Exploring the Binding Domain of EmrE, the Smallest Multidrug Transporter*

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EmrE is a small multidrug transporter in Escherichia coli that extrudes various positively charged drugs across the plasma membrane in exchange for protons, thereby rendering cells resistant to these compounds. Biochemical experiments indicate that the basic functional unit of EmrE is a dimer where the common binding site for protons and substrate is formed by the interaction of an essential charged residue (Glu14) from both EmrE monomers. Previous studies implied that other residues in the vicinity of Glu14 are part of the binding domain. Alkylation of Cys replacements in the same transmembrane domain inhibits the activity of the protein and this inhibition is fully prevented by substrates of EmrE. To monitor directly the reaction we tested also the extent of modification using fluorescein-5-maleimide. Furthermore, preincubation with tetraphenylphosphonium reduces the reaction of Y4C, I5C, L7C, and A10C, were modified at least 80%. Furthermore, a comparative analysis reveals that the face of transmembrane domain 1 (TM1) containing Glu14 is conserved, displaying a helical periodicity (Fig. 1A and Ref. 5). Previously, using site-directed mutagenesis of this TM1 face we identified a cluster of five amino acids that play a role in substrate and H+ recognition and/or translocation with substitutions at most positions yielding either inactive mutants or mutants with modified affinity to substrates (12, 13).

We now use Cys replacements in TM1 to study accessibility of the residues in TM1 to alkylating reagents and the effect of these agents on function. This method was first established to identify residues exposed to the aqueous translocation pathway of ligand-activated channels and the cystic fibrosis transmembrane conductance regulator chloride channel (14–16). The reactive residues are thought to delineate water-filled cavities that may represent the substrate translocation pathway. In several studies with ion-coupled transporters reactive residues were shown to be located in the vicinity of the binding site of the hydrophilic substrates (17–19). Here we describe a combined approach where sensitivity of activity to alkylation is correlated with a direct quantitation of the exposure of the residues to alkylation. While most residues are not accessible or only partially accessible, some are quantitatively modified. Moreover, TPP- and other substrates reduce modification at selected positions by more than 80% indicating that residues from each monomer in the functional unit are close enough to the binding site that substrate prevents their modification or they might be equivalently protected in an allosteric manner. The significance of these observations to a recent mechanistic model (20) is discussed.

A novel approach to study the effect on inactive mutants is described. The observations presented here allow for a tentative identification of residues in the substrate binding domain of EmrE.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli TA15 (21) was transformed with plasmid pPGI-2, which codes for the T7 polymerase under the inducible control of the ApL promoter (22). The plasmids used for EmrE expression are pT7-7 derivatives (22) with or without histidine tag (EmrE-His) (4). The construction and characterization of most of the single Cys mutants were described previously (12, 23–25). Mutants Y4C, I5C, G8C, G9C, T19C, and L20C were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Except for Y4C, phenotype, binding, and transport properties of these mutants were essentially identical to those of wild type and will be described in detail elsewhere. The Y4C mutant binds TPP- to levels practically identical to those of the wild type but does not transport and does not confer resistance against toxic cations.3

Expression and Isolation of Membranes—E. coli TA15 cells transformed with plasmid pPGI-2 and the appropriate pT7-7 derivatives

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2The abbreviations used are: SMR, small multidrug resistance; TM1, transmembrane domain 1; EmrE, Escherichia coli multidrug resistance protein; CLA, untagged EmrE; in which the three native Cys residues have been replaced with Ala; TPP+, tetraphenylphosphonium; DDM, dodecyl-β-o-maltopyranoside; NEM, N-ethylmaleimide; NEM-fluorescein, fluorescein-5-maleimide; Ni-NTA, nickel-nitrilotriacetic acid; MTSEA, 2-aminoethylmethanethiosulfonate hydrobromide; MTSES, 2-sulfonatoethylmethanethiosulfonate; MTSET, 2-(trimethylammonium)ethylmethanethiosulfonate bromide. Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue, followed by a letter denoting the replacement at that position.

3D. Rotem, S. Steiner-Mordoch, and S. Schuldiner, unpublished data.
were grown at 30 °C in minimal medium A supplemented with glycerol (0.5%), thiamine (2.5 μg/ml), ampicillin (100 μg/ml), and kanamycin (50 μg/ml). When the culture reached an A₆₀₀ = 0.9, it was transferred to 42 °C for 15 min to induce the T7 polymerase. Then the culture was shifted back to 30 °C, and 2 h later the cells were harvested by centrifugation. Cells were resuspended in buffer containing 50 mM sucrose, 0.5 mM dithiothreitol, 150 mM NaCl, 15 mM Tris-Cl, pH 7.5, 2.5 mM MgSO₄, 15 μg/ml deoxyribonuclease (Sigma) and broken by French press. The membrane fraction was collected by ultracentrifugation at 240,000 × g for 20 min 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.

**TTP⁺ Binding Assay**—Binding of TTP⁺ was assayed essentially as described (4). Ni-NTA beads (20 μl/assay) (Qiagen GmbH, Hilden, Germany) were washed twice in distilled water and once in buffer containing 0.08% dodecyl maltoside (DDM, Glycon Biochemicals GmbH, Luckenwalde, Germany), 150 mM NaCl, 15 mM Tris-Cl, pH 7.5 (DDM/Na-buffer). Membranes (0.2–1.0 mg protein/ml) were solubilized in 0.8% DDM/Na-buffer (15 min at room temperature) and incubated with the washed beads at 4 °C for 1 h. The unbound material was discarded, and EmrE-His bound to beads was washed once with 0.08% DDM/Na-buffer and resuspended in 200 μl of the same buffer containing 5 mM [³H]TTP⁺ (27 Ci/mmol, Amersham Biosciences). The samples were incubated for 30 min at 4 °C with shaking. In each experiment, the values obtained in a control reaction, with 25 μM unlabeled TTP⁺, were subtracted. The reaction was stopped by pulse centrifugation. The bead fraction was then incubated for 10 min at room temperature with 450 μl of 0.08% DDM/Na-buffer containing 150 mM imidazole to release the EmrE-His and [³H]TTP⁺ bound to it from the beads. After spinning down the beads, the [³H]TTP⁺-associated radioactivity was measured by liquid scintillation. All binding reactions were performed in duplicates. Each experiment was performed at least twice.

L7C was previously reported to be inactive in [³H]TTP⁺ binding (12), but further experiments with higher amounts of protein demonstrated that it has a decreased affinity, 15-fold lower than that of wild type EmrE.

When inhibition of binding was studied, experiments were performed with the following modifications: N-ethylmaleimide (NEM; Sigma) and thiosulfonate derivatives (Anatrace Inc., Maumee, OH) such as 2-aminoethylmethanethiosulfonate hydrobromide (MTSEA) or ethylmaleimide (NEM; Sigma) and thiosulfonate derivatives (Anatrace Inc., Maumee, OH) were added at the indicated concentrations for 10 min at 25 °C before labeling with [³H]TTP⁺. In some experiments, substrates were added at the indicated concentrations for 10 min at 25 °C before labeling with NEM-fluorescein. The reaction was stopped by dilution with the same buffer containing β-mercaptoethanol at a final concentration of 5 mM. The samples were then added to beads washed as described above and incubated in the dark at 25 °C for 1 h. Unbound material was discarded and EmrE-His bound to beads was washed twice with 0.5% SDS, 6 M urea, and 5 mM β-mercaptoethanol.

The protein was eluted from the beads using a buffer containing 200 mM β-mercaptoethanol, 100 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.2% bromphenol blue, and 450 mM imidazole and analyzed by SDS-PAGE. Fluorescence labeling analysis of the gel was done using a Fujifilm LAS-1000 imaging system and digitally analyzed with Image Gauge 3.46 Fujifilm software. The results were calibrated to protein amount after staining of the same gels with Coomassie, scanning, and quantitation using the same software. Each experiment was performed at least three times.

**Formation of Mixed Oligomers**—Membranes (60–100 μg) from cells expressing His-tagged Cys-less-Ala (CLA) EmrE E14C, G17C, Y40C, or W63C and membranes (320 μg) from cells expressing untagged CLA EmrE were mixed and solubilized at 80 °C for 15 min with at least 10 volumes of 0.8% DDM/Na-buffer as described (8). The extract was centrifuged for 1 min at 14,000 rpm to discard precipitates. Methane thiosulfonates (1 mM MTSEA and MTSET and 2 mM MTSES) were added 10 min before addition of washed Ni-NTA beads (20 μl of beads/assay) and incubated at 4 °C for 1 h. The unbound material was discarded, and EmrE-His bound to beads was washed three times with 0.08% DDM/Na-buffer. Binding activity was tested as described above. To test the extent of reaction with the methane thiosulfonates the beads were incubated with SDS-urea and reacted with NEM-fluorescein, and labeling was evaluated as described above.

## RESULTS

Previously, using site-directed mutagenesis we identified a cluster of five amino acids in TM1 that may play a role in substrate and H⁺ recognition and/or translocation (12). This was based on the fact that substitutions at the five positions yielded proteins that could not confer resistance to any of the substrates of EmrE. When tested further one mutant was found incapable of substrate binding (Gly⁷), three (Leu⁷, Thr¹⁸, and Ile¹⁸) bound it with a decreased affinity, and one (Ala¹⁸) displayed a modified affinity to H⁺ and, as a consequence, was impaired in the coupling of the proton gradient to substrate fluxes (12). Here we use site-directed chemical modification of the cysteine substitutions to further probe the role of the above residues in binding substrate.

**Alkylation of Selected Residues in TM1 Inhibits Activity and Substrate Prevents the Effect**—First we tested the effect of NEM, a small alkylating agent, on the ability of Cys substitutions in TM1 to bind TTP⁺, a substrate of EmrE that binds with high affinity. In these experiments, detergent-solubilized EmrE mutants were treated with NEM, immobilized on Ni-NTA beads, and assayed for their ability to bind TTP⁺ relative to the untreated protein. As shown in Fig. 1B, after treatment with 1 mM NEM, TTP⁺ binding of three of the Cys mutants is inhibited. Binding by L7C and A10C is inhibited more than 90%, while binding by I11C decreases to about 50% of the value in the absence of NEM. To further characterize the inhibition at these positions we tested the dependence on NEM concentration (Fig. 1C). The binding activity of both L7C and A10C is very sensitive to the modification, and 50% inhibition is observed at about 3 and 10 μM, respectively. The activity of I11C decreases at low concentrations of NEM but rapidly levels off, and maximal inhibition is 50% even at 1 mM NEM. Interestingly, these three residues are three of the five residues identified in our studies using site-directed mutagenesis. The other two residues (Gly⁷ and T18C) display a modified affinity to H⁺ and, as a consequence, were impaired in the coupling of the proton gradient to substrate fluxes (12). Here we use site-directed chemical modification of the cysteine substitutions to further probe the role of the above residues in binding substrate.
Accessibility of Cys Substitutions in TM1 to NEM-fluorescein—The results presented above supply us with an important insight on the residues that may play a role in substrate binding. To find out whether the residues in both monomers are reacting and whether other residues are reacting without effect on the activity, we tested the accessibility of the Cys substitutions to NEM-fluorescein, an alkylating reagent that can be easily detected after reaction with the protein. In these experiments, detergent-solubilized EmrE mutants were allowed to react with NEM-fluorescein. After purification using metal-chelate chromatography, the protein was separated by SDS-PAGE, and the amount of fluorescence associated with it was quantitated. A representative result is shown in Fig. 2 where the fluorescence of NEM-fluorescein-labeled E14C (lanes 1–3) and A10C (lanes 4–6) protein is shown. The E14C protein solu-

FIGURE 1. The effect of NEM on TPP$^+$ binding. A, sequence alignment of residues in TM1 of 242 members of the SMR family represented as sequence logos (38). The scale gives the certainty of finding a particular amino acid at a given position and is determined by multiplying the frequency of that amino acid by the total information at that position. The residues at each position are arranged in order of predominance from top to bottom, with the highest frequency residue on top. Red color is used for negatively charged amino acids, light blue for aromatic, gray for hydrophobic, and yellow for small or hydrophilic ones. Sequence logos were generated using the “Web Logo” facility of Steven E. Brenner at www.bio.cam.ac.uk/seqlogo. B, effect on activity of Cys replacements in TM1 mutants. [3H]TPP$^+$ binding was assayed as described under “Experimental Procedures,” with and without treatment with 1 mM NEM. The results are shown as percent of TPP$^+$ binding of each mutant without NEM. The different mutants were previously shown to vary in their ability to bind [3H]TPP$^+$ (12, 14, 15, 17, 19, 21, 23, 25, 27, 29, and 31 pmol/μg, respectively). L7C, I11C, G17C, and T18C bind [3H]TPP$^+$ poorly (2, 2, 1, and 2 pmol/μg, respectively). E14C displays no binding activity at all (NA). C, effect of different concentrations of NEM on activity of L7C (●), A10C (○), and I11C (■). NEM was added at the indicated concentrations at 25 °C for 5 min before performing the TPP$^+$ binding assay. The results are shown as percent of TPP$^+$ binding of each mutant without NEM. D, effect of different concentrations of TPP$^+$ on NEM inhibition. The indicated concentrations of unlabeled TPP$^+$ were added at 25 °C to L7C (●), A10C (○), and I11C (■) 10 min prior to the addition of 1 mM NEM. The [3H]TPP$^+$ binding assay was essentially as described under “Experimental Procedures” except that the immobilized protein on the beads was washed three times to remove unlabeled TPP$^+$. The results are shown as a percent of the activity of each mutant with no treatment (no TPP$^+$ and no NEM).
Figure 2. Accessibility to NEM-fluorescein of single cysteine replacements in TM1. A, representative gel showing accessibility to E14C and A10C. Membranes of both mutants were solubilized either in Na-buffer supplemented with 0.5% SDS, 6 M urea solution (lanes 7 and 4) or in 0.8% DDM/Na-buffer. 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 3 and 6) TPP+ (25 μM) was added 10 min prior to the NEM-fluorescein. The reactions were stopped after 30 s by the addition of protein in the gel. The level of labeling of all the mutants solubilized in 0.5% SDS, 6 M urea was practically identical (data not shown). The dashed line represents 20% accessibility threshold.

The harsh denaturation conditions were essential for a full exposure of the Cys residues at all positions. Without heating or urea some mutant proteins were only partially accessible/reactive supporting again the notion that EmrE is a very well packed and stable protein (data not shown). A protocol similar to that described in the legend to Fig. 2A was carried out with all the mutants in TM1, and the results are shown in Figs. 2B and 3. In Fig. 2B, the fluorescence intensity of the label incorporated to the DDM-solubilized protein is shown as a percent of the intensity incorporated to the SDS-urea-treated protein. Cys residues at positions 4, 5, 7, and 10 are fully accessible to the reagent. At positions 6, 11, 12, and 17 about 40–60% of each residue react, while at the other positions tested (8, 9, 13–16, and 18–21) less than 20% do. The results suggest a tight packing of the protein in certain domains. Most of the fully accessible ones are in the same face of the helix as Glu14. The effect of substrate on the accessibility to NEM-fluorescein was tested and the results are shown in Fig. 3. In these experiments a high concentration of substrate is used so its effects are detectable even with those residues that display low affinity. Substrate has no detectable effects on labeling of the three residues at the N terminus (Y4C, I5C, and Y6C). Notably, substrate dramatically reduces the level of labeling of L7C and A10C, two of the most accessible ones. Also some of those that are only partially accessible display a significant degree of protection. Remarkably, T18C displays an increased exposure upon addition of TPP+ (Fig. 3A), ethidium, and acriflavine (data not shown). To further analyze the effect of substrates on the alkylation reaction some of the residues were further investigated. The results in Fig. 3B show the concentration dependence of the protective effects of substrate. Similarly to what we showed above for the effect on activity, concentrations below 1 μM almost fully (~85%) protect the alkylation of A10C (a mutant with a high affinity to TPP+), and higher concentrations are needed to fully protect about 80% of the labeling of L7C, a mutant with a lower affinity to TPP+. In the case of G17C, a mutant with a very low affinity (Kd >100 nM), the behavior is quite different; a very small protection of about 20% is detected at 1–2 μM TPP+, and it does not further increase even at 25 μM TPP+. Labeling of Y4C, I5C, and Y6C (only the latter is shown) is not affected by substrate suggesting that, although exposed to NEM, they are not in the binding domain. As mentioned previously, the accessibility of T18C is increased by substrate. The dependence on the substrate concentration shown in Fig. 3B may reflect previous findings that the affinity of T18C to TPP+ is lower than that of the wild type or A10C (12). To further probe the phenomenon of protection from labeling, the effect of other substrates was tested. Representative results are shown in Fig. 3C for the effect on labeling of A10C with two substrates: ethidium bromide and acriflavine. Both reduce more than 80% of the level of labeling at concentrations of 50–100 μM, well in line with the fact that the affinity of EmrE to these substrates is lower than that to TPP+

Generation of Functional Hetero-oligomers Allows Studying Otherwise Inactive Proteins—Glu14 is one of the essential residues in the binding domain. The Cys replacement at position 14 is inaccessible to alkylation (Fig. 2, A and B). The conclusions that can be reached with this mutant are limited by the fact that it is completely inactive, and we therefore do not know whether it is correctly folded. However, we have shown previously that mixing with a wild type protein can functionally complement the inactive mutant and generates a hetero-oligomer that binds TPP+ in a mode that suggests that the functional properties of the dimer are a result of the interaction between individual monomers (7, 8). In one of these methods, heating to 80 °C separates the dimers and mixing occurs upon cooling (8). One of the monomers is tagged and inactive and is used to pull down the active untagged monomer and form the functional hetero-oligomer. Here we take advantage of this method to chemically modify the Cys residue at position 14 after the dimer was formed. We generated hetero-oligomers between tagged E14C-CLA and untagged CLA (with a Glu residue at position 14) and assayed the binding activity of the hetero-oligomer after treatment with several thiol reagents (Fig. 4A). We used thiosulfonate derivatives that insert a positive charge (MTSET), a negative one (MTSES), or an aliphatic amine (MTSEA) that is only partially protonated at the pH of the reaction (8.5). As shown previously the hetero-oligomer binds TPP+ to considerable levels (Fig. 4A and Ref. 8). The absolute levels of binding
depend on the ratios of CLA and tagged protein and can reach up to 3 pmol/μg of tagged protein (wild type binds up to 15 pmol/μg). The lower levels of binding are due to an almost 10-fold decrease in the affinity of the hetero-oligomer to TPP⁺. MTSEA and MTSET inhibit the activity implying that a positive charge in one monomer at position 14 has a deleterious effect on the activity of the dimer even though in the other monomer at the equivalent position there is a carboxyl. Strikingly, MTSES stimulates binding 20–30% in a reproducible way suggesting that an additional negative charge at position 14 may improve the affinity of the hetero-oligomer.

The above-described protocol provides a strategy for studying the effect of chemical modification at positions that are assumed to be essential and therefore inactive when replaced with Cys. We took advantage of this approach to study three other residues suggested to be essential from mutagenesis studies (12). Mutants with Cys replacements at positions 17, 40, and 63 do not bind [³H]TPP⁺ and do not display transport activity. The hetero-oligomers between the tagged inactive mutants at positions 40 and 63 and the untagged CLA display robust levels of binding (Fig. 4, A and C), while the Gly¹⁷ hetero-oligomer does not show functional complementation (data not shown). In the functional heterodimers formed by Y₄₀C and W₆₃C with CLA, the three thiosulfonate derivatives inhibit binding (Fig. 4, B and C). The results suggest that modifications at positions 40 and 63 have a deleterious effect on activity and support the hypothesis that they play important roles in substrate binding.

**DISCUSSION**

Previously the importance of TM1 in the function of EmrE was highlighted because of the central role of Glu¹⁴ in catalysis (4, 27, 28). A phylogenetic analysis also pointed to the fact that a clear helical periodicity of conservation is observed in TM1 indicating the importance of one face of the helix (Fig. 1A and Ref. 5). Using site-directed mutagenesis of this TM1 face we identified a cluster of five amino acids that play a role in substrate recognition and/or transport activity. We assessed the effects of alkylation with NEM, a small maleimide, on binding activity of the detergent solubilized protein. In parallel, we analyzed the inhibition using the data from the accessibility of the residues to NEM-fluorescein, a maleimide that can be easily detected because of its fluorescence. Activity of mutants with Cys replacements at three positions, L₇C, A₁₀C, and I₁₁C, is very sensitive to alkylation with NEM. Activity of mutants L₇C and A₁₀C is practically completely inhibited. Strikingly, the inhibition is totally prevented by substrates of EmrE at concentrations expected from their relative affinities. A possible explanation for this mutual exclusion is supplied by our accessibility studies where we show that the three residues react with NEM-fluorescein, but binding of substrate limits the accessibility to the site of action. Accessibility to NEM-fluorescein is high near the N terminus and decreases toward the core of the protein with residues 11 and 12 reacting below 20% except for residue Gly¹⁷, a protein with practically no activity (Kₐ > 100 mM). The residues close to the N terminus, Y₄C and I₅C, are fully accessible to NEM-fluorescein, and the reaction is not prevented by substrate. In addition,
NEM does not inhibit the activity of these mutants. These findings are in good agreement with the model proposed by Koteiche et al. (29) using site-directed spin labeling. The analysis of the spin-spin interactions is consistent with a scissor-like packing of the two TM1s. This results in a V-shaped chamber that is in contact with the aqueous phase near the N terminus (29). Our definition of “accessible residues” is based on differential rates of labeling that may be due to different reactivity or accessibility. To highlight the differences between the mutants we chose the shortest time at which the accessible residues are practically fully labeled. Even after longer times (10–30 min), differences are still observed (data not shown). After overnight labeling, as shown by Koteiche et al. (29), all the residues show some measurable, but not necessarily quantitative, degree of accessibility. The fact that three residues in a row, 10, 11, and 12, react with NEM-fluorescein, even if to a different degree, suggests a possible break in the helical structure in this area. The partial effect of NEM on the activity of the I11C replacement may be due to the fact that this residue is only partially accessible to the alkylating reagent. We have measured labeling of I11C over a time course, and we found that 50% labeling is already detected after 30 s, and it does not significantly increase even after 30 min reaction (data not shown). Only after several hours exposure an increased labeling can be observed. It is possible that the asymmetry of the dimer detected in the structure determined by electron crystallography (9, 30) results in only one of the residues exposed to the aqueous chamber under the conditions tested. In the same way also a Cys residue at position 12 is only partially accessible. However, in the case of L12C, reaction of NEM does not result in inhibition. Since the alkylation with NEM-fluorescein is partially inhibited by substrate, it is possible that substrate and NEM-fluorescein share a common pathway, but it is unlikely that Leu12 is in a position important for binding. The fully exposed residue at positions 4 and 5 seems to be at the entrance of the hydrophilic cavity and does not interact directly with substrate as judged by the results presented.

We showed here for A10C (and data not shown for L7C) that three different substrates have the same effect on accessibility. This finding suggests the existence of a common binding site to the various substrates or at least that the determinants tested here are shared by different substrates. T18C is very unique in its reaction to the modifiers. It becomes more accessible in the presence of substrate and displays a slightly improved affinity toward the substrate after the position is alkylated. A possible reason for this behavior is that Thr18 becomes more accessible in the presence of substrate and displays a partial effect of NEM on the activity of the I11C replacement may be due to the fact that this residue is only partially accessible to the alkylating reagent. We have measured labeling of I11C over a time course, and we found that 50% labeling is already detected after 30 s, and it does not significantly increase even after 30 min reaction (data not shown). Only after several hours exposure an increased labeling can be observed. It is possible that the asymmetry of the dimer detected in the structure determined by electron crystallography (9, 30) results in only one of the residues exposed to the aqueous chamber under the conditions tested. In the same way also a Cys residue at position 12 is only partially accessible. However, in the case of L12C, reaction of NEM does not result in inhibition. Since the alkylation with NEM-fluorescein is partially inhibited by substrate, it is possible that substrate and NEM-fluorescein share a common pathway, but it is unlikely that Leu12 is in a position important for binding. The fully exposed residue at positions 4 and 5 seems to be at the entrance of the hydrophilic cavity and does not interact directly with substrate as judged by the results presented.

A limitation of the Cys accessibility studies is encountered when studying essential residues and, as a consequence, inactive proteins when replaced with Cys. In the case of an oligomeric protein such as EmrE we describe here an approach to circumvent this problem by generating hetero-oligomers. This results in functional complementation displayed by G17C may suggest a role in conformational changes rather than in substrate binding. Residues 19 and 20 displayed a very low accessibility, and even though a consistent increase in the accessibility of T19C was detected in the presence of substrate, it was very small and it did not increase beyond 10% of the SDS value (data not shown).

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FIGURE 4. Exploring essential residues with functional hetero-oligomers. Membranes from cells expressing CLA (untagged Cys-less EmrE) were solubilized with 0.8% DDM/Na-buffer and mixed at 80 °C with solubilized membranes of E14C (A), Y40C (B), or W63C (C), three inactive mutants tagged with Myc-His. The extract was centrifuged for 1 min at 14,000 rpm to discard precipitates. Methane thiosulfonates (1 mM of MTSEA and MTSET and 2 mM of MTSES) were added 10 min before addition of washed Ni-NTA beads (20 µl of beads/assay) and incubated at 4 °C for 1 h. The unbound material was discarded, and EmrE-His bound to beads was washed three times with 0.08% DDM/Na-buffer. Binding activity was tested as described above (5 nM [3H]TPP in a 200-µl reaction) and exceeded the following values: 0.65, 0.44, and 0.62 pmol/µg of E14C, Y40C, and W63C, respectively. To test the extent of reaction with the methanethiosulfonates the beads were incubated with SDS-urea and reacted with NEM-fluorescein, and labeling was evaluated as described above. The results are shown for each mutant in the inset as percent labeling in the absence of any thiosulfonate.
dose-dependent inhibition is observed that is due to a decrease in the affinity to TPP\(^+\) (8). Here we used thiosulfonate derivatives that insert a positive charge (MTSET), a negative one (MTSES), or an aliphatic amine (MTSEA) that is only partially protonated at the pH of the reaction (8.5). Introduction of a positive charge in the cavity completely inhibited activity, while a negative charge improved it slightly, but consistently, possibly by replacing the missing negative charge and increasing the affinity to substrates. We extended this approach to other residues as well, Trp\(^{43}\), a fully conserved and essential residue (23, 26, 33) and Tyr\(^{40}\), a residue conserved in a large subset of proteins in the SMR family (5). In both cases, thiosulfonates fully inhibited activity supporting the involvement of these residues in substrate recognition. However, we cannot rule out the possibility that the modification might inhibit by preventing proper interaction of the monomers or by interacting with a different residue in the active subunit. Gl17C is the only one of four inactive mutants tested that did not show a significant functional complementation. This may be due to incorrect folding of the heterodimer but may also suggest that the role of Gl17 is related to the flexibility of the protein rather than substrate binding.

Based on crystals of EmrE that diffract to 3.8 Å, an atomic model was recently proposed by Ma and Chang (20). 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. In each conformational heterodimer, each TM1 is in a different environment, one solvent is exposed while the other lies in a putatively hydrophobic environment. A transport mechanism is suggested in which the functional unit is a tetramer formed by two heterodimers. Within each heterodimer, one Glu\(^{14}\) takes part in drug binding, while the other is responsible for proton translocation resulting in coupling of the drug and proton fluxes. Critically, such a model indicates that there are two equally sized subpopulations of TM1 residues, each residing in a significantly different environment, most likely with different accessibility to substrate and alkylating reagents. Our studies here with alkylating reagents and previously with carbodiimides (34) do not support the existence of two equally sized subpopulations.

The three-dimensional structure of EmrE with bound TPP\(^+\) was determined at 7.5 Å resolution by electron cryomicroscopy of two-dimensional crystals (9). 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 from six out of the eight α-helices, confirming the suggestion that TPP\(^+\) binds near the center of the dimer (9).

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