Substrate-Induced Tryptophan Fluorescence Changes in EmrE, the Smallest Ion-Coupled Multidrug Transporter†

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Received February 25, 2005; Revised Manuscript Received March 22, 2005

ABSTRACT: Tryptophan residues may play several roles in integral membrane proteins including direct interaction with substrates. In this work we studied the contribution of tryptophan residues to substrate binding in EmrE, a small multidrug transporter of *Escherichia coli* that extrudes various positively charged drugs across the plasma membrane in exchange with protons. Each of the four tryptophan residues was replaced by site-directed mutagenesis. The only single substitutions that affected the protein's activity were those in position 63. While cysteine and tyrosine replacements yielded a completely inactive protein, the replacement of Trp63 with phenylalanine brought about a protein that, although it could not confer any resistance against the toxicants tested, could bind substrate with an affinity 2 orders of magnitude lower than that of the wild-type protein. Double or multiple cysteine replacements at the other positions generate proteins that are inactive in vivo but regain their activity upon solubilization and reconstitution. The findings suggest a possible role of the tryptophan residues in folding and/or insertion. Substrate binding to the wild-type protein and to a mutant with a single tryptophan residue in position 63 induced a very substantial fluorescence quenching that is not observed in inactive mutants or chemically modified protein. The reaction is dependent on the concentration of the substrate and saturates at a concentration of 2.57 μM with the protein concentration of 5 μM supporting the contention that the functional unit is a dimer. These findings strongly suggest the existence of an interaction between Trp63 and substrate, and the nature of this interaction can now be studied in more detail with the tools developed in this work.

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 (*1*, *4*−*6*). 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 suggests that the basic EmrE oligomer is a dimer (*7*−*10*). EmrE has only one membrane-embedded charged residue, Glu14, that is conserved in more than 100 homologous proteins (*5*, *11*) and plays a central role in substrate and proton binding (*12*−*15*). Deprotonation of Glu14 is required for substrate binding (*4*, *14*). Substrate-induced proton release has been observed directly in a detergent-solubilized preparation of EmrE (*16*). From the pH dependence of the magnitude of the release we have estimated a p*K* for Glu14 of 8.3−8.5. The unusually elevated pK_a suggests that the environment around Glu14 is chemically unique. To further probe this environment, carbodiimide modification of Glu14 has been studied using functional assays and directly monitored using electrospray ionization mass spectrometry (ESI-MS) (*17*). Hydrophobic carbodiimides such as diisopropylcarbodiimide (DiPC), but not hydrophilic ones, modified ∼80% of Glu14, and the reaction was fully prevented by preincubation with substrates (*17*). The results indicate that each Glu14 residue in the oligomer is accessible to DiPC and in an hydrophobic domain. Taken together with other biochemical data, the findings support a mechanism in which both Glu14 residues in a dimer are involved in TPP^+ [1] and H^+ binding.

Systematic cysteine scanning mutagenesis of the neighbors of residue Glu14 in TM1 revealed an amino acid cluster surrounding Glu14 that is part of the substrate-binding domain (*18*). Correspondingly, a homologue of EmrE from the archaeon *Halobacterium salinarum* (Hsmr) is built of over 40% valine and alanine residues and remains relatively unaltered on the face containing Glu14; however, Val and Ala residues are clustered on the face opposite Glu14, in domains that do not seem important for activity (*11*).

Recent advances in the structural analysis of multidrug recognizing transcription factors (*19*−*21*) and transporters (*22*, *23*) suggest that these proteins possess large hydrophobic binding sites and bind their substrates through a combination

† This work was supported by Grant NS16708 from the National Institutes of Health and Grant 119/04 from the Israel Science Foundation. S.S. is a Mathilda Marks-Kennedy Professor of Biochemistry at the Hebrew University of Jerusalem.

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[1] Abbreviations: TMS, transmembrane segment; DDM, *n*-dodecyl *β*-maltoside; TPP^+, tetraphenylphosphonium; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Na buffer, 150 mM NaCl, 15 mM Tris-HCl, pH 7.5 [when DDM was added, its concentration was 0.08% (DDM-Na buffer)].

10.1021/bi050356t CCC: $30.25
Published on Web 04/23/2005

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 (*24*).

In this work we studied the contribution of tryptophan residues to substrate binding in EmrE. Close interaction between substrates and aromatic residues has been observed in the binding sites of multidrug recognizing transcription factors (*19*−*21*). Aromatic residues such as tryptophan can interact with cations through a strong, noncovalent force termed the cation/π interaction (for a review see ref *25*). 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 (*25*).

EmrE has four Trp residues, and only one of them (W63) is fully conserved (*5*) and, as shown here, essential for activity. We have mutagenized each one of the Trp residues and constructed functional proteins with two Trp in different combinations and with a single Trp at position 63. The single Trp63 protein is functional provided that at least two of the other Trp residues are replaced with another aromatic residue (Tyr). Using purified wild-type and single Trp63 proteins, we have detected a substrate-induced change in Trp fluorescence.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. E. coli DH5α and TA15 (*26*) strains were used throughout this work. TA15 strain was previously transformed with plasmid pGP1-2, which codes for the T7 polymerase under the inducible control of the λ PL promoter (*27*). The plasmids used for EmrE gene expression are pT7-7 (*27*) derivatives with a Myc epitope and a six-His tag (EmrE-His, for simplicity, will be called EmrE throughout this paper) (*4*).

Mutagenesis. The construction of the mutants was as previously described (*28*). Mutants were obtained by polymerase chain reaction, using the overlap extension procedure as described by Ho et al. (*29*). The template used was CLA-His [a cysteine-less EmrE that was built with alanine replacements (*18*)].

Resistance to Toxic Compounds. For testing resistance to toxic compounds, cells were grown overnight at 37 °C in LB-amp medium containing 30 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) titrated to pH 7.5 and the following different concentrations of the toxic compounds ethidium bromide, acriflavin, and methyl viologen: 0, 7.75, 31.5, 62.5, 125, 250, and 500 μM. Cell density was estimated from absorbance at 600 nm.

Transport of Ethidium in Whole Cells. Transport was assayed essentially as described (*1*). *E. coli* DH5α cells bearing the appropriate plasmids were grown in LB medium at 37 °C to $A_{600} = 0.4$, were collected by centrifugation, and were resuspended to $A_{600} = 0.1$ in minimal medium A (*30*) supplemented with 0.05% NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.36% glucose. At this point, ethidium and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were added to a final concentration of 1 μg/mL and 40 μM, respectively, and the cells were incubated for 60 min at 37 °C. The cells were collected by centrifugation and quickly resuspended in medium containing the same ethidium concentration with or without CCCP. Fluorescence was measured in a PerkinElmer fluorometer (LS 50 B luminescence spectrometer) using FL WinLab software with exciting wavelength at 545 nm and emission at 610 nm.

Expression, Purification, and Reconstitution of EmrE into Proteoliposomes. E. coli TA15 cells that bear plasmids pGP1-2 and pT7-7 containing His-tagged EmrE constructs were grown at 30 °C in minimal medium A supplemented with 0.5% glycerol, 2.5 μg/mL thiamin, 100 μg/mL ampicillin, and 50 μg/mL kanamycin. When the culture reached an $A_{600} = 1$, it was transferred to 42 °C for 15 min to induce the T7 polymerase. Then the culture was shifted back to 30 °C. Two hours later the cells were harvested by centrifugation. Cells were resuspended with buffer containing 250 mM sucrose, 1 mM dithiothreitol, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM $MgSO_4$, 15 μg/mL DNase I (Sigma), and 1 mM phenylmethanesulfonyl fluoride and broken by a French press. The membrane fraction was collected by ultracentrifugation at 213500*g* for 1 h at 4 °C and resuspended in the above buffer without dithiothreitol and without DNase. The membranes were frozen in liquid nitrogen and stored at −70 °C.

Reconstitution was performed essentially as described (*15*). Four hundred microliters of membranes (containing 120 μg of His-tagged protein) were solubilized in 2 mL of buffer containing 150 mM NaCl, 15 mM Tris-HCl, pH 7.5 (Na buffer), 1% *n*-dodecyl β-maltoside (DDM), 0.5 mM phenylmethanesulfonyl fluoride, and 15 mM β-mercaptoethanol. After removal of unsolubilized material by centrifugation (20000*g* for 30 min), imidazole was added to 20 mM, and the His-tagged protein was incubated with Ni^{2+}-nitrilotriacetic acid (Ni-NTA) beads (Qiagen, Hilden, Germany) for 1 h at 4 °C. The beads were washed with at least 4 mL of Na buffer containing 1% *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* phospholipids mix (10 mg of phospholipids, 1.2% *n*-octyl β-D-glucopyranoside, 15 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Eluted protein and phospholipids were sonicated together in a bath-type sonicator to clarity and diluted in buffer containing 0.19 M NH_4Cl, 15 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol. After 20 min at room temperature samples were centrifuged at 250000*g* for 60 min, and the pellet was resuspended in 100 μL of the same buffer, frozen in liquid nitrogen, and stored at −70 °C. Prior to the transport assay, the proteoliposomes were thawed at room temperature and sonicated lightly to clarity.

TPP^+ Binding Assay. Tetraphenylphosphonium (TPP^+) binding was assayed essentially as described (*4*). Ni-NTA beads (10 μL/assay) were washed once in distilled water and twice in 0.08% DDM-Na buffer. Membranes, solubilized in 0.8% DDM-Na buffer, were added to the washed beads and incubated at 4 °C for 1 h. The unbound material was discarded, and the His-tagged protein bound to beads was washed three times with 0.08% DDM-Na buffer. Buffer containing 5 nM [^{3}H]TPP^+ (27 Ci/mmol; Amersham Biosciences) was added, and the samples were incubated for 20 min at 4 °C. In each experiment, the values obtained in a control reaction with 25 μM unlabeled TPP^+ were subtracted. Separating the beads from the supernatant by pulse centrifugation stopped the binding reaction. The bead fraction was then incubated for 10 min at room temperature with 450 μL

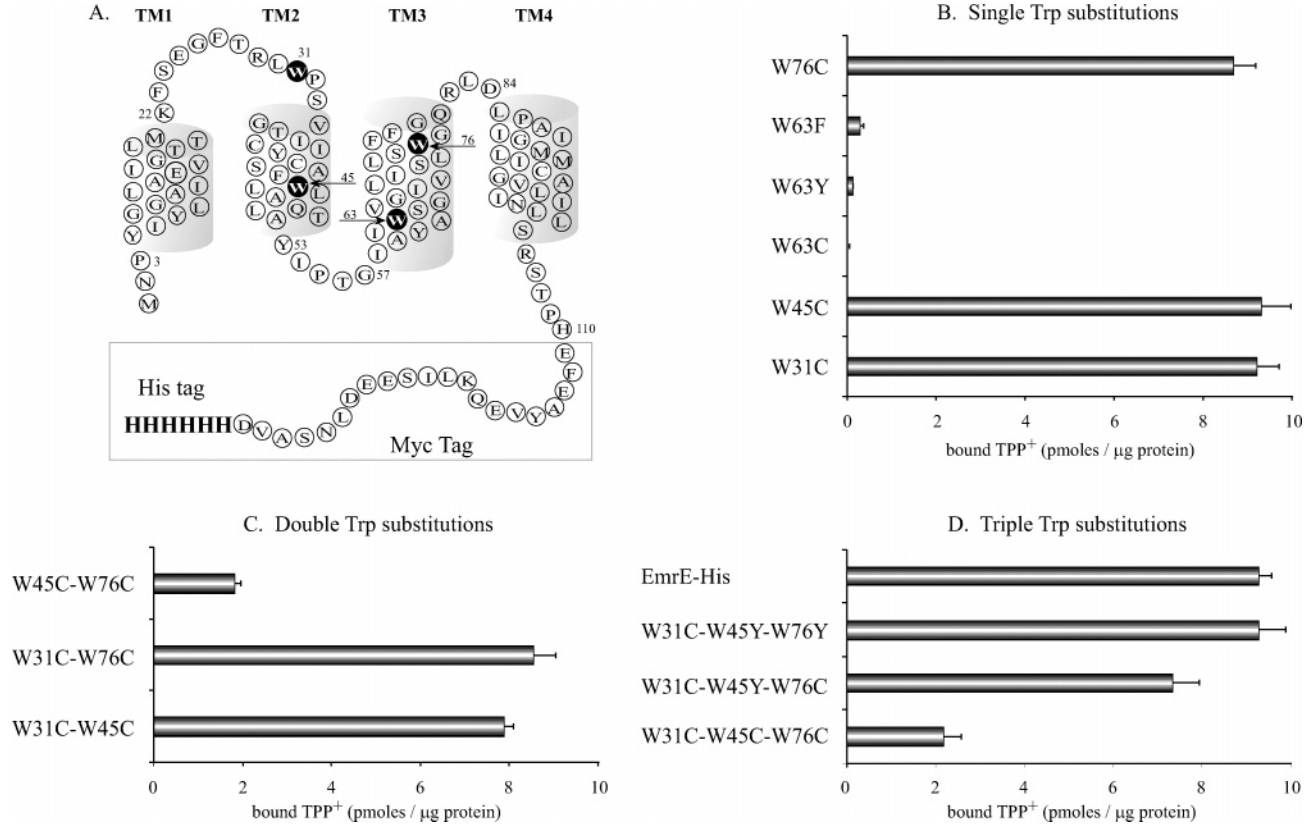

FIGURE 1: TPP^+ binding activity of the Trp substitution mutants. (A) Secondary structure model of EmrE-His. The residues in the square are the Myc-His tag (*4*). Trp residues that were mutated throughout this work are shown in black. The results of $[^3H]TPP^+$ binding of the various Trp substitutions mutants are presented in histograms. The mutants are divided into three groups: single Trp substitutions (B), double Trp substitutions (C), and triple Trp substitutions (D). The results of $[^3H]TPP^+$ binding activity of EmrE are presented in (D). The $[^3H]TPP^+$ binding assay was performed as described under Experimental Procedures.

of 0.08% DDM-Na buffer containing 150 mM imidazole in order to release the His-tagged protein and $[^3H]TPP^+$ bound to it from the beads. After spinning down the beads, the $[^3H]$-TPP^+ associated radioactivity was measured by liquid scintillation. All binding reactions were performed in duplicate.

Transport Assay. Uptake of $[^{14}C]$methyl viologen into proteoliposomes was assayed at 25 °C by dilution of 3 μL of the ammonium chloride-containing proteoliposomes (100 ng of His-tagged protein) into 200 μL of an ammonium-free solution (*1*, *15*). The latter contained 20 μM $[^{14}C]$methyl viologen (11.9 mCi/mmol, Sigma), 140 mM KCl, 10 mM Tricine, 5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.5. At given times, the reaction was stopped by dilution with 2 mL of the same ice-cold solution, filtering through Millipore GSWP filters (0.22 μm) and washing with an additional 2 mL of solution. The radioactivity on the filters was estimated by liquid scintillation. In each experiment, the values obtained in a control reaction, with 15 μM nigericin, were subtracted from all experimental points.

Fluorescence Measurements. Fluorescence was recorded by a LS 50 B luminescence spectrometer (PerkinElmer Ltd., Beaconsfield, England) using FL WinLab software. Protein used for these experiments was overexpressed and purified as previously described (*16*). Purification steps that included metal chelate and size exclusion chromatography yielded a highly purified preparation. Protein concentration was calculated from the absorption of the protein at 280 nm (extinction coefficient 30440 cm^{-1} M^{-1} for the wild-type protein and 15930 cm^{-1} M^{-1} for the W31C-W45Y-W76Y[2] mutant). All experiments were carried out in a 1 × 1 cm quartz cuvette in a 0.08% DDM-Na buffer. To obtain similar fluorescence intensities, "tryptophan concentration" was kept constant at 20 μM. For this purpose, the following final protein concentrations were used: EmrE and E14C at 5 μM and W31C-W45Y-W76Y at 20 μM. At the sensitivity used for these measurements the blank with no protein showed no fluorescence in the range tested. During measurements samples were constantly stirred using a magnetic stirrer. When TPP^+ was added to samples, it was added to a final concentration of 3 μM for EmrE and E14C and 12 μM for W31C-W45Y-W76Y.

For DCCD inhibition, 5 μM EmrE in 0.08% DDM-Na buffer was incubated with 500 μM DCCD (Sigma) for 30 min at 25 °C with magnetic stirring before the measurement.

RESULTS

Phenotype of Tryptophan Substitution Mutants. In the amino acid sequence of EmrE there are four tryptophan residues at positions 31, 45, 63, and 76 (Figure 1A). According to the secondary structure model of EmrE (*6*, *31*) Trp31 is predicted to be located on the first loop, facing the periplasm (*32*), while the other three tryptophan residues are located in the transmembrane region: Trp45 is located in

[2] Site-directed mutants are named as follows: single amino acid replacements are named with the letter of the original amino acid, then its position in the protein, and the letter of the new amino acid.

Table 1: Growth Phenotype of Cells Expressing Trp Mutants[a]

tryptophan substitution	mutation	growth in presence of substrate: methyl viologen	ethidium bromide	acriflavin
single	W31C	+	+	+/−
	W45C	+	+	+/−
	W63C/Y/F	−	−	−
	W76C	+	+	+/−
double	W31C-W45C	+	+/−	−
	W31C-W76C	+	+/−	−
	W45C-W76C	−	−	−
triple	W31C-W45C-W76C	−	−	−
	W31C-W45Y-W76C	+	+/−	−
	W31C-W45Y-W76Y	+	+	+/−

[a] For testing resistance to toxic compounds, *E. coli* DH5α cells transformed with the various mutants were grown overnight at 37 °C in the presence of EmrE substrates as described under Experimental Procedures. Growth was estimated according to cell density measurement from absorbance at 600 nm. The growth level of the cells was estimated compared to cell growth of cells transformed with wild-type EmrE as positive control and pT7-7 (empty vector) as negative control. The symbol (+) is assigned to cells able to grow as well as wild-type EmrE under the same conditions. The lack of growth under these conditions is defined as (−). The growth level between that of wild-type EmrE and that of the empty vector transformed cells is marked by (+/−).

TMS 2 and Trp63 and Trp76 are located in TMS 3. Trp63 is fully conserved within the small multidrug resistance (SMR) family (*5*) and essential for the protein's activity. Even the conservative replacement of Trp63 with an aromatic residue resulted in a nonfunctional protein (*33*, *34*).

In this study, we aimed at characterizing the role of tryptophan residues in the function of EmrE. To achieve that, we constructed a set of mutants using site-directed mutagenesis. Each of the tryptophan residues was replaced with cysteine, except for Trp63 that was also replaced in a conservative manner with tyrosine and phenylalanine.

The ability of the mutated proteins to confer resistance against toxicants was assessed by testing the ability of cells expressing them to grow under otherwise nonpermissive conditions. Cells were grown in liquid media containing different concentrations of methyl viologen, ethidium bromide, or acriflavin. Cells carrying the vector plasmid without any insert cannot grow in these media at those concentrations of toxicant while cells expressing either EmrE or CLA-His (the Cys-less template used throughout this paper) were able to grow at all concentrations of toxicants. This assay provides a qualitative estimate of the activity of the mutants generated.

Three of the single tryptophan substitutions, W31C, W45C, and W76C, presented a similar phenotype and conferred resistance when grown in the presence of methyl viologen and ethidium bromide (Table 1). In the presence of acriflavin, cell growth was partially inhibited. Replacements of Trp63 by Cys, Tyr, or Phe totally abolished the protein's ability to confer resistance to either of the toxicants mentioned above.

To further test the role of the only essential residue, double tryptophan substitutions and triple tryptophan substitutions were constructed. Since only Trp63 is essential, the other three positions were changed in a sequential manner. Phenotype screen revealed that cells expressing W31C-W45C or W31C-W76C were resistant to methyl viologen, but their growth was partially inhibited by ethidium bromide and completely inhibited by acriflavin (Table 1). W45C-W76C expressing cells lost the capability to grow in the presence of any of the three toxicants and so did cells expressing W31C-W45C-W76C, a mutant protein whose sequence consists of only one tryptophan residue. We conclude that even though each one of the residues can be mutated individually, replacement of more than one Trp residue has a deleterious effect on the phenotype. Interestingly, however, when a tyrosine residue was introduced at position 45, to yield the mutant protein W31C-W45Y-W76C, resistance to methyl viologen was regained, as well as the ability to partially grow in the presence of ethidium bromide. Moreover, introducing a second tyrosine residue, at position 76, restored completely the protein's ability to confer resistance to ethidium bromide. W31C-W45Y-W76Y expressing cells were able to grow in the presence of methyl viologen and ethidium bromide. Cell growth in the presence of acriflavin was partially inhibited. The findings suggest a possible structural role of the tryptophan residues in the membrane domains that can be fulfilled by tyrosine as well.

TPP^+ Binding Activity of Tryptophan Substitution Mutants. To assess expression levels and to study function in more detail, all mutant proteins were purified by Ni-NTA affinity chromatography and were then assayed for their ability to bind TPP^+, a high-affinity substrate of EmrE (*4*). All of the mutants tested, including those who failed to confer any resistance, expressed to similar levels (not shown). As expected, all of the single substitutions that supported growth in the presence of the toxic compounds bound [^{3}H]TPP^+ to considerable levels compared to that of the EmrE control (Figure 1B). The mutants W63C and W63Y, in which tryptophan in position 63 was replaced by either cysteine or tyrosine, respectively, displayed no measurable binding activity. However, a replacement by phenylalanine in this position yielded a protein with a low, yet detectable, level of TPP^+ binding (Figure 1B). Further analysis revealed that the affinity of W63F to TPP^+ is about 2 orders of magnitude lower than that of the wild-type protein: $K_d = 383 \pm 29$ nM as compared with K_d of 1.9 nM for the wild-type protein (*18*).

Mutant proteins W31C-W45C, W31C-W76C, W31C-W45Y-W76C, and W31C-W45Y-W76Y that conferred at least some degree of resistance in the in vivo phenotype assay described above bound [^{3}H]TPP^+ to levels similar to those displayed by the wild type (Figure 1C,D). Interestingly, mutants W45C-W76C and W31C-W45C-W76C bound TPP^+ although they did not confer any resistance in the above phenotype screen. The degree of binding was lower than that of EmrE. The affinity of W31C-W45C-W76C to TPP^+ was assessed and found to be 25.5 ± 1.6 nM, 10 times lower than that displayed by the wild-type protein.

The results of TPP^+ binding activity of the triple tryptophan substitution mutants repeat a tendency already observed in the phenotype screen results. Namely, the mutant W31C-W45C-W76C exhibits little binding activity, but introducing tyrosine residues in positions 45 and then also 76 results in an increase of TPP^+ binding ability. The mutant W31C-W45Y-W76Y displays a level of TPP^+ binding similar to that of the wild-type protein with an affinity of 3.5 ± 0.7 nM. Since this mutant is fully functional while the W31C-W45C-W76C is not, we will use the former for

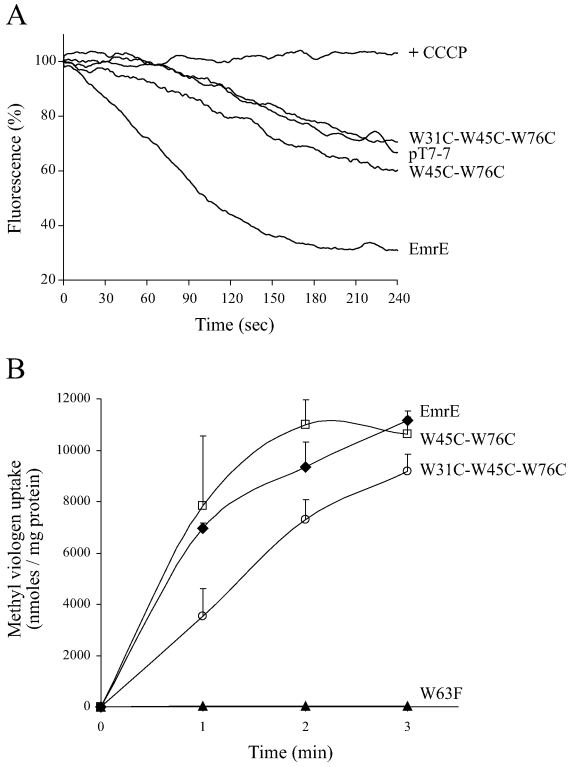

FIGURE 2: Transport activity of mutants that did not confer resistance to toxic compounds yet bound [^{3}H]TPP$^+$ to a detectable level. (A) Energy-dependent transport of ethidium assayed in whole cells. *E. coli* DH5α cells with the corresponding plasmids were grown in LB medium to $A_{600} = 0.4$ and then resuspended and incubated for 60 min at 37 °C in minimal medium containing CCCP (40 μM) and ethidium (1 μg/mL) as described under Experimental Procedures. Cells were then centrifuged and quickly resuspended in medium containing the same ethidium concentration with (+CCCP) or without CCCP. (B) $\Delta\mu_{H^+}$-driven methyl viologen uptake was assayed in proteoliposomes reconstituted with W63F (▲), W45C-W76C (□), W31C-W45C-W76C (○), and EmrE (◆) as described in Experimental Procedures.

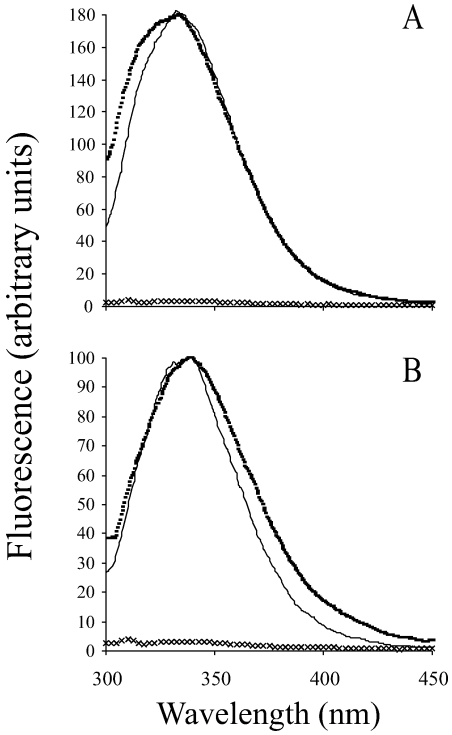

FIGURE 3: Emission spectra of EmrE (continuous line) and single Trp63 (fragmented line) recorded after excitation at 280 nm (A) or 295 nm (B). Protein concentrations were 5 μM EmrE and 20 μM single Trp63 per assay. The spectrum without any protein (×) is shown for both wavelengths. Fluorescence measurements were performed as described under Experimental Procedures.

other studies described below and we will coin it "single Trp63".

Transport Activity. The mutants that displayed normal phenotype expression and TPP$^+$ binding were not further characterized. The three mutants that failed to confer resistance, but still managed to bind TPP$^+$, were further investigated. Even though the phenotype is, in general, a good reflection of the activity of the protein in vivo, we directly assayed this function by assessment of the ability of the various mutants to remove ethidium from cells against its concentration gradient. In these experiments cells are passively loaded with ethidium in the presence of CCCP, a protonophore that collapses the proton electrochemical gradient. Ethidium interacts with nucleic acids in the cell, and this is monitored by the increase in its fluorescence. Cells are then collected by centrifugation and rapidly resuspended in a medium containing the same concentration of ethidium but devoid of uncoupler. Upon removal of the uncoupler in the cells expressing EmrE, a rapid decrease in the fluorescence is observed and is completed in slightly more than 2 min (Figure 2A, EmrE). This decrease in the fluorescence represents removal of ethidium from the cell against a concentration gradient and was completely prevented when the cells were resuspended in a medium with CCCP (Figure 2A, +CCCP). The rate of extrusion in the cells transformed with vector alone (pT7-7) or with mutants W45C-W76C and W31C-W45C-W76C is much slower, most likely due to the activity of other multidrug transporters, and reaches a new equilibrium only after about 20 min (not shown).

Strikingly, the results were very different when the mutants W45C-W76C and W31C-W45C-W76C, as well as EmrE, were solubilized, purified, and reconstituted into proteoliposomes, and their transport activity was measured. The ΔpH-driven [^{14}C]methyl viologen uptake into the proteoliposomes is presented in Figure 2B. The mutants that were inactive in the whole cell assay display now substantial activity: W45C-W76C accumulates methyl viologen against its concentration gradient at rates similar to those of wild-type EmrE. In proteoliposomes reconstituted with W31C-W45C-W76C methyl viologen accumulates at a slower initial rate but eventually reaches the same steady state. As expected, the mutant W63F that bound [^{3}H]TPP$^+$ with very poor affinity did not catalyze methyl viologen uptake.

Fluorescence Measurements. The indole group of tryptophan can serve as an intrinsic fluorophore in proteins. Since the emission spectrum is highly sensitive to solvent polarity, it can also reveal information about its environment, conformational changes in the protein, and/or direct interaction with substrates. The tryptophan emission spectrum of detergent-solubilized EmrE has a maximum at ~335 nm (Figure 3A), much lower than that of the water-soluble *N*-acetyl-L-tryptophanamide (NATA) at ~358 nm (data not shown), suggesting that they are exposed to an hydrophobic environment. The environment of Trp63, the only essential Trp in EmrE, was probed using single Trp63, the mutant that bears a single tryptophan residue at position 63 and yet is fully active. Its emission maximum is practically identical

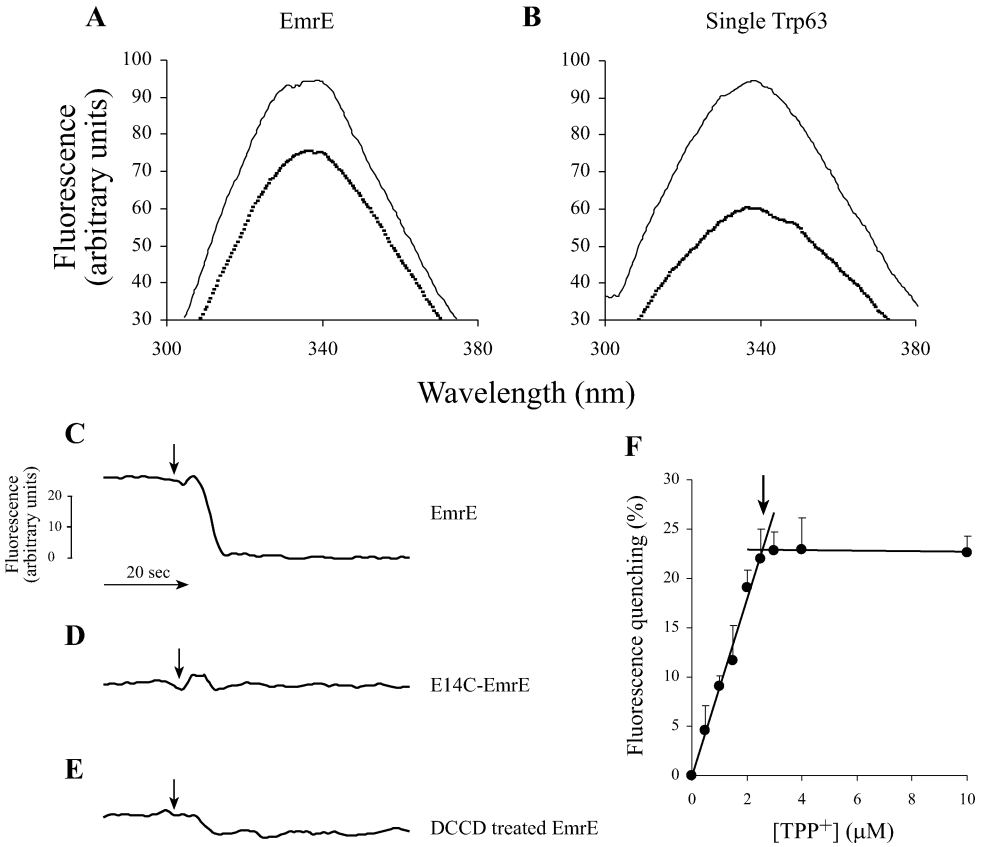

FIGURE 4: Emission spectra of EmrE (A) and single Trp63 (B) before (continuous line) and after addition of TPP^+ (fragmented line). TPP^+ was added to EmrE and single Trp63 at saturating concentrations (3 and 12 μM, respectively). (C) Fluorescence of EmrE was recorded as a function of time. TPP^+ was added at the down arrow. (D) and (E) present the results of the same experiment except that the protein used was E14C-EmrE and DCCD-pretreated EmrE, respectively. (F) Fluorescence quenching of EmrE is plotted against the concentration of TPP^+. The excitation wavelength used in experiments presented in (A), (B), and (F) was 295 nm and in (C), (D), and (E) 280 nm. The emission wavelength was 335 nm. Fluorescence was recorded as described under Experimental Procedures.

to that of the wild-type protein. An interesting difference between the two emission spectra is observed: in that of the single Trp63 a shoulder is clearly visible. To further understand the nature of this shoulder, the spectrum was recorded with excitation at 295 nm (Figure 3B) where only Trp, but not Tyr, absorbs. When excited at this wavelength, the shoulder disappears and can thus be tentatively assigned to tyrosine fluorescence. Even though tyrosine fluorescence is usually at shorter wavelengths (303 nm), tyrosinate and complexes of tyrosine with compounds such as phosphate fluoresce at longer wavelength (*35*). At this excitation wavelength a very small red shift of Trp63 is apparent (Figure 3B).

To assess whether changes in fluorescence are detected upon substrate binding, we recorded the emission spectra in the presence of TPP^+, one of the substrates of EmrE. Addition of saturating concentrations of a substrate such as TPP^+ induces a significant quenching ($\sim$21.7 $\pm$ 1.4%) of the Trp fluorescence of EmrE with no detectable shift in the emission maximum (Figure 4A). To test whether this quenching is related to the function of the protein, the quenching was tested on E14C, an inactive EmrE mutant (*4*). Addition of TPP^+ to detergent-solubilized E14C caused no change in the fluorescence intensity (Figure 4D) or on the position of the emission maximum (not shown). In addition, also wild-type protein that was pretreated with DCCD, a carbodiimide that inhibits substrate binding and transport (*15*), showed no change in fluorescence upon substrate addition (Figure 4E). The concentration dependence of the fluorescence quenching is shown in Figure 4F. As expected, the quenching increases with substrate concentration and saturates. The intersection between the two linear parts of the graph (arrowed) identifies the concentration of $[^3H]TPP^+$ (2.57 μM) that saturates binding to all the EmrE molecules in the assay (5 μM) giving a molar ratio of TPP^+:EmrE of 1:2, in line with the idea that the dimer is the functional unit (*4*, *10*). Also, other substrates such as ethidium and acriflavin induce quenching of a similar nature (not shown). However, TPP^+ is the substrate used for these experiments since it does not absorb in the wavelength range used here and, therefore, does not require corrections of any kind. In control experiments with the water-soluble *N*-acetyl-L-tryptophanamide (NATA), addition of TPP^+ had no effect on the fluorescence intensity or on the position of the peak.

To identify which Trp residue(s) is (are) responsible for the phenomenon described, we tested substrate-induced quenching in the single Trp63 mutant. Also in this mutant, addition of saturating concentrations of TPP^+ induced quenching, but the magnitude was 37.8 $\pm$ 2.7% of the fluorescence, a degree of quenching twice as high as that detected in the wild type (Figure 4A,B). Also in this case, there is no detectable change in the emission maximum upon addition of substrate.

These results demonstrate that Trp63, the only essential Trp residue in EmrE, is also a sensor for ligand binding and/or conformational changes following the binding reaction.

DISCUSSION

Tryptophan residues may play several roles in integral membrane proteins. They maintain interactions between a protein and its substrate using hydrophobic stacking interactions (*36*). They may also participate in cation/π interactions between proteins and their ligands (*37*). In addition, tryptophan residues of membrane proteins are nonrandomly distributed and tend to localize at the ends of transmembrane α-helices (*38*). This preference of tryptophan residues for the interface region reflects the amphipathic nature of a tryptophan residue that has the ability to form hydrogen bonds, as well as to exhibit hydrophobic character (*38*).

EmrE is a well-characterized multidrug transporter from *E. coli*. The amino acid sequence of EmrE contains four tryptophan residues, three of which are located in the transmembrane region, based on hydropathy predictions of the secondary structure.

Each of the four tryptophan residues was replaced by site-directed mutagenesis. The only single substitutions that affected the protein's activity were those in position 63. Replacement of Trp63 to cysteine yielded an inactive protein that conferred no resistance against known substrates of EmrE in the in vivo activity test. This mutant also did not bind TPP^+. A more conservative replacement of Trp63 to tyrosine yielded again an inactive mutant. Only the replacement of Trp63 to phenylalanine brought about a protein that, although it could not confer any resistance against the toxicants tested, could bind TPP^+ to a low, yet detectable degree. The affinity of this mutant was assayed and found to be 2 orders of magnitude lower than that of the wild-type protein ($K_d = 383 \pm 29$ nM as compared with K_d of 1.9 nM). These findings strongly suggest involvement of Trp63 in the function of the protein, most likely in substrate binding.

Double and triple tryptophan substitutions reveal possible roles of the other tryptophans in EmrE, mainly Trp45 and Trp76. Of the three double tryptophan substitutions, the mutant that exhibited the lowest activity was W45C-W76C. Cells expressing this mutant displayed no resistance against tested toxicants and no transport of ethidium in intact cells. TPP^+ binding activity of the detergent-solubilized protein was only 20−25% of that of wild-type protein. However, when W45C-W76C was reconstituted into proteoliposomes and assayed for proton gradient-dependent [^{14}C]methyl viologen uptake, it displayed levels of uptake similar to those of the wild-type protein. This may imply that tryptophan residues at positions 45 and 76 have a role either in folding or in insertion in vivo to the membrane. The detergent-solubilized protein binds ligand with a lower affinity, and when reconstituted artificially into liposomes, it regains activity. The findings suggest that the process of insertion in vitro, which differs from that in vivo, fulfills a chaperone-like function correcting the protein folding.

The results with the triple tryptophan substitutions support the above hypothesis. W31C-W45C-W76C could not confer resistance against any of the toxicants tested and did not transport ethidium in intact cells. But, in a manner similar to that of the double mutant W45C-W76C, it did bind TPP^+ with an affinity 10 times lower ($K_d = 25.5 \pm 1.6$ nM) than that of the wild-type protein. When reconstituted into proteoliposomes, the mutant W31C-W45C-W76C displayed transport activity that reached a steady-state level similar to that of the wild type, but with a slower initial rate. The role of the tryptophan residues at positions 45 and 76, in contributing to the correct insertion or fold of the protein in the membrane, can be fulfilled also by tyrosine. When the tryptophan at position 45 was replaced with tyrosine instead of cysteine (W31C-W45Y-W76C), resistant phenotype was partially restored and TPP^+ binding activity improved. Replacement of an additional tryptophan (W76) with tyrosine as well (W31C-W45Y-W76Y) brought about full restoration of activity. Cells expressing the mutant W31C-W45Y-W76Y displayed resistance against methyl viologen and ethidium bromide and were partially resistant to acriflavin. TPP^+ binding activity was as high as the wild type's, with similar affinity ($K_d = 3.5 \pm 0.7$ nM). A similar phenomenon was reported when constructing Trp-less LacY and MelB sugar transporters where the tryptophans were replaced with Phe in order to maintain full activity (*39*, *40*). Also in the case of LacY, single but not multiple Cys replacements of Trp were well tolerated (*39*). In the case of EmrE we show here that the multiple replacements that are not functional in intact cells can be "rescued" by solubilization and reconstitution. This phenomenon will be further investigated because it may provide a tool for the study of the different mechanisms of insertion in vivo and in vitro.

In contrast, any of the substitutions made at position 63, to cysteine, tyrosine, or phenylalanine, yielded an inactive protein or, in the case of W63F, a protein with a much lower affinity to substrates. To test more directly whether this irreplaceable tryptophan at position 63 is involved in the binding of substrates, we took advantage of the fact that the indole group of tryptophan serves as an intrinsic fluorophore in proteins, and changes in fluorescence as a result of substrate addition can be used to study interactions between transporter and its substrates and/or changes of conformation associated with this interaction.

The emission spectrum of single Trp63, which contains a single tryptophan residue, was compared to that of the wild type and found to be practically identical and typical of membrane-embedded Trp residues (see, for example, refs *41* and *42*). Upon addition of a substrate to the wild type there is a very substantial ($21.7 \pm 1.4\%$) fluorescence quenching that is only observed in the functional protein since neither E14C, an inactive mutant, nor DCCD-treated protein display substrate-induced quenching. The reaction is dependent on the concentration of the substrate and saturates at a concentration of 2.57 μM with the protein concentration of 5 μM. This finding strongly supports the contention that the functional unit is a dimer (*7*, *10*, *17*). Based on crystals of EmrE that diffract to 3.8 Å, an atomic model was recently proposed by Ma and Chang (*43*). In this model, the structure of EmrE is a tetramer composed of two conformational heterodimers related by a pseudo-2-fold symmetry axis perpendicular to the membrane surface. A transport mechanism is suggested in which the functional unit is a tetramer formed by two heterodimers. Within each heterodimer, one Glu14 takes part in drug binding while the other is responsible for proton translocation resulting in coupling of the drug and proton fluxes. Although it would be possible to invoke binding of two substrate molecules per tetramer, the model suggested by Ma and Chang (*43*) indicates that there is one substrate-binding site per tetramer

and is not in agreement with the findings presented in this work.

In the single Trp63 an even larger quenching (37.8 ± 2.7%) is observed upon addition of substrate. This can be explained by the fact that the emission spectrum observed with the wild type is actually the combined spectra of four tryptophan residues, while the mutant has only a single tryptophan residue. We cannot rule out at present that other tryptophans contribute also to the substrate-induced quenching. In any case, the single Trp63 provides a powerful tool to measure the interaction between substrate and EmrE. It also provides a tool for FRET measurements in a protein with only one tryptophan, measurements that will allow for a critical test of existing structural models. The results presented here strongly suggest the existence of an interaction between Trp63 and TPP^+, and the nature of this interaction, whether direct or mediated by conformational changes, can now be studied in more detail with the tools developed in this work.

ACKNOWLEDGMENT

We thank Misha Soskine for support and help in the purification of the various mutants.

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BI050356T