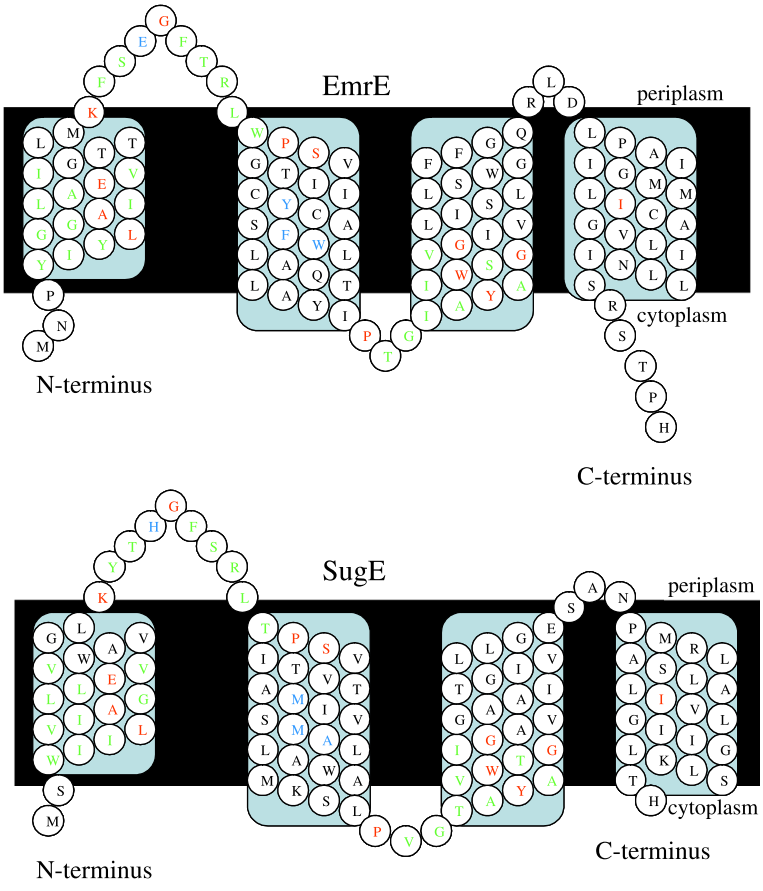

Fig. 1. The sequence and topology of *E. coli* SugE and EmrE. Residues that are similar motifs for SMR proteins are indicated in green. Residues that are conserved between all members of the SMR family are highlighted in red. Residues in blue are more conserved within each of the Smp and Sug subfamilies of SMR proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

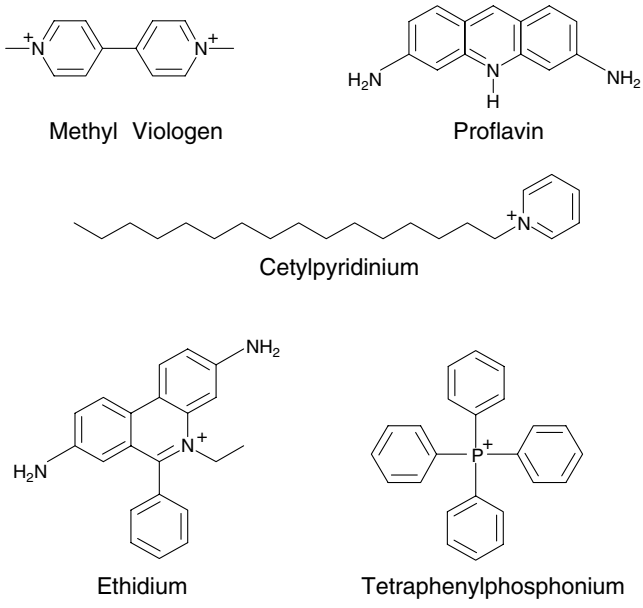

Fig. 2. Structures of compounds used in this study.

study drug interactions with SugE in different membrane mimetic environments.

Experimental procedures

Purification of SugE. The preparation of SugE was processed similar to the protocol established for EmrE in our group [7]. SugE protein was expressed from *E. coli* strain LE392Δ*unc* containing the expression plasmid pMS119EH. Cells were grown to a density of 0.5 (A_{600}) in 1 L terrific broth [8] inoculated at 37 °C and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The cells were incubated for three more hours, harvested by centrifugation, and thoroughly washed with SMR A buffer (50 mM Mops, 8% glycerol, 5 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol, pH 7). Cells were resuspended in SMR A buffer with 100 μM phenylmethylsulfonyl fluoride and lysed by two passes through a French press (16,000 psi). A low speed centrifugation (9000*g* for 15 min) removed the heavy cellular constituents and unlysed cells, followed by a high speed spin (110,000*g* for 1.5 h) to collect the membrane fraction. The membranes were resuspended in SMR A buffer and diluted to a final protein concentration of 10 mg/mL.

Ten milliliters of the membrane fraction was extracted with 300 mL of 3:1 chloroform/methanol (C:M). Fifty milliliters of double distilled water was added to separate the water soluble constituents from the organic phase. The organic phase was collected and centrifuged to

further separate remaining water from the organics. The organic phase was then evaporated below 6 mL using a rotovap. The protein was further purified and separated from any extracted lipid using Sephadex LH-20 hydrophobic chromatography in 1:1 C:M solvent using an Aktä purifier. Solvent from the purified SugE was removed under N_2 gas and the dried protein was stored at −70 °C until use. EmrE purified in this fashion can be reconstituted into small unilamellar vesicles (SUVs) and demonstrates transport activity [7].

Solubilization of SugE in detergent. Both sodium dodecyl sulfate (SDS) (from Bio-Rad) and *N*-dodecyl-β-D-maltoside (DM) (from Anatrace) were found to solubilize SugE at useful levels. Stock detergent solutions were made with 8% w/v SDS detergent in SMR B buffer (5 mM Mops, 10 mM NaCl, and 10 μM dithiothreitol (DTT), pH 7) and 2% w/v DM in SMR B buffer. Each tube of dried SugE was exposed to 300 μL of the desired detergent solution. The SugE suspension was vortexed for two hours at room temperature followed by an overnight freeze–thaw cycle at −20 °C. The sample was then centrifuged at 14,000*g* for 10 min to remove any insolubles. The pellet was discarded and the protein concentration of the supernatant was determined by a modified Lowry assay [9]. The supernatant was diluted with its respective detergent solution in SMR B buffer so that the SugE concentration was 2 mg/mL. This sample was stored at −70 °C for later use.

Reconstitution of SugE into small unilamellar vesicles. A tube of dried SugE was resuspended in 110 μL of 3:1 C:M. Of the 110 μL, 10 μL was set aside and dried under N_2 gas. The dry pellet was resuspended in SDS solution as previously described and a modified Lowry assay [9] was carried out on this sample to determine the protein content in the remaining 100 μL of SugE solution. Two milligrams of SugE was removed from this suspension, added to 1.5 mL of 25 mg/mL *E. coli* polar lipid extract (Avanti), and the mixture dried under N_2 gas to remove organic solvent. One milliliter of SMR C buffer (0.5 mM Mops, pH 7) was added to the dried pellet. The sample was vortexed for 20 min at room temperature to resuspend the pellet. Five freeze–thaw cycles were carried out on this sample at −70 °C. Three cycles of sonication were carried out on the sample for 3 s each at 25% power (5 μA amplitude), and stored at −70 °C for later use. SUVs were also constructed similarly in the absence of SugE. The prepared SUV's were checked using a microscope and/or dynamic light scattering for uniformity of size and shape.

ITC calorimetry of SugE. Prepared samples of SugE in detergent or SUV solutions were thawed. To exchange into a buffer lacking salt and reducing agent, 0.5 mL SugE sample and 1.5 mL SMR C buffer (0.5 mM Mops, pH 7) with detergent or SUVs were loaded on a 5 mL Hi Trap Desalting Column (from Phamacia). The column had previously been equilibrated with 6 column volumes of SMR C buffer prior to loading of SugE. The column was eluted with SMR C buffer, and the first 2 mL of elution containing the SugE protein was collected. The eluted SugE was diluted to a final concentration of 0.480 mg/mL (40 μM), and degassed in a thermovac at room temperature for 5 min. The degassed sample was injected into the sample cell of a Microcal isothermal titration calorimetry (ITC) calorimeter. Calorimetry trials were carried out at 25 °C.

Ligand (ethidium (Et), proflavin (Pro), cetylpyridinium (CTPC), methyl viologen (MV) or tetraphenylphosphonium (TPP)) solubilized in SMR C buffer containing the same concentration and type of detergent or lipid mixture (SDS, DM or SUV) used in the protein sample was prepared as the titrant. Sixty injections of this titrant containing either 0.5 mM ligand for strong interactions or 2.0 mM ligand for weaker interactions were injected into the ITC sample cell.

Injections occurred at intervals of 240 s, and the duration time of each injection was 8 s. Heat transfer (μcal/s) was measured as a function of elapsed time (s). Heats of dilution were subtracted from the heats collected in the corresponding experiments. Independent preparations of SugE were used in this experiment to verify that binding trends were consistent.

Calorimetry trials were also carried out in the absence of SugE in the same experimental conditions as described above. No change in heat released was observed in the injections throughout the experiment.

Isothermal titration calorimetry analysis. From analysis of each generated thermogram, the data was corrected to account for heats of dilution. The remaining heat was measured to calculate the reaction enthalpy ($\Delta \boldsymbol{H}$) using the equation

$$\Delta \boldsymbol{H} = \sum_i \delta \boldsymbol{h}_i / \boldsymbol{n}_{tp}, \tag{1}$$

where $\boldsymbol{n}_{tp}$ is the total moles of protein in the cell, and $\delta \boldsymbol{h}_i$ is the heat evolved from each injection. A plot of $\Delta \boldsymbol{H}$ against the molecular ligand:protein ratio was used to construct a binding isotherm.

Non-linear regression fitting to the binding isotherm (ORIGIN software; MicroCal Software) was used to determine the equilibrium dissociation constant (K_D) of the ligand–protein binding interaction. From the value of K_D, the free energy of binding (ΔG) and entropy of binding (ΔS) can be calculated from the following equation:

$$\Delta G = -RT \ln(1/K_D) = \Delta H - T\Delta S, \tag{2}$$

where T is 278 K and R is 1.9872 cal/K mol.

Results

It was found using EmrE that a ratio of SMR protein molecules to ligand molecules in the range of 4–10 was suitable for measuring the heats evolved from SMR protein-binding interactions with drug [10]. Similar ratios worked here with SugE. Thirty injections of an 8 μL solution containing 2 mM ethidium into a 1.2 mL solution of 40 μM SugE were made (Fig. 3A). Both the titrant and sample solutions contained SUVs in SMR C buffer as a membrane mimetic. Each peak in the thermogram (Fig. 3A) represents an injection of drug. Negative deflections from the baseline upon addition of drug indicate that the reaction was exothermic.

Binding isotherms are displayed in Fig. 3B with a curve of best fit through the compiled data points of three independent trials. Binding and thermodynamic values extracted from binding isotherms are summarized in Table 1. Independent preparations of SugE were used to obtain this data.

The values of K_D were in the micromolar range similar to values reported for EmrE [10] and other multidrug resistant proteins to these drugs [11–13]. The stoichiometry of the interaction under the conditions studies was 1 drug bound per SugE subunit. SugE reconstituted in SUVs and in DM environments bound ethidium and proflavin ligands with greater affinity than tetraphenylphosphonium and methyl viologen (Table 1). In the SDS mimetic, each of the ligands bound with similar affinities. In the case of using cetypyridinium (CTPC) ligand, binding was not detected in any given membrane mimetic by our chosen method of ITC.

Control experiments were previously performed to investigate drug–membrane mimetic interactions [10]. In these controls, each ligand in each of the membrane mimetics was injected into the ITC sample cell containing the same membrane mimetic in the absence of

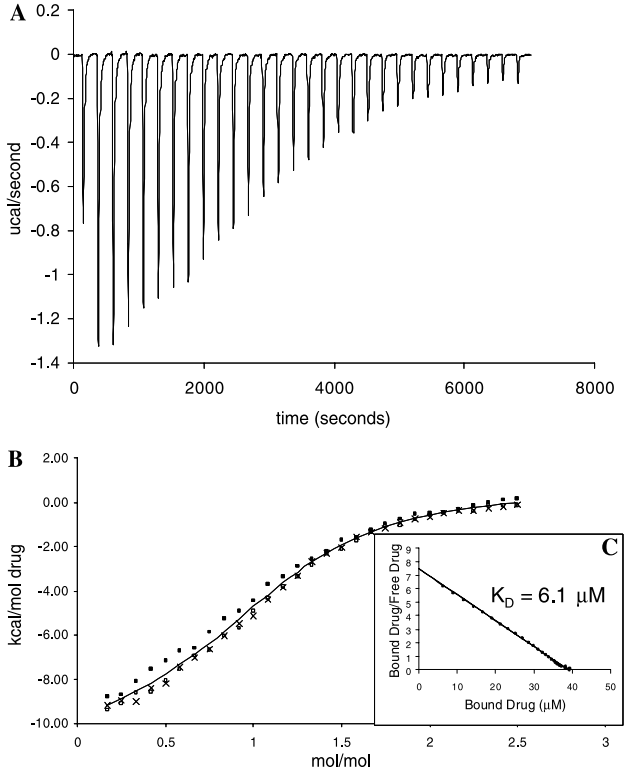

Fig. 3. Representative titration calorimetry of SugE in SUVs with ethidium. (A) Each peak corresponds to the injection of 8 μL of 0.5 mM ethidium in SUVs into the reaction cell containing 40 μM SugE in SUVs. The concentration of *E. coli* polar lipid that formed SUVs in this experiment was 37.5 mg/mL. (B) Cumulative heat of reaction is displayed as a function of the injection number. The solid line is the least squares fit to the experimental data of separate trials. (C) Linearization of the data from a single trial in a Scatchard plot.

protein. These trials were carried out using the same concentrations and injection volumes as carried out previously in the presence of protein. There was not any observable heat loss or gain observed in these control experiments.

The concentration of detergents used was well above the critical micelle concentrations for SDS and DM. Aggregation numbers of 62–101 for SDS (Anatrace, measurement) and 78–149 for DM ([14], Anatrace measurement) were used to determine the ratio of micelles:-SugE subunits. This ratio is on an order of 40 in SDS, and 4 in DM.

Discussion

Two types of residues in multidrug resistance proteins are necessary in binding to the compounds illustrated in Fig. 2. The first essential residue is a negatively charged acidic residue for binding to the positive charge of the ligand [1,15–18]. A glutamate residue in the first transmembrane helix of SugE and EmrE (Fig. 1) is present for this function [16]. This residue is highly conserved amongst multidrug resistant proteins [1]. Aromatic residues are also involved in protein–drug interactions [19,20]. Aromatic residues are involved in van der Waal and π–π interactions with the aromatic rings of the ligand. They also establish π interactions with the positive charge on the ligand [18,21]. SugE contains aromatic residues Y59 and W62 that are highly conserved in SMR proteins ([2], Fig. 1) which may assist in this interaction. When these residues are mutated in EmrE, the protein is non-functional [22,23].

Each of the drugs illustrated in Fig. 2 has been shown to partition into the membrane mimetics used in this experiment [10]. This suggests the possibility of drug first partitioning into the lipid, and then entering the binding pocket of SugE from the lipid environment. It was observed that drug binds to membrane mimetics

Table 1
Dissociation constants and thermodynamic data for binding of drug to SugE in various mimetic environments at 25 °C

Environment	Drug	K_D (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)
SUVs	Ethidium	6.1 ± 1.5[a]	-7.1 ± 0.1[b]	-10.8 ± 0.2[a]	-12.4 ± 1.1[c]
	Methyl viologen	24.9 ± 5.8	-6.3 ± 0.1	-7.9 ± 0.5	-5.5 ± 1.9
	Proflavin	7.4 ± 0.2	-7.0 ± 0.0	-9.2 ± 0.0	-7.4 ± 0.0
	TPP			(not detected)	
SDS	Ethidium	3.7 ± 0.4	-7.4 ± 0.1	-12.1 ± 0.0	-15.8 ± 0.2
	Methyl viologen	5.3 ± 1.4	-7.2 ± 0.2	-9.6 ± 0.2	-8.0 ± 1.1
	Proflavin	4.2 ± 1.0	-7.3 ± 0.1	-10.9 ± 0.1	-11.8 ± 0.3
	TPP	4.3 ± 0.9	-7.3 ± 0.1	-11.6 ± 0.1	-14.5 ± 0.6
DM	Ethidium	6.2 ± 1.0	-7.1 ± 0.1	-10.6 ± 0.1	-12.1 ± 0.5
	Methyl viologen	54 ± 16	-5.8 ± 0.2	-7.1 ± 1.7	-4.3 ± 6.3
	Proflavin	4.5 ± 0.7	-7.3 ± 0.1	-9.3 ± 0.1	-6.6 ± 0.3
	TPP	43 ± 12	-6.0 ± 0.2	-9.9 ± 1.8	-13.3 ± 5.5

[a] Average and standard deviation from 3 trials of independent SugE sample preparations.
[b] 3 values of ΔG were calculated from K_D values obtained from 3 trials. The average and standard deviation of the calculated ΔG values are included.
[c] 3 values of ΔS were calculated using measured ΔH values and calculated ΔG values from the 3 separate trials. The average and standard deviation of calculated ΔS values are what is reported.

with dissociation constants in the millimolar range. This is considerably weaker than drug binding to SugE (Table 1) with dissociation constants in the micromolar range. This suggests that it is energetically favorable for drug to partition from lipid into the binding site of SugE.

Various multidrug resistant proteins recruit drug solubilized in the inner leaflet of the membrane such as MexA-MexB-OmpR, QacA, Lmr, and P-glycoprotein which act as flipases to relocate drug from the inner leaflet to the outer leaflet of the membrane [15,24–30]. Electron microscopy studies have suggested accessibility of drug to EmrE from both the membrane and cytoplasm [31], so there is a possibility that SugE may recruit drug from both locations as well.

Certain trends were observed between drug binding to SugE studied here and EmrE [10]. First, the SUV and DM environments do not seem to alter the binding of SugE to ligand in any way with the exception of TPP. Ethidium (Et) and proflavin (Pro) ligands showed stronger binding than the tetraphenylphosphonium (TPP) and methyl viologen (MV) ligands. Steric hindrance from the phenyl rings on the TPP substrate, and the second positive charge on MV may limit their binding and accessibility to the binding pocket of SugE. This may explain why both TPP and MV bound with weaker affinity to SugE than the other drugs.

TPP binding to SugE was not detected in SUVs. It was shown that TPP bound to SUVs with a K_D of 0.2 mM [10], but binding of TPP to SugE or EmrE [10] in SUVs was not observed. This inability of TPP to bind EmrE and SugE in SUVs was also observed in fluorescence studies (K. Duncalf and R.J. Turner, unpublished results). SUVs have a greater curvature than the inner membrane of *E. coli*, which could constrict the transmembrane segments towards the inner leaflet to a greater extent. This may explain the limited access of TPP in this membrane mimetic. Tighter lipid–helix and helix–helix packing in SUVs may also limit TPP entry into the SugE-binding site.

SDS is an environment that denatures many soluble proteins. Unlike soluble proteins, many membrane proteins retain their structure and have been studied in this mimetic [32–35]. Circular dichroism and fluorescence studies performed in SDS, DM, and SUVs have shown that EmrE in each of these environments has a similar structure [36]. It should be noted that EmrE is in a slightly more open and potentially more flexible conformation in SDS than DM and SUVs [36]. TPP and MV bound to both EmrE [10] and SugE (Table 1) with a similar affinity as Et and Pro ligands in SDS. A more open conformation of SMR proteins in SDS compared with the other membrane mimetics may explain the increased affinity of TPP and MV to SugE and EmrE. A more open conformation could improve accessibility of drug to the binding site and form a larger binding pocket that can better accommodate these drugs.

In the case of CTPC, binding was detected to the membrane mimetics (K_D in SUV $= 8.4 \times 10^{-4}$ mM) [10] but binding could not be sensed by ITC for EmrE [10] or SugE studied here. The heat evolved from this drug binding to protein could not be observed amongst the heat evolved from drug–membrane mimetic interactions. However, SugE has demonstrated resistance to CTPC [37] so it is has the ability to bind this drug even though this interaction cannot be detected using ITC methods.

Subtle enthalpy and entropy differences amongst the environments were also observed. Enthalpy contributions were similar amongst all drugs in DM or SUVs and slightly higher in SDS (Table 1). The major enthalpic contributor of binding is likely the interaction between the positive charge on the drug and negatively charged glutamic acid [16]. Since EmrE has a more open conformation in SDS [36], it may better accommodate this ionic interaction. Another subtle trend is that drug containing more aromatic ring structures such as Et and TPP (Fig. 2) had a higher entropic contribution to binding.

The binding pocket of each SugE subunit was shown to bind a single molecule of drug, similarly as EmrE [10]. This suggests each subunit of SugE has the ability to bind drug on its own without having to form a larger binding pocket composed of residues from multiple subunits, similar to EmrE [10]. EmrE has been shown to be present either as monomers [10,38,39], or form a variety of different multimers [16,29,40–47]. EmrE monomers have the ability to bind drug [10], therefore multimerization is not required for such an interaction to occur. However, multimerization may be required for the subsequent transport of drug. It is still unknown whether or not SugE has the ability to multimerize. It has only been shown to be monomeric in 6:6:1 chloroform/methanol/water [38], and its oligomeric state has not been investigated in other environments. Perhaps differences in multimerization or an inability of SugE to multimerize account for its differences in functioning compared to EmrE.

SugE and EmrE share 30% sequence identity, 60% sequence similarity (Fig. 1) and bind to drug with similar affinity. Both proteins contain a glutamic acid residue on transmembrane segment 1, and a tryptophan and tyrosine residue on transmembrane segment 3 (Fig. 1). These residues are highly conserved in SMR proteins and responsible for binding to the drug ligand substrate [1,15–20]. Although both EmrE and SugE bind the drugs in Fig. 2 similarly, the events to follow binding are different with each protein. Only EmrE demonstrates resistance to all of these toxins, whereas SugE has only been shown to promote resistance to CTPC [37]. Other compounds SugE has demonstrated resistance to are cetyldimethylethyl ammonium bromide and hexadecyltrimethyl ammonium bromide [37]. A feature unique to these drugs is a long acyl chain. As this study has shown, drugs lacking an acyl chain have the ability to bind to SugE, although the presence of an acyl chain ap-

pears to be required for transport to take place. The roles of residues conserved and exclusive to Sug proteins (Fig. 1) may be accountable for interacting with the acyl chain of the drug.

In considering the residues conserved amongst Sug proteins, it was thought that mutation of these residues to those that are conserved amongst Smp proteins would cause Sug proteins to function as Smp proteins. Residues in SugE conserved in Sug proteins (highlighted in blue in Fig. 1) were mutated to those found in EmrE (highlighted in blue in Fig. 1) in a past study [6]. Upon mutation, instead of promoting resistance to the compounds illustrated in Fig. 2, SugE promoted bacterial hyper sensitivity to these toxins. It suggested SugE was functioning as an importer. It should be noted that topologies of both proteins are oriented with N and C termini on the cytoplasmic side of the membrane [48,49]. Therefore, these results were not due to different orientations of the protein in the membrane. This ITC study has verified that each of the drugs in Fig. 2 bind to both SugE and EmrE with similar strength and stoichiometry. This suggests that the differences amongst these proteins are the mechanism occurring after binding has taken place.

In conclusion, this study of isothermal titration calorimetry has demonstrated the weak, non-specific binding of SugE to a variety of lipophilic cationic drugs. It has demonstrated that this interaction is similar in various membrane mimetics. Also the binding stoichiometry of drug to SugE has been shown to be 1:1. This study has shown binding of drug to SugE or EmrE to be similar in strength and stoichiometry in any of the membrane mimetics. Therefore, many of the conserved residues amongst these proteins (Fig. 1) are most likely involved in drug binding. The events that take place after binding are different as only EmrE demonstrates resistance to all of the drugs used in this study. Further investigation is required to determine the fate of drug bound to SugE.

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