Identification of Tyrosine Residues Critical for the Function of an Ion-coupled Multidrug Transporter

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Aromatic residues may play several roles in integral membrane proteins, including direct interaction with substrates. In this work, we studied the contribution of tyrosine residues to the activity of EmrE, a small multidrug transporter from *Escherichia coli* that extrudes various drugs across the plasma membrane in exchange with protons. Each of five tyrosine residues was replaced by site-directed mutagenesis. Two of these residues, Tyr-40 and Tyr-60, can be partially replaced with hydroxyamino acids, but in the case of Tyr-40, replacement with either Ser or Thr generates a protein with modified substrate specificity. Replacement of Tyr-4 with either Trp or Phe generates a functional transporter. A Cys replacement at this position generates an uncoupled protein; it binds substrate and protons and transports the substrate downhill but is impaired in uphill substrate transport in the presence of a proton gradient. The role of these residues is discussed in the context of the published structures of EmrE.

EmrE, a protein from *Escherichia coli*, provides a unique model for the study of polytopic membrane proteins. It is a small (110-residue) multidrug transporter that extrudes various positively charged drugs in exchange for protons, thus rendering bacteria resistant to these drugs (1–3). The protein has been characterized, purified, and reconstituted in a functional form (3–5). High affinity substrate binding has been established as a reliable and sensitive assay for activity of the detergent-solubilized transporter (4). Structural and biochemical evidence suggest that the basic EmrE oligomer is a dimer (6–9). EmrE has only one membrane-embedded charged residue, Glu-14, that is conserved in more than 200 homologous proteins (10) and plays a central role in substrate and proton binding (11–14). Deprotonation of Glu-14 is required for substrate binding (4, 14). Substrate-induced proton release has been observed directly in a detergent-solubilized preparation of EmrE (15). From the pH dependence of the magnitude of the release, we have estimated a pKₐ for Glu-14 of 8.3–8.5. The unusually elevated pKₐ suggests that the environment around Glu-14 is chemically unique.

The question of multiple drug recognition has been addressed with several approaches. Recent advances in the structural analysis of multidrug-recognition transcription factors (16–18) and transporters (19, 20) suggest that these proteins possess large hydrophobic binding sites and bind their substrates through a combination of hydrophobic and electrostatic interactions. Negative charges in these hydrophobic domains are stabilized by hydrogen bonding to other residues such as the hydroxyls in tyrosines (21). Close interaction between substrates and aromatic residues has been observed in the binding sites of multidrug-recognizing transcription factors (16–18). In addition, aromatic residues can interact with cations through a strong, noncovalent force termed the cation/π interaction (for a review, see Ref. 22). It has been suggested that aromatic residues in a protein can pull a cationic substrate (or a proton) out of water and into a nominally hydrophobic environment (22).

Recently it was demonstrated that the fully conserved Trp-63 is an essential residue, and substrate binding induces quenching of its fluorescence, suggesting that it may be interacting with substrate (23). In this work, we further explore the role of aromatic residues in the activity of EmrE by studying the contribution of the tyrosine residues to its activity. EmrE has five tyrosine residues, at positions 4, 6, 40, 53, and 60. One of them, Tyr-60, is fully conserved in the small multidrug resistance family; Tyr-40 is conserved in a branch of this family (24). Position 4 is highly conserved as an aromatic amino acid. We have mutagenized each of the tyrosine residues and examined the effect of the mutation on the protein function. Although replacement of the tyrosine residues in positions 6 and 53 had no effect on the protein function, the replacements of the residues in positions 4, 40, and 60 affected the activity of the protein. The results are discussed in the context of the published structures of EmrE.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—*E. coli* DH5α (Invitrogen) and TA15 (25) strains were used throughout this work. The TA15 strain was previously transformed with plasmid pGP1–2, which codes for the T7 polymerase under the inducible control of the α PL promoter (26). The plasmids used for EmrE gene expression are pT7–7 (26) derivatives with a Myc epitope and a His tag at the C terminus (EmrE-His, for simplicity, will be called EmrE throughout this paper) (4).

**Mutagenesis**—The construction of the mutants was as previously described (27). Mutants were obtained by polymerase chain reaction using the overlap extension procedure as described by Ho et al. (28) or using another procedure mainly as described by Li et al. (29). The templates used for mutagenesis were pT7–7 EmrE or pT7–7 CAMY (a cysteine-less EmrE with alanine replacements (30)).

**Resistance to Toxic Compounds**—*E. coli* DH5α cells transformed with either pT7–7–EmrE, pT7–7 (vector), or with the various mutants were grown overnight at 37 °C in LB-ampicillin medium. 5 μl of serial dilutions of the culture were spotted on a series of LB-ampicillin plates containing 30 mM BisTris propane titrated to pH 7 with various EmrE substrates (100 μg/ml ethidium bromide, 50 μg/ml acriflavine, and 0.2 mM methyl viologen) or on a plate with no addition. Growth was analyzed after overnight incubation at 37 °C.

**Expression, Purification, and Reconstitution of EmrE into Proteoliposomes**—*E. coli* TA15 cells bearing plasmids pGP1–2 and pT7–7 containing His-tagged EmrE constructs were grown at 30 °C in...
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minimal medium A supplemented with 0.5% glycerol, 2.5 μg/ml thiamine, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. When the culture reached O600 = 1, it was transferred to 42 °C for 15 min to induce the T7 polymerase. Thereafter, the culture was shifted back to 30 °C. 2 h later, the cells were harvested by centrifugation. The cells were resuspended with buffer containing 250 mM sucrose, 1 mM dithiothreitol, 150 mM NaCl, 15 mM Tris-Cl, pH 7.5, 2.5 mM MgSO4, 15 μg/ml DNase-I (Sigma), and 1 mM phenylmethylsulfonyl fluoride and broken by a French press. The membrane fraction was collected by ultracentrifugation at 213,500 × g for 1 h at 4 °C and resuspended in the above buffer without dithiothreitol, DNase, and phenylmethylsulfonyl fluoride. The membranes were frozen in liquid nitrogen and stored at −70 °C.

Reconstitution was performed essentially as described previously (11). 400 μl of membranes were solubilized in 2 ml of buffer containing 150 mM NaCl, 15 mM Tris-Cl, pH 7.5 (Na-buffer), 1% DDM3 (Glycon GmbH), 0.5 mM phenylmethylsulfonyl fluoride, and 15 mM β-mercaptoethanol. After removal of unsolubilized material by centrifugation (20,000 × g for 30 min), imidazole was added to 20 mM, and the His-tagged protein was incubated with Ni-NTA beads (Qiagen, Hilden, Germany) for 1 h at 4° C. The beads were washed with at least 4 ml of buffer containing 250 mM sucrose, 1 mM dithiothreitol, 150 mM NaCl, 15 mM Tris-Cl, pH 7.5, 2.5 mM MgSO4, 15 μg/ml DNase-I (Sigma), and 1 mM phenylmethylsulfonyl fluoride and broken by a French press. The membrane fraction was collected by ultracentrifugation at 213,500 × g for 1 h at 4 °C and resuspended in the above buffer without dithiothreitol, DNase, and phenylmethylsulfonyl fluoride. The membranes were frozen in liquid nitrogen and stored at −70 °C.

To determine the protein concentration in the proteoliposomes, the proteoliposomes were solubilized in buffer containing 15 mM Tris-Cl, pH 7.5, 6 M urea, and 2% SDS (SDS-urea buffer) for 15 min. The solubilized proteins were immobilized on Ni-NTA beads for 1 h at room temperature. The unbound material was discarded, and the His-tagged protein bound to beads was washed twice with SDS-urea buffer. The protein was eluted from the beads using a buffer containing 200 mM imidazole and mixed with 375 μl of E. coli phospholipid mix (10 mg of E. coli lipids (Avanti Polar Lipids, Alabaster, AL), 1.2% n-octyl-β-D-glucopyranoside (Glycon GmbH), 30 mM imidazole, and 15 mM β-mercaptoethanol. The protein was eluted with 500 μl of the same buffer containing 200 mM imidazole and mixed with 375 μl of E. coli phospholipid mix (10 mg of E. coli lipids (Avanti Polar Lipids, Alabaster, AL), 1.2% n-octyl-β-D-glucopyranoside, 15 mM Tris-Cl, pH 7.5, and 150 mM NaCl). Eluted protein and phospholipids were sonicated together in a bath-type sonicator to clarity and n

RESULTS

Tyrosine Replacements at Positions 6 and 53 Are Functional—In the amino acid sequence of EmrE, there are five tyrosine residues at positions 4, 6, 40, 53, and 60 (Fig. 1). According to the secondary structure model of EmrE (31, 32), Tyr-4 and -6 are predicted to be located at the beginning of transmembrane segment (TM1) facing the cytoplasm (33); Tyr-53 is located in loop 2 connecting TM2 and TM3, whereas the other two tyrosine residues are located in the transmembrane regions Tyr-40 in TM 2 and Tyr-60 in TM 3. Tyr-60 is the only one fully conserved within the small multidrug resistance family (34). In previous studies, the conservative replacement of Tyr-60 with Phe, an aromatic residue, resulted in a nonfunctional protein (35).

In this study, we characterized the role of tyrosine residues in the function of EmrE with a set of mutants constructed using site-directed mutagenesis. The basic screen included assessment of the ability of the mutated proteins to confer resistance against toxicants. To evaluate expression levels and to study their function in more detail, all mutant proteins were purified by Ni-NTA affinity chromatography and assayed

3 The abbreviations used are: DDM, n-dodecyl-β-maltoside; Ni-NTA, N9-N3- nitroso-2-2-acetic acid; TPP, tetraphenylphosphonium; MV2-, methyl viologen; Tricine, N(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; NEM, N-ethylmaleimide; TM, transmembrane segment.

4 The abbreviations used are: DDM, n-dodecyl-β-maltoside; Ni-NTA, N9-N3- nitroso-2-2-acetic acid; TPP, tetraphenylphosphonium; MV2-, methyl viologen; Tricine, N(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; NEM, N-ethylmaleimide; TM, transmembrane segment.
for their ability to bind TPP\(^+\), a high-affinity substrate of EmrE (4).

Upon induction of expression, all of the mutants tested, including those that failed to confer any resistance, expressed to similar levels (not shown). Previously, the Y40C mutant did not express to detectable levels. However, at the time, a different vector and a different purification protocol were used, and the three wild-type cysteine residues were replaced with Ser (27). When the mutant was expressed in pT7-7 with a Myc-His tag in the background, where the Cys residues were replaced with Ala (30), the expression level of Y40C was normal. All of the other mutants used in this work and in our recent papers are in the same background.

The ability of the mutated proteins to confer resistance against toxicants was assessed by testing the ability of the cells expressing such mutated proteins to grow on otherwise nonpermissive conditions. This was achieved in solid media containing either ethidium bromide (100 \(\mu\)g/ml), acriflavine (50 \(\mu\)g/ml), or methyl viologen (0.2 mM). Cells carrying the vector plasmid without any insert cannot grow on these media at any of the dilutions tested. Cells expressing either EmrE or CAMY were able to grow at each of the dilutions (Fig. 1). This assay provides a highly dynamic range to qualitatively analyze the activity of the mutants generated.

Mutations at two positions were well tolerated. At position 6, two replacements that were designed based on evolutionary conservation observed at this position, Y6L and Y6F, displayed phenotypes almost indistinguishable from wild type (Fig. 1). Even a Cys replacement yielded a protein that conferred the same resistance. A Cys replacement at position 53 also yielded a protein with a robust phenotype (Fig. 1). All of the substitutions at these positions also bound \([\text{H}]\text{TPP}^+\) to considerable levels compared with that of EmrE control (Fig. 2). Because they are practically indistinguishable from wild type, these mutants were not studied further.

An Aromatic Residue at Position 4 Is Required for Proper Coupling

—At position 4, aromatic residues are evolutionarily conserved in >200 homologues (10). Conservative replacements Y4F and Y4W displayed wild-type phenotype (Fig. 1) and robust binding activity (Fig. 2). Cysteine replacement at position 4 generated a protein that did not confer resistance to any of the toxicants tested (Fig. 1). Unexpectedly, the Y4C mutant displayed high levels of binding despite the fact that it did not confer resistance. To further investigate this apparent contradiction, the purified mutant protein was reconstituted into proteoliposomes, and \(\Delta p\text{H}\)-driven uptake of MV\(^{2+}\) was measured quantitatively using a radiolabeled substrate (3). In these experiments, a pH gradient (acid inside) was generated by diluting ammonium-loaded proteoliposomes.

**FIGURE 1. Growth phenotype of cells expressing Tyr mutants.** On a secondary structure model of EmrE, the Tyr residues are shown in black. Insets, the ability of mutants at the indicated positions to grow on solid media containing the toxic compounds 0.2 mM methyl viologen (black bars), 50 \(\mu\)g/ml acriflavine (dark gray bars), and 100 \(\mu\)g/ml ethidium (light gray bars) is shown. The assay is described under “Experimental Procedures.” WT, wild type.
into an ammonium-free medium. The wild-type protein accumulated radiolabeled MV2\(^+\) against its concentration gradient in a process that involves exchange of 2H\(^+\) with one substrate molecule (Fig. 3A). When proteoliposomes reconstituted with Y4C were used, no MV2\(^+\) accumulation was detected to any measurable degree (Fig. 3A). Furthermore, the mutant did not transport TPP\(^-\) against its concentration gradient (data not shown), even though it bound it with high affinity (Fig. 2). Because Y4C binds substrate but does not transport it, we then asked whether it catalyzes downhill transport in the absence of a proton electrochemical gradient. To answer this question, proteoliposomes were loaded with radiolabeled MV2\(^+\) and diluted in the same medium (no H\(^+\) gradients generated) but without substrate. In such an experiment, the substrate rapidly effluxed from the proteoliposome in a transporter-mediated process (Fig. 3B). As shown in the figure, the rate of downhill transport catalyzed by Y4C was similar to that displayed by wild-type EmrE. These results suggest that an aromatic residue in this position is required for proper coupling between the substrate transport and the proton gradient.

**Mutants at Position 40 Display Modified Specificity**—Several replacements at position 40 (Cys, Ser, Thr, Met, Phe, Leu, and Val) conferred resistance to MV2\(^+\) but not to acriflavine or ethidium (Fig. 1). None of the substitutions at position 40 bound TPP\(^-\) to significant levels (Fig. 2). Even though none of them bound any TPP\(^-\), the reconstituted proteins transported MV2\(^+\) against its concentration gradient, although at very different rates (Fig. 3C). Replacements with hydroxyamino acids, Y40T and Y40S, displayed significant MV2\(^+\) uptake activity at rates 40 and 22% of those of the wild type, respectively, whereas the activity of mutants Y40C and Y40F was lower (5% of wild type) but measurable. Therefore, the hydroxyamino acids can substitute for the tyrosine at this position much more efficiently, suggesting that the hydroxyl moiety is important for the transport activity. However, this is not an absolute requirement, because both Cys and Phe substitutions display low but measurable activity. The low activity of these mutants (and also replacements with Leu, Val, and Met) is sufficient to confer resistance in the phenotype assay described above. We have previously shown with other mutants that MV2\(^+\) uptake levels as low as 5% of the wild type can sustain growth under conditions that are nonpermissive to cells carrying the vector alone (27).

As we have demonstrated above, replacements with hydroxyamino acids at position 40 generate proteins that display significant MV2\(^+\) uptake activity even though they do not bind TPP\(^-\). Another way to explore the interaction of TPP\(^-\) with the protein is by testing its effect on MV2\(^+\) uptake. In such an experiment, proteoliposomes reconstituted with the corresponding protein were assayed for MV2\(^+\) uptake at various concentrations of TPP\(^-\). The results shown in Fig. 4 demonstrate that the IC\(_{50}\) value for TPP\(^-\) inhibition of the MV2\(^+\) uptake activity of Y40S was \(\sim 55\) times higher (\(\sim 4\) \(\mu\)M) than the IC\(_{50}\) value for inhibition of the wild type. These results imply that the affinity to TPP\(^-\) is greatly decreased in the mutant and practically immeasurable with the direct binding assay. This observation suggests that the aromatic ring in position 40 has a significant role in TPP\(^-\) binding, whereas it is not essential for MV2\(^+\) recognition. To characterize the substrate specificity of this mutant, the ability of several drugs to inhibit MV2\(^+\) uptake of wild-type and Y40S EmrE was measured (Table 1). The results demonstrated that the MV2\(^+\) uptake activity of the mutant was affected differentially by different substrates. Although with most substrates, the differences in the IC\(_{50}\) values were moderate (from 2.6 to 7-fold), with the phosphonium derivatives, the increase in the IC\(_{50}\) values was dramatic; the IC\(_{50}\) values of diphenylmethylphosphonium, triphenylmethylphosphonium, and TPP\(^-\) were 19, 37, and 55 times higher for Y40S compared with wild-type EmrE, respectively. The higher the number of aromatic rings on the tetrahedral phosphonium ion, the larger the detrimental effect of the Tyr mutation.

**Accessibility/Reactivity of Cys Replacements at Position 60 Is Affected by Substrate**—Tyrosine at position 60 is evolutionarily highly conserved and, as anticipated, three of the replacements tested, Cys, Phe, and Ser, did not yield a functional phenotype on solid media. Surprisingly, however, replacement with Thr yielded a mutant that conferred resistance to MV2\(^+\) (Fig. 1). Although only the latter mutant conferred resistance, all of the replacements tested displayed low but measurable activity. The low activity of these mutants (and also replacements with Leu, Val, and Met) is sufficient to confer resistance in the phenotype assay described above. We have previously shown with other mutants that MV2\(^+\) uptake levels as low as 5% of the wild type can sustain growth under conditions that are nonpermissive to cells carrying the vector alone (27).
The data presented above for mutants at positions 40 and 60 suggest that they play a role in substrate binding. To further substantiate this conclusion, we previously showed that a hetero-oligomer generated in vitro, where one protomer is a Cys-less construct and the other one is Y40C, binds TPP/H11001. In this protein, binding was abolished by alkylation of the only cysteine in the inactive protomer in position 40 (10). A similar phenomenon was reported also for a mixed hetero-oligomer where the Cys residue was at position 14 in the inactive protomer (8). Although in this type of experiment, an indirect effect could not be ruled out, the findings suggested a direct role of the involved residues in binding.

Because Y60C binds TPP/H11001, we tested its role in binding by exploring accessibility to alkylating reagents and the effect of substrates on this reaction. In Fig. 5, we see the results of an experiment where two EmrE mutants, the previously characterized L7C, and the here-described Y60C were challenged with NEM-fluorescein under various conditions. After purification using metal-chelate chromatography, the protein was separated by SDS-PAGE, and the amount of fluorescence associated with it was quantitated. Both mutants were labeled to a similar degree, whether solubilized in DDM (lanes 1 and 4) or in SDS-urea (lanes 3 and 6). When the reaction was carried out after preincubation with substrate (TPP+/H11001, 25 μM), the modification was prevented to a large degree (lanes 2 and 5). To further analyze the effect of substrates on the alkylation reaction, we tested the concentration dependence of the protective effects of substrate. Similar to what we showed previously, concentrations of ~5 μM TPP+ almost fully (~85%) protected the alkylation of L7C. Higher concentrations were needed to protect ~60% of the labeling of Y60C, a mutant with a lower affinity to TPP+.

These findings demonstrate that Y60C is accessible to alkylation and the reaction is prevented by substrate. As such, they provide further support for a role of Tyr-60 in substrate recognition.

**DISCUSSION**

Tyrosine residues can fulfill diverse roles in protein-ligand interactions. They can participate in ligand binding through hydrophobic bonds (36), hydrogen bonds (37), or cation/π interactions (22). They can also stabilize the binding site in the absence of ligand (21). In this work, we examined the effect of mutagenesis of each of the tyrosine residues on the activity of the multidrug transporter EmrE. We showed that three of five tyrosine residues in EmrE have significant roles in substrate binding and/or translocation.

Aromatic residues are evolutionarily conserved at position 4 in >200 Smr homologues (10), and not surprisingly, replacements in EmrE with other aromatic residues do not affect the protein activity. Other residues in the same TM, namely Leu-7, Ala-10, and Glu-14, have been shown to play a part in the substrate and proton binding site of the protein (10, 11).
30). Activity of mutants with Cys replacements at positions Leu-7 and Ala-10 is very sensitive to alkylation with NEM, and the inhibition is totally prevented by substrates of EmrE. A possible explanation for this mutual exclusion is supplied by accessibility studies where we showed that the residues react with NEM-fluorescein, but binding of substrate limits the accessibility to the site of action. Accessibility of a Cys replacement at position 60 is affected by pH. NEM-fluorescein was added for 20 min prior to the NEM-fluorescein. The reactions were stopped after 30 s by the addition of β-mercaptoethanol to a final concentration of 5 mM followed by immobilization and analysis by SDS-PAGE as described under “Experimental Procedures.” In the upper panel, the fluorescent signal is shown prior to staining with Coomassie shown in the lower panel. B, different concentrations (0–25 μM) of TPP⁺ were added for 20 min at 25 °C prior to labeling with NEM-fluorescein of the mutants.

FIGURE 5. Accessibility/reactivity of Cys replacements at position 60 is affected by substrate. A, representative gel showing accessibility to L7C and Y60C. Membranes of both mutants were solubilized either in Na-buffer supplemented with 0.5% (6 mM) SDS-urea solution or in 0.8% DDM/Na-buffer as indicated. After a brief centrifugation to remove unsolubilized material (see “Experimental Procedures”), NEM-fluorescein was added to a final concentration of 0.5 mM. Where indicated (lanes 2 and 3), TPP⁺ (25 μM) was added 20 min prior to the NEM-fluorescein. The reactions were stopped after 30 s by the addition of β-mercaptoethanol to a final concentration of 5 mM followed by immobilization and analysis by SDS-PAGE as described under “Experimental Procedures.” In the lower panel, the fluorescent signal is shown prior to staining with Coomassie shown in the lower panel. B, different concentrations (0–25 μM) of TPP⁺ were added for 20 min at 25 °C prior to labeling with NEM-fluorescein of the mutants.

FIGURE 4. Inhibition of [14C]methyl viologen transport with TPP⁺. EmrE (■) and the mutants Y40S (○) or Y60S (▲) were purified and reconstituted as described under “Experimental Procedures.” Ammonium-loaded proteoliposomes (2 μl) were diluted into an ammonium-free medium containing [14C]methyl viologen, and the indicated concentrations of TPP⁺ and radioactivity incorporated after 2 min were measured. WT, wild type.

<table>
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<th>Ligand</th>
<th>EmrE-His IC₅₀ (μM)</th>
<th>Y40S IC₅₀ (μM)</th>
<th>Ratio (Y40S/EmrE-His)</th>
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<td>TPP⁺</td>
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<td>3.8 ± 0.8</td>
<td>54.3</td>
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<td>TPPM⁺</td>
<td>1.2 ± 0.2</td>
<td>44.1 ± 7.2</td>
<td>36.8</td>
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<td>DPPMP⁺</td>
<td>17.6 ± 0.7</td>
<td>337.1 ± 110</td>
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<td>Acriflavin⁺</td>
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<td>31.8 ± 4.7</td>
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<td>111.5 ± 14.9</td>
<td>490.6 ± 138</td>
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<tr>
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TABLE 1

Inhibition of [14C]methyl viologen transport activity of EmrE and Y40S by a variety of drugs

EmrE and the mutant protein Y40S were purified and reconstituted as described under “Experimental Procedures.” Ammonium-loaded proteoliposomes (2 μl) were diluted into an ammonium-free medium containing [14C]methyl viologen and different concentrations of the indicated drugs, and radioactivity incorporated after 2 min was measured. IC₅₀ values were calculated with Origin 7.0 software (OriginLab, Northampton, MA).
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FIGURE 6. The location of tyrosyl residues in one of the structures of EmrE. The location and possible interactions of Tyr residues in the second structure of EmrE (44) (Protein Data Bank code 2Z2M) was analyzed. A, schematic representation of the asymmetric unit composed of two EmrE subunits. Shown are subunits A in gold (with its tyrosyl residues in green) and B in blue (with tyrosyl residues in pink). Most of the tyrosyl residues are facing the lipid. B, possible interaction between Tyr-40 from monomer 1 (green) and Glu-14 of monomer 2 (pink). Note that Glu-14 of monomer 1 is facing the lipidic milieu. C, Tyr-60 and Trp-63, two fully conserved and critical residues in EmrE, are facing the lipid.

role in MV\textsuperscript{2+} transport. Replacement of the tyrosine to serine causes a major reduction in the affinity of the protein to TPP\textsuperscript{+} and other phenylphosphonions and a milder reduction in the affinity of other ligands that were examined. The most dramatic effects are seen with the nonplanar substrates, such as the phosphonium derivatives, suggesting a direct interaction between the aromatic rings of the tyrosine and the substrate. Also for other multidrug transporters, it has been demonstrated that the identity of a single residue can determine the specificity of the transporter. For instance, it was shown that substitutions in the Phe-143 and Phe-306 residues of the Bacillus subtilis multidrug transporter Bmr changes its substrate specificity (39). More recently, high resolution structures of proteins that interact with multiple drugs confirm the hypothesis that different substrates may interact with different residues in a large binding pocket (16, 17, 40). Our results cannot rule out the possibility that replacement of a single tyrosine modifies the specificity profile of EmrE due to an indirect conformational change in the protein that affects, somehow, the binding domain. However, the notion that position 40 is part of the substrate binding domain is supported by the finding that a hetero-oligomer generated in vitro, where one protomer is a Cys-less construct and the other one is Y40C, binds TPP\textsuperscript{+} and binding is abolished by alkylation of the only cysteine in position 40 of the inactive protomer (10).

Tyr-60 is fully conserved in the small multidrug resistance family (34). Its replacement by serine or threonine yields mutants that display robust ΔpH-driven uptake of MV\textsuperscript{2+} after reconstitution into proteoliposomes. The Y60S uptake activity was inhibited by other EmrE ligands at the same level as the wild-type protein (Fig. 4). Even though we cannot rule out the indirect effects of the mutations on binding and transport, we conclude that the body of evidence presented here and in previous work suggests a role for Tyr-60 in substrate binding. These results hint that the hydroxyl group of Tyr-60 has a major role in the transport activity of EmrE. The fact that Y60S and Y60T have lower activity compared with the wild-type protein may suggest that the aromatic ring has a role in the determination of the positioning of the hydroxyl group in the exact place where its influence is optimal.

Mutations in both Tyr-40 and Tyr-60 suggest that the hydroxyl group plays a major, but not unique, role in the activity of EmrE. In addition to its potential role in direct interaction with ligand through a hydrogen bond (37), the hydroxyl group of tyrosine can fulfill a role in the stabilization of the binding site in the absence of ligand (21). So far, we cannot determine the exact role of the hydroxyl group of these residues. As suggested previously, residues in TM1 of EmrE play a direct role in a single binding site shared by protons and substrate (10, 30). It is tempting, therefore, to speculate that TM1 residues from both protomers contribute to the binding site. As it is shown here and elsewhere (10, 23), aromatic residues from TM2 and TM3 have also been identified as part of the binding site. We speculate that, in the absence of substrates, the carboxyls of Glu-14 in the binding cavity are stabilized by interaction with protons or with at least part of the aromatic residues contributed by TM2 and TM3. The aromatic residues provide an environment that may explain the unusually high pK values of these carboxyls (15) and allow for interaction with the hydrophobic substrates, as has been documented in other proteins that bind similar substrates (21, 41). As it is shown here, the aromatic ring of Tyr-40 has an effect in the binding of some substrates. This finding suggests that, in EmrE, as in other proteins interacting with multiple substrates, the size of the binding pocket is...
large enough to allow different molecules to reside in it in different orientations and to establish interactions with different sets of residues on the pocket walls.

The three-dimensional structure of EmrE with and without bound TPP⁺ was determined at 7.5 Å resolution by electron cryomicroscopy of two-dimensional crystals (9, 42). The two structures are practically indistinguishable. The minimal structural unit is an asymmetric homodimer composed of eight transmembrane α-helices, i.e. four helices from each monomer, with density for TPP⁺ in a binding chamber formed by six of the eight α-helices, confirming the suggestion that TPP⁺ binds near the center of the dimer (9). Two x-ray structures of EmrE have been published, and the two structures are very different (43, 44). The presence of substrate in the second one may be responsible for the large differences between the two structures, although similar substrate-induced conformational changes were not observed in the two-dimensional crystals. The x-ray structure also shows an asymmetric dimer, and the protomers have antiparallel topological orientation. This finding has obvious and exciting similarities to the internal structural repeat found in the neurotransmitter transporter homologue LeuT (45–47). How- ever, it is at odds with biochemical data that have demonstrated the suggestion that TPP⁺ binds near the center of the dimer (9).

Modifying Substrate Specificity in a Multidrug Transporter

TPP⁺

The three-dimensional structure of EmrE with and without bound TPP⁺ was determined at 7.5 Å resolution by electron cryomicroscopy of two-dimensional crystals (9, 42). The two structures are practically indistinguishable. The minimal structural unit is an asymmetric homodimer composed of eight transmembrane α-helices, i.e. four helices from each monomer, with density for TPP⁺ in a binding chamber formed by six of the eight α-helices, confirming the suggestion that TPP⁺ binds near the center of the dimer (9). Two x-ray structures of EmrE have been published, and the two structures are very different (43, 44). The presence of substrate in the second one may be responsible for the large differences between the two structures, although similar substrate-induced conformational changes were not observed in the two-dimensional crystals. The x-ray structure also shows an asymmetric dimer, and the protomers have antiparallel topological orientation. This finding has obvious and exciting similarities to the internal structural repeat found in the neurotransmitter transporter homologue LeuT (45–47). However, it is at odds with biochemical data that have demonstrated the suggestion that TPP⁺ binds near the center of the dimer (9).

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REFERENCES