The Assembly Motif of a Bacterial Small Multidrug Resistance Protein*

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Bacteria can evade the toxic effects of antimicrobials via several mechanisms including development of drug efflux proteins (1). The small multidrug resistance (SMR) family of bacterial integral membrane proteins represents one group of these drug efflux proteins, with 52% of currently sequenced eubacteria and archaea containing at least one SMR homologue (1). SMR proteins exhibit low substrate specificities; they are capable of conferring clinically significant resistance to large hydrophobic and cationic molecules, among them dyes, sanitizing agents, and antibiotics (2–7). SMRs act as antiporters, coupling the efflux of one drug molecule from the cytosol to the import of protons using the proton motive force (2, 8–10). The structural and mechanistic details of this process, however, may exhibit substrate-specific differences (11); this plasticity represents a significant challenge to the design of a single small molecule inhibitor that could circumvent SMR-mediated drug resistance.

Individual SMR molecules must assemble into homo-oligomeric structures to transport substrates, a requirement likely related to the relatively small size of the individual SMR molecule (∼110 residues divided into four transmembrane (TM) segments (3–6, 12)). The exact size of a fully assembled SMR structure remains an area of active investigation, with evidence for both dimeric (6, 13–19) and tetrameric (14, 17, 19–23) stoichiometry; however, SMR dimers are thought to represent the minimal functional unit (6, 13–19). Structural studies of dimers of the Escherichia coli SMR EmrE indicate that substrates bind a pocket formed by TMs 1–3 of two monomers (21, 24). This pocket is lined with aromatic and hydrophobic residues from TMs 1–3 that interact with substrates and a conserved Glu residue in TM1 that coordinates protons and positive charges on substrate molecules (21).

To date, a role for residues in TM4 in substrate binding and translocation has not been recognized (24), and these segments are paired and separated from the binding site (21). This pair of TM4 helices is in close contact along the entire helix length and remains in approximately identical conformations and positions in both the free and the substrate-bound dimer (11, 24). The TM4 helix pair has accordingly been proposed to represent a strong intermonomer association that does not contribute to conformational change during ligand binding but instead stabilizes the dimer interface (24). This potential dependence of SMR function on TM4 self-assembly suggests that inhibition of the self-interaction of this segment may provide a straightforward means of controlling drug efflux.

Although these EmrE structural studies have provided invaluable information regarding the overall geometry of SMR self-interaction, atomic resolution data capable of identifying the specific side chains and interactions that assemble SMRs are not yet available. Mutagenesis data from our laboratory and others (25, 26), however, are beginning to pinpoint the residues and interactions that could stabilize TM4-mediated inter-
monomer contacts in SMR family members. In a previous study of peptides that correspond to TM4 of the *Halobacterium salinarum* SMR protein, Hsmr, we noted that TM4 had a propensity to strongly self-interact in vitro (25). We also found that the oligomerization of Hsmr TM4 peptides was sensitive to residue replacements of Gly, Gly, and Ile that altered side chain volume. Disruption of dimerization by replacement of the TM4 residues Gly, and Gly, with Cys or Pro was also noted in the *E. coli* SMR, EmrE (26). The six-residue separation of these Gly residues suggested that they form a GG motif (27, 28) which mediates “knobs-into-holes” packing across the TM4–TM4 interface (25, 26). However, the “knob” residues involved in such an interaction have not yet been identified, although roles for Leu, and Ile, SMR self-assembly have been suggested (26).

With the goal of cataloguing the residues that may be required for TM4-mediated SMR oligomerization, in the present work, we have constructed a library of 12 mutants that scan the central portion of TM4 segment of Hsmr. This SMR homolog was selected because it is capable of self-assembly in the presence of SDS (23), allowing for rapid screening and quantitation of oligomerization on PAGE. We find that three key residues, Gly, Gly, and Val, define an assembly “hot spot” where replacements are highly disruptive to Hsmr-based drug resistance.

**EXPERIMENTAL PROCEDURES**

**Production of Wild-type and Mutant Hsmr Proteins**—The pT7-7 Hsmr-His, plasmid was kindly provided by S. Schuldiner. Hsmr were produced using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmids were transformed into *E. coli* BL21(DE3) cells and grown at 37°C to an OD value of 0.7. Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM while shaking for 1 h. Cells were then pelleted by centrifugation and lysed with 4 ml/g of cells of 2% SDS, 6 M urea, 10 mM Tris, 10 mM imidazole, pH 8 (lysis buffer). Cell debris was pelleted by centrifugation at 10,000 × g, and the supernatant was incubated with nickel–nitrilotriacetic acid beads for 1 h. The beads were washed three times with lysis buffer, and Hsmr was eluted with lysis buffer containing 400 mM imidazole. Eluted Hsmr was dialyzed against 0.3% SDS, 50 mM buffer, and Hsmr was eluted with lysis buffer containing 400 beads for 1 h. The beads were mutagenized in the present study.

**Ethidium Resistance Assays**—The ability of wild-type and mutant Hsmr proteins to confer resistance to the cytotoxic compound ethidium bromide (EtBr) was performed essentially as described (23). *E. coli* BL21(DE3) cells harboring the Hsmr plasmid were grown on LB plates in the presence of 100 µg/ml ampicillin. Individual colonies were picked and added to 2× YT broth (w/v: 1.6% Tryptone, 1% yeast extract, and 0.5% NaCl) containing 100 µg/ml ampicillin, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and 0, 200, or 500 µM EtBr. Cell growth was assayed by an OD value of 0.7 after 1 h of growth with shaking at 37°C.

**Gel Electrophoresis and Densitometry**—100 ng of purified Hsmr proteins were analyzed by SDS-PAGE on 4–12% NuPAGE gels (Invitrogen) in MES buffer according to the manufacturer’s directions. Unstained Mark-12 protein standards (Invitrogen) were used for molecular weight estimation. Purified Hsmr molecules were analyzed by SDS-PAGE on 4–12% NuPAGE gels (Invitrogen) in MES buffer according to the manufacturer’s directions. Unstained Mark-12 protein standards (Invitrogen) were used for molecular weight estimation.

**FIGURE 1. TM4 residue conservation in the SMR protein family.** A, topological diagram of Hsmr. Each TM segment and the intervening loops are indicated relative to their approximate position in the bilayer (shaded yellow). B, residue conservation in the SMR protein family. The TM4 sequences of Hsmr and the SMR family members EmrE (from *E. coli*) and Pasmr (the *Pseudomonas aeruginosa* SMR protein) are shown. Residues boxed in blue are conserved in the SMR family subclade to which all three sequences belong (termed SMP; see Ref. 1 for details). The characteristics of the two most commonly observed residues at each position (at >60% conservation levels) are indicated by the schematic below the sequences. Small residues (Ala, Gly, Ser) are indicated by a small dot; large residues (Ile, Leu, Val) are indicated by a large dot; and polar residues (Asn, Gln, His) are indicated by an asterisk. Non-conserved positions are denoted with X, and positions where conservation data were not available are denoted by dashes. Hsmr residues indicated in bold were mutagenized in the present study.
formed using ImageJ (NIH). The percentage of dimer was estimated by dividing the density of the dimer band by the total density of the dimer and monomer bands.

Modeling of Hsmr TM4 Dimers—Potential dimerization sites for the TM4 sequence were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS) as described elsewhere (29–31). Briefly, two identical α-helices were generated from the primary sequence of Hsmr TM4 (residues 85–105, inclusive), and their potential interactions were identified via global computational searching. TM4–TM4 helix interactions were independently searched in parallel and antiparallel orientations. All simulations were conducted in vacuo, a partial mimic of the low dielectric of the membrane. Residues mediating the close approach of TM4 helices in each of the CHI-generated TM4 dimers as well as those in contact between chain B residues 42–64 and 134–155 of the cyanobacterial photosystem I (PSI; Protein Data Bank (PDB) ID 1jb0) were determined by contact analysis in CNS; structures were queried for side chain and Ca atoms on each monomer found within 4.5 Å of side chain and/or Ca atoms on the other monomer. The 4.5 Å cutoff used represents approximately one-half the center-to-center distance of the pair of EmrE TM segments that could represent TM4 (11), as well as approximately one-half the average distance between helices packed by small side heptad repeats (32). Ca atoms were included in residue contact calculations in order for Gly residues to be considered. CNS was also used to determine intermonomer contact areas in the TM4 dimer models using a probe size of 1.88 Å. The contacting residue pairs in each model were compared with our present mutagenesis data and those of previous studies (25, 26). Dimer models were excluded from consideration if they did not include all of the residues identified as fully disruptive or disruptive at the TM4–TM4 interface or if the interface encompassed one or more of the residues identified as non-disruptive in our present mutagenesis study.

RESULTS

Design of Residue Substitutions in Hsmr TM4—An inspection of the Hsmr sequence and comparison of TM4 sequences among family members (Fig. 1) was followed by mutagenesis to full-length Hsmr at 12 TM4 positions that were targeted for replacement based on their approximate localization to the central portion of the segment and their degree of conservation (Fig. 1). Because the intention was to disrupt any potential helix–helix interactions at TM4, the residue substitutions selected were designed to alter side chain volume. Those with larger side chains (Val, Leu, Ile, Asn) were replaced with Ala; conversely, small side chains (Gly and Ala) were mutated to Val or Met. These replacements were anticipated to disrupt any close-packed or knobs-into-hole type interactions in which TM4 may participate.

Mutations at Conserved Sites in Hsmr TM4 Compromise Protein Function—The ability of each TM4 mutant to confer resistance to cytotoxic compounds was evaluated by measuring bacterial growth in the presence of either 200 or 500 μM EtBr. Here, cell survival depends on the ability of Hsmr to pump EtBr out of the cell with the degree of resistance proportional to protein activity (23). EtBr resistance in the presence of Hsmr TM4 mutants ranged from 0 to 100% versus WT at the two EtBr concentrations tested (Fig. 2, A and B). The changes in apparent Hsmr activity observed in the WT and mutant proteins were not expected to arise from altered SMR expression levels as protein recovery from this E. coli strain was found to be consistent among Hsmr variants (not shown).

Assembly Motif of Bacterial SMRs

FIGURE 2. Resistance activity and dimerization profile of WT and TM4 mutant Hsmr proteins. The growth of E. coli expressing WT Hsmr or the indicated mutant is shown normalized to WT growth values in the presence of 200 μM (A) and 500 μM (B) ethidium bromide. The growth of untransformed BL21(DE3) cells (–) was used as a negative control. Mutants are arranged from left to right in order of decreasing resistance activity versus WT and were classified according to activity level as described under “Experimental Procedures.” Error bars represent differences propagated from the standard deviations of at least three experiments. Note that the resistance activities of the highly disruptive mutants at 200 μM EtBr and the highly disruptive and disruptive mutants at 500 μM EtBr were indistinguishable from the activity of untransformed cells. C, representative silver-stained SDS-PAGE and quantitation of dimerization efficiency of WT and mutant Hsmr proteins. Each lane contains 100 ng of purified protein. Positions of monomer and dimer are indicated to the left on the gel by single and double dots, respectively. The histogram displays the mean percentage of dimerization relative to WT on SDS-PAGE for each mutant. Error bars were propagated from the standard deviation of three experiments. The significance levels of unpaired t tests comparing the dimerization efficiency of each mutant with WT are: *, p ≤ 0.05; **, p ≤ 0.01. The gel migration patterns and dimerization efficiency of all partially disruptive and non-disruptive mutants, and were classified according to activity level as described under “Experimental Procedures.” Substituted residues are indicated as follows: red, all disruptive mutants, and green, all non-disruptive mutants. Note that all disruptive mutants localize to the evolutionarily conserved face of the TM helix (blue shaded region) and vice versa for the non-disruptive mutants.
The Hsmr mutants were classified into groups based on resistance phenotype at the two EtBr concentrations tested; cells expressing the fully disruptive G90V, G97V, and V98A mutants could not grow at 200 μM EtBr; those expressing the disruptive mutants L91A, L93A, and I94A could not grow at 500 μM EtBr; those expressing the partially disruptive mutants V100A and L101A had less than 70% of WT activity at 500 μM EtBr; and those expressing the non-disruptive mutants V89A, A92M, V95A, and N102A retained at least 80% of WT activity at 500 μM EtBr (Fig. 2, A and B).

Disruptive TM4 Mutants Compromise Hsmr Oligomerization—To determine whether the activity trends observed could be traced to disrupted helix-helix contacts, we exploited the ability of the Hsmr protein to self-associate in the detergent SDS (23) and separated monomeric and oligomeric Hsmr molecules using PAGE (Fig. 2C). The two partially disruptive (V100A, L101A) and four non-disruptive (V89A, A92M, V95A, N102A) mutants ran as WT (A92M shown as an example). Conversely, each of the three fully disruptive mutants significantly disrupted the self-association of Hsmr, with the G90V- and G97V-substituted molecules dimerizing at ~60% WT levels and the V98A mutant dimerizing with ~40% WT efficiency. Two of the three disruptive mutants, L91A and L93A, reduced Hsmr dimerization to ~50–60% of the WT value. However, the disruptive I94A replacement had no effect on Hsmr self-association on SDS-PAGE, although this mutant exhibited reduced resistance to EtBr in vivo.

Upon mapping these residue groups onto a projection of TM4 (Fig. 2D), it was noted that the fully disruptive, disruptive, and partially disruptive mutants all lie on the evolutionarily conserved face of the TM4 helix. Additionally, the fully disruptive and disruptive mutants (G90V, L91A, L93A, I94A, G97V, and V98A) localize toward the central portion of the TM4 segment, whereas the two partially disruptive mutants (V100A, L101A) are positioned toward its C-terminal end. It can therefore be concluded that the minimum sequence on the TM4 helix required for SMR-mediated resistance at high toxicant levels could be represented as 90GLXLIXXGV98.

Modeling of Hsmr TM4-TM4 Dimers—Because structural and biochemical data suggest a role for TM4-TM4 interactions in SMR assembly, computational modeling of TM4 homodimers was used to visualize how the 90GLXLIXXGV98 sequence might mediate Hsmr assembly. The modeling of Hsmr TM4 self-interaction was accomplished using CHI, an algorithm developed to model the self-assembly of the TM domains of glycoporphin A (GpA) and phospholamban (29–31). Hsmr TM4 dimers were modeled in both parallel and antiparallel orientations because evidence exists to support each mode of SMR oligomerization (see Refs. 33 and Refs. 13, 19, 21, 34, and 35, respectively). Independent parallel and antiparallel simulations produced 24 TM4-TM4 association models (data not shown). Two of these (Fig. 3) were compatible with our present mutagenesis data and would not be expected to aggregate indefinitely (36).

**DISCUSSION**

The 90GLXLIXXGV98 Sequence Is Crucial for Hsmr Drug Efflux Activity and Self-assembly—The six residues found to be individually essential for Hsmr drug resistance activity each localize to the conserved surface of the TM4 helix. These conserved residues, including Gly90, Leu91, Leu93, Ile94, Gly97, and Val98, are unable to confer resistance to high concentrations of ethidium when mutated to oppositely sized residues (Fig. 2, A and B). Mutants at Gly90 and Gly97 in the TM4 of the related EmrE SMR were similarly found to be defective in self-association as well as activity, implying that abrogated function arises from disruption of TM4-mediated intermonomer interactions.

![FIGURE 3. Model of SMR assembly mediated by TM4-TM4 contacts.](image-url)
Assembly Motif of Bacterial SMRs

| GG4: GpA |
|-----------------------------------------------|
| IT LI I F G V M A G V I G T I L I S Y G I R L |
| Hsmr TM4 |
| V A G V X L A I V A G V V L N V A |
| Heptad: |
| PSl B134-155 |
| H L W G A F L A S L A I L L F I A G Q Y L |
| Psl B42-64 |
| Y Q K I E S H I G H A I I E L W V S G S |
| Mst K Gly |
| B Q T L I L G L L I L G I L I V |
| MHC α |
| T V V C A L G S V G L G Y I V V G T I F I I Q G L R S |
| MHC β |
| K L S G I G C V G L G V I F G L G L F I R E S Q K |

![Sequence alignment of Hsmr TM4 and TM segments that associate via GG4 or small residue heptad repeats.](image)

**FIGURE 4.** Sequence alignment of Hsmr TM4 and TM segments that associate via GG4 or small residue heptad repeats. The two small residues critical for GpA self-association (38) are indicated in bold. Small residues at positions α and d are indicated in bold for heptad repeats in the Hsmr TM4 sequence (this work); an example of the GASLeft motif (chain B residues 134–155 and 42–64 of cyanobacterial PSI (32)); the class II major histocompatibility complex (MHC α- and β-chains (40); and the de novo designed peptide MS1-Gly with β = β-alanine (41). Residues in contact at the GpA dimer interface (38), in contact between the PSI helix pair (Ref. 38 and this work), and key to Hsmr function (this work) are underlined. The mean propensity of small residues (Gly, Ala, Ser) to occur at the indicated positions in the heptad GASLeft packing motif (32) was aligned to the small residue periodicity of Hsmr TM4, where white (no shading) indicates a propensity of 1; light gray indicates >1 to <1.25; dark gray indicates 1.25 to <1.5; and black indicates ≥1.5. Note that the patterning of key Gly residues in the Hsmr TM4 sequence corresponds to that of other small residue heptad repeat motifs and to the propensity values, whereas the patterns of large residues required for function are similar to those in contact across the PSI helix pair. All sequences are presented in an N- to C-terminal direction, with the exception of the PSI B134–155 sequence, which is aligned in a C- to N-orientation.

(26). The SDS-PAGE results presented here also implicate the 90GLXLIIXGV98 sequence in dimer assembly, with five of six individual mutants that exhibit impaired drug resistance also disrupting Hsmr oligomerization. We therefore conclude that the minimum TM4 sequence required for Hsmr drug resistance activity can be defined as 90GLXLIIXGV98. This sequence encompasses the Gly90 and Gly97 residues previously identified as important for TM4-mediated SMR self-assembly and function in EmrE (26) and two of the key residues to strong self-association of Hsmr TM4 peptides (25).

**TM4-mediated Intermonomer Interactions Are Stabilized by Close Packing between Residues**—The pattern of large and small residues in the 90GLXLIIXGV98 sequence of TM4 defined in this work by mutagenesis matches the conservation of residue size along the TM4 helix (Fig. 1), suggesting that knobs-into-holes packing may stabilize SMR assembly via this segment. Two sequence motifs described in the literature are capable of mediating such interactions: the GG4 motif (alternately termed GX4O or GASRight) and the small residue heptad motif (also known as GASLeft) (reviewed in Ref. 37). The former exhibits a characteristic i, i + 4 small residue periodicity and is exemplified by the GpA homodimer, where two small Gly residues create a shallow groove into which the large Val residues of individual monomers intercalate at the dimer interface (38, 39). Small residue heptad packing motifs, on the other hand, can be defined by a seven-residue repeat (typically designated by the letters a–g, see Fig. 4), where the a and d positions most directly contact the opposing helix and are frequently occupied by small residues (32). Helix pairs packed by the GG4 versus heptad repeat motif also differ in their geometry (reviewed in Ref. 37), with the former crossing in a right-handed manner at an angle of ~40° and the latter crossing in a left-handed fashion at ~20° (32).

Comparison of the patterning of the fully disruptive, disruptive, partially disruptive, and non-disruptive mutations in Hsmr TM4 to that of the interfacial residues of the GpA dimer (GG4) or to that of sequences assembled via small residue heptad motifs (Fig. 4) suggests that the periodicity of the 90GLXLIIXGV98 sequence required for Hsmr self-assembly and/or function more closely approximates the latter, with the two key Gly90 and Gly97 residues occupying the α positions. The positioning of the Leu93, Leu93, and Val98 residues is also consistent with heptad repeat packing; analogous positions adjacent to small residues are occupied by large side chains and/or are in close contact in a helix pair packed via a small residue heptad repeat (Fig. 4). Placement of the Ile94 residue at position α in the TM4 heptad repeat, however, does not match the expected small residue pattern (Fig. 4), although we note that the functionally disruptive I94A mutation uniquely maintains dimerization relatively equal to WT (Fig. 2C). The large-to-small substitution at Ile94 may therefore disrupt function by altering, rather than preventing, residue intercalation between Hsmr monomers.

Identification of the 90GLXLIIXGV98 sequence defined in this work as a small residue heptad repeat is consistent with the left-handed geometry of antiparallel TM4-TM4 association observed in the EmrE crystal structure (21). Intercalation of the key small and large residues of the 90GLXLIIXGV98 sequence additionally occurs in each of our parallel and antiparallel orientations modeled in silico, and each is packed in a left-handed manner (Fig. 3). The geometry of the antiparallel model, however, appears to more closely approximate the estimates of residue location along the TM segments provided by selenomethionine labeling of EmrE (21). For example, the crystallographic antiparallel EmrE dimer has its two Leu93 residues located adjacent to one another on the boundary between the dimer interface and the surrounding lipid, similar to the Leu93 positioning of the antiparallel model of Hsmr TM4 presented in this work (Fig. 3A). Such an arrangement would be inconsistent with the parallel model, where the Leu93 residues localize to opposite sides of the dimer (Fig. 3B). Superimposition of our antiparallel Hsmr TM4 model with the x-ray structure of the antiparallel TM4 portion of the EmrE dimer indicates that the TM4 helices of the model are appropriately oriented for dimerization to occur at the 90GLXLIIXGV98 sequence (Fig. 3C); for example, Gly90 and Gly97 residues on one helix are proximal to Gly97 and Gly90, respectively, across the interface between the TM4 helix pair. The Hsmr TM4 model and the EmrE structure show some deviation at the helix termini, potentially due to truncated helix ends in EmrE (21) and/or to sequence differences such as the presence of Pro96 in EmrE (Fig. 1B).

The mutational analysis presented here does not specifically implicate oligomeric states higher than dimer in Hsmr function; however, the TM4 surfaces exposed in each of our model dimers are compatible with additional self-interaction. The potential of SMRs to form tetramers via “two-faced” TM4-TM4 interactions has also been suggested to utilize some of the Ala and Val residues on the non-conserved face of TM4 (25), and monomer pairings that involve Ala92 and Val95 in helix-helix packing are observed in our parallel computational models (data not shown). We note that a dimer-tetramer model of SMR
assembly mediated solely by TM4 could satisfy both parallel and antiparallel assembly constraints, with antiparallel contacts mediating dimerization at one surface and parallel at the other (Fig. 3D).

Although our results cannot unequivocally exclude the possibility that the interactions mediated by the 90GLXXLXXGV98 small residue heptad repeat motif on TM4 involve an Hsmr helix other than TM4, many lines of evidence support the role for TM4 self-interaction in SMR assembly. TM4 remains in a relatively constant conformation during SMR binding by a variety of substrates, acting as an anchor for the oligomer (11), and peptides corresponding to the Hsmr TM4 sequence self-associate strongly in a manner dependent on Gly90 and Ile94 (25).

Implications for SMR Inhibitor Design—Given the broad specificity and relatively inefficient binding of toxin substrates by small multidrug resistance proteins, strategies to inhibit their action that involve small molecule inhibitors targeted at the substrate binding pocket are unlikely to be consistently successful. The requirement of oligomerization for function in the SMR family, however, provides an additional opportunity for the design of inhibitors that disrupt this resistance pathway. The SMR resistance/assembly motif defined in this work as the TM4 residues 90GLXXLXXGV98 thus provides the starting point for the design of peptide or small molecules that inhibit SMR oligomerization through competition with critical TM4-TM4 interactions.

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