Exploring the Role of a Unique Carboxyl Residue in EmrE by Mass Spectrometry

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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, whereby 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 (Glu-14) from both EmrE monomers. Carboxyl modification of EmrE has been studied using functional assays, and the evidence suggests that Glu-14 is the target of the reaction. Here we exploited electrospray ionization mass spectrometry to directly monitor the reaction with each monomer rather than following inactivation of the functional unit. A cyanogen bromide peptide containing Glu-14 allows the extent of modification by the carboxyl-specific modification reagent diisopropylcarbodiimide (DiPC) to be monitored and reveals that peptide 2-NPYIYLGAILAEVITLTM21 is ~80% modified in a time-dependent fashion, indicating that each Glu-14 residue in the oligomer is accessible to DiPC. Furthermore, preincubation with tetrphenylphosphonium reduces the reaction of Glu-14 with DiPC by up to 80%. Taken together with other biochemical data, the findings support a "time sharing" mechanism in which both Glu-14 residues in a dimer are involved in tetrphenylphosphonium and H+ binding.

EmrE, an Escherichia coli multidrug resistance protein, 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 (Fig. 1, A and B) (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, Glu-14, which is conserved in more than 100 homologous proteins (5). Acidic side chains embedded in the membrane have been shown to be important for activity in various ion-coupled transporters (for a review, see Ref. 11). In some cases, these acidic side chains are involved in substrate recognition and binding; in others, they comprise part of the coupling ion translocation pathway. EmrE is unique in that the same acidic side chain (the carboxyl group of Glu-14) is involved in recognition of both substrate and the coupling ion. Furthermore, because replacement of the two other acidic side chains in EmrE (E2S/C/D84C) does not detrimentally effect the activity of the protein, it is concluded that the only carboxyl group required for catalysis is that of Glu-14 (12, 13).

Within the small multidrug resistance family of transporters, a comparative analysis reveals that the face of transmembrane domain 1 containing Glu-14 is conserved, displaying a helical periodicity. Site-directed mutagenesis of this transmembrane domain 1 face identifies 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 (14). 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 Glu-14. However, Val and Ala residues are clustered on the face opposite Glu-14 in domains that do not seem important for activity (15).

Previously, we have shown that the hydrophobic carbodiimides (carboxyl-specific modification reagents) dicyclohexylcarbodiimide and diisopropylcarbodiimide (DiPC) inhibit the binding and transport of substrates by EmrE (13), whereas dimethylaminopropyl-N-ethylcarbodiimide (EDAC), a hydrophilic carbodiimide, does not. Moreover, because dicyclohexyl-carbodiimide also inactivated an EmrE mutant containing only a single carboxyl residue at position 14, it was inferred that
Glu-14 is covalently modified by the carbodiimide (12). However, no information was available as to the number of Glu-14 residues reacting in the functional unit. In the present study, by exploiting the ability of reverse phase (RP)-high performance liquid chromatography (HPLC) in conjunction with ESI-MS to resolve complex mixtures (16–18), it was directly demonstrated that Glu-14 is modified by DiPC. Moreover, tetraraphenylphosphonium (TPP\(^+\)) reduces modification of Glu-14 by \(-80\%\), indicating that both Glu-14 residues in the functional unit are close enough to the binding site that TPP\(^+\) prevents their modification or that they might be equivalently protected in an allosteric manner by TPP\(^+\). These observations are not consistent with a recent mechanistic model based on three-dimensional crystals diffracting to 3.8 Å (19). In this model, the functional unit of EmrE is a dimer of structural heterodimers. However, within each heterodimer the two Glu-14s are in structurally distinct positions leading to functionally distinct roles in proton and substrate translocation. The observations presented here are more consistent with a “time-sharing” model where the Glu-14 residues in both monomers are in a similar environment, have similar reactivity to carbodiimides, and are both involved in substrate and proton recognition.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—**E. coli TA15 (20) was used for EmrE expression. Cells were transformed with plasmid pGPI-2, which encodes for the regulated expression of T7 polymerase under the inducible control of the AP\(_1\) promoter (21). The plasmids used for EmrE gene expression are pT7-7 (21) derivatives with the hexahistidine tag using a Myc epitope as linker (4). Throughout this report, for simplicity, Myc- and His-tagged protein is named EmrE. E25C, D84C, and E25C/D84C-EmrE (SC-EmrE, single carboxyl construct) mutants were previously characterized (12, 13).

**Overexpression and Purification of EmrE—**E. coli TA15 cells that bear plasmids pGPI-2 and pT7-7 containing His-tagged EmrE constructs were grown at 30 °C in minimal medium A supplemented with 2.5 \(\mu\)g/ml thiamine, 0.5% glycerol, 100 \(\mu\)g/ml ampicillin, and 50 \(\mu\)g/ml kanamycin. When the culture reached \(A_{600} = 1\), the temperature was elevated rapidly to 42 °C to allow for T7 polymerase expression for 15 min and then was decreased to 30 °C. Two hours later the cells were harvested by centrifugation and washed once with buffer containing 150 mM NaCl, 15 mM Tris, pH 7.5, 250 mM sucrose before storage at \(-70^\circ\)C. For membrane preparation, cells were resuspended in the same buffer containing 2.5 mM MgSO\(_4\), 1 mM dithiothreitol, 15 \(\mu\)g/ml DNaseI, and 1 mM phenylmethylsulfonyl fluoride (5 ml of buffer/g cells). Membranes were prepared by disrupting the cells using a Microfluidics microfluidizer processor (M-110EHH). Nondisrupted cells were discarded by centrifugation (4225 \(\times\) g for 5 min at 4 °C), and the membranes were collected by centrifugation at 240,000 \(\times\) g for 90 min at 4 °C. The membrane pellet was washed and resuspended in 150 mM NaCl, 15 mM Tris, pH 7.5, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (membrane protein concentration \(-20 \mu\)g/ml), frozen in liquid air, and stored at \(-70^\circ\)C. Wild type EmrE and the mutants were purified by solubilizing membranes (\(-5 \mu\)g of protein/ml) in 1% DDM, 150 mM NaCl, 15 mM Tris-HCl, pH 7.5, 50 mM imidazole, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride for 40 min at 25 °C. Unsolubilized material was removed by centrifugation at 240,000 \(\times\) g for 45 min at 4 °C. In the case of single carboxyl-EmrE, \(\beta\)-mercaptoethanol (15 mM) was added throughout purification to prevent cysteine cross-linking.

The solubilized protein was loaded on a HiTrap™ chelating HP column (Amersham Biosciences) mounted on an Akta Explorer (Amersham Biosciences) and washed with 0.08% DDM, 150 mM NaCl, 15 mM Tris-HCl, and 50 mM imidazole, pH 7.5 till \(A_{280}\) of the flowthrough decreased below 0.05. EmrE was eluted with a gradient of up to 300 mM imidazole. Major peak fractions were pooled, and the protein solution was brought to \(-70^\circ\)C. Wild-type EmrE and the mutants were purified by size exclusion chromatography (SEC). SEC of the purified protein was performed on a Superase-12 HR (HR 16/60) column (Amersham Biosciences). The protein solution was dialyzed against 1X saline and then was concentrated in a Centricon-10 concentrator (Millipore). The purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight was calculated using the ImageJ software (ImageJ version 1.43u). The molecular weight of the protein was determined to be 34 kDa. The purified protein was stored at \(-70^\circ\)C. For experiments, the protein was thawed and stored on ice until use. For analysis, the protein was diluted to 50 \(\mu\)g/ml in 50 mM NaPi (pH 7.5), 100 mM NaCl, 0.02% DDM, and 1 mM phenylmethylsulfonyl fluoride before storage at \(-20^\circ\)C. The protein was thawed and stored on ice until use. For analysis, the protein was diluted to 50 \(\mu\)g/ml in 50 mM NaPi (pH 7.5), 100 mM NaCl, 0.02% DDM, and 1 mM phenylmethylsulfonyl fluoride before storage at \(-20^\circ\)C.
Phase separation was accomplished by adding water (200 μl) and mixing vigorously. The phases were separated by centrifugation (10,000 × g for 2 min), yielding a precipitate at the interface. Although EmrE is soluble in CHCl3/MeOH (1:1, v/v), at the ratio used here it was possible to isolate the precipitate by rapidly removing the bulk of the upper aqueous methanol phase by careful aspiration, followed by tilting of the tube allowing removal of the lower chloroform phase in a similar fashion. For size exclusion HPLC of the full-length protein, the pellet was air dried for 1–2 min with the tube inverted and resuspended in 90% formic acid. For cleavage of the protein, after drying the pellet it was resuspended in a saturated solution of CNBr in 90% formic acid and left for 12 h in the dark. Formic acid was removed in a vacuum centrifuge, and the pellet was left in 0.5 ml of 0.1% trifluoroacetic acid in water overnight. The sample was dried again and resuspended in 60% formic acid immediately prior to RP-HPLC.

HPLC—For final purification of EmrE after covalent modification with DiPC, HPLC was used prior to ESI-MS in an in-line setup (liquid chromatography/MS). To separate the CNBr peptides generated from EmrE, a polystyrene/divinylbenzene column (PLRP/S, Polymer Labs, 5 μm, 300 Å, 150 mm × 2.1 mm) at 40 °C was used for reverse phase liquid chromatography/MS (23). Following equilibration in 95% solvent A, 5% solvent B for 5 min (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in CH3CN/isopropanol (1:1)), the percentage of solvent B was increased to 40% over the next 25 min and further to 100% B over the subsequent 120 min at a flow rate of 0.1 ml/min. All chromatographic separations were performed at 40 °C using a modified ABI 120A dual-syringe pump HPLC equipped with a post-detector (A280) splitter for back pressure regulation.

Electrospray Ionization Mass Spectrometry—ESI-MS was performed using a PerkinElmer Sciex API III triple-quadrupole instrument operating in the positive ion mode as described. The orifice potential was 75 V for full-length EmrE and ramped from 60 to 120 V across the mass range (600–2300) for CNBr fragments. MS/MS spectrometry fragment ion spectra were obtained by splitting the flow into the API-III during RP-HPLC and collecting peptides of interest for infusion (3 μl/min) into a ThermoFinnigan LCQ Deca ion trap instrument with a 33-gauge stainless steel needle source at 3.3 kV. The nomenclature used for fragment ions is N-terminal fragments (b type) and C-terminal fragments (y type).

RESULTS

ESI-MS of EmrE—Following electrospray ionization, EmrE ions carrying from 7 to 16 positive charges (H+) are readily detected (Fig. 1C). Molecular mass reconstruction of wild type EmrE with a carboxyl-terminal Myc- and His6 tag reveals that

**FIG. 2. Recovery of a CNBr peptide containing Glu-14 from EmrE.** CNBr peptides from 100 μg of purified EmrE were separated as described under “Materials and Methods.” A, schematic representation of EmrE. The four transmembrane α-helices (rectangles), one essential residue (Glu-14), and CNBr cleavage sites (arrows) are shown. Predicted peptides are indicated by lines. B, collision-induced dissociation spectrum of 2-NPYIYLGAILAEVIGTTL-21. Annotated peaks in the spectra include the doubly charged parent (m/z 1031.9) and the b- and y-ion series.
formation of a protonated O-aclyisourea that rearranges to form a stable N-aclyisourea with no further change in mass (Refs. 24, 25 and Fig. 3A). Following reaction of EmrE with DiPC, RP-HPLC-ESI-MS of CNBr fragments resolved by RP-HPLC revealed a doubly charged parent ion at \( m/z \) 1094.9 corresponding to a molecule of molecular mass 2187.8 Da with a measured 126.7 Da mass increase (Fig. 3B, compare I and II). Collision-activated dissociation of the doubly charged parent ion at \( m/z \) 1094.9 revealed fragment ions consistent with the sequence \(^2\)NPYIYLGGAILA modified at position 14 by DiPC (data not shown). Notably, reaction of EmrE with a 10-fold higher concentration of the hydrophilic carbodiimide EDAC leads to only 8% modification of Glu-14 over an extended time period (Fig. 4A, II, 120 min), demonstrating once again that hydrophobicity of the reagent governs access to Glu-14.

Previously published results (13) demonstrate that substrates protect EmrE from inhibition by hydrophobic carbodiimides, and the results presented here by RP-HPLC in-line with ESI-MS reveal that TPP\(^+\) protects EmrE by reducing the amount of modification of Glu-14 by DiPC (Fig. 3B, compare II and III). At pH 6.5, DiPC reacts in a concentration-dependent manner with the carbonyl group of Glu-14 and the reaction terminates at at ~80% modification, most probably because of the instability of carbodiimides in aqueous solution (Fig. 4A, I). This information identified the linear range of DiPC modification and served as the foundation of a quantitative experiment using different concentrations of TPP\(^+\). Although the experimental setup did not allow the use of EmrE concentrations lower than 40 \( \mu \)M, thus preventing us from studying the concentration dependence of TPP\(^+\) protection against DiPC modification at low concentrations, higher concentrations of TPP\(^+\) clearly reduce the extent of DiPC modification by ~80% (Fig. 4B).

**DISCUSSION**

Previously, carbodiimide modification of EmrE has been studied using functional assays monitoring EmrE inactivation. Two lines of evidence suggest that Glu-14 is the target of this carbodiimide-mediated inactivation: (i) EmrE is inactivated by hydrophobic, but not hydrophilic, carbodiimides; (ii) an EmrE mutant possessing a single carboxyl at position 14 is inhibited by dicyclohexylcarbodiimide in a similar manner to wild type. A remarkable aspect of the inhibition is that it can be virtually completely prevented by substrates of EmrE. Now, by exploiting ESI-MS, it is possible to monitor the reaction of carbodiimide with each EmrE monomer directly rather than following inactivation of the functional unit.

Practically all (~80%) of the detergent-solubilized EmrE is covalently modified by the hydrophobic carbodiimide DiPC. CNBr cleavage of EmrE reveals that DiPC reacts extensively with the peptide containing Glu-14, leading to the expected change in mass because of the formation of an N-aclyisourea. In contrast, as expected from functional studies (13), the hydrophilic carbodiimide EDAC fails to react significantly at concentrations up to 10-fold higher (20 \( \mu \)M). Because the carbodiimide moiety reacts with the protonated form of a carbonyl group, it is concluded that all Glu-14 residues in the EmrE functional unit are accessible to DiPC, inaccessible to EDAC, and predominantly protonated at pH 6.5. These findings agree with previous studies demonstrating that Glu-14 is buried with an anomalously high \( pK_a \) of ~8.5 (26). Furthermore, the observation that Glu-14 is accessible to relatively large bulky hydrophobic reagents (e.g., dicyclohexylcarbodiimide, DiPC), but inaccessible to similarly sized hydrophilic reagents (e.g., EDAC), strengthens the hypothesis that Glu-14 is located inside a large hydrophobic drug binding pocket. Interestingly, EmrE mutants with either (E25C or D84C) or both (single carboxyl-EmrE) of...
the carboxyl residues located in the loops removed undergo DiPC modification in a fashion very similar to the wild type EmrE, supporting the idea that the Glu-14 carboxyl is not networked with other acidic residues (data not shown).

Structural evidence from two-dimensional crystals led to the proposal that the minimal functional unit of EmrE was likely to be a dimer (27). This was confirmed by determination of the three-dimensional structure of EmrE with TPP$^+$ bound at 7.5 Å resolution by electron cryomicroscopy of two-dimensional crystals (9). The minimal structural unit is an asymmetric homodimer composed of 8 transmembrane $\alpha$-helices, i.e. 4 helices from each monomer, with density for TPP$^+$ in a binding chamber formed from 6 of the 8 $\alpha$-helices, confirming the suggestion that TPP$^+$ binds near the center of the dimer (9). However, these findings do not rule out the existence of higher order oligomers. In fact, co-expression of two plasmids in a cell-free system allowed demonstration of functional complementation, and pulldown experiments suggested that the basic functional unit is the dimer (7), whereas additional weak interactions between dimers detected using cross-linking implies the existence of a dimer of dimers (7).

Based on crystals of EmrE that diffract to 3.8 Å, an atomic model was recently proposed by Ma and Chang (19). 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

![Quantitative analysis of Glu-14 modification](image)

**Fig. 4.** Quantitative analysis of Glu-14 modification. A, time course of $^{2}$NPYLYGLGAILAEVGTTLM$^{21}$ modification by DiPC. Purified EmrE (∼40 μM) in DDM at pH 6.5 was incubated with the shown concentrations of DiPC (I) or EDAC (II) at 30 °C for varying amounts of time, leading to a mass shift of 126 or 155 atomic mass units, respectively. The total ion current from peptide $^{2}$NPYLYGLGAILAEVGTTLM$^{21}$ modified by DiPC divided by the total ion current from unmodified and modified $^{2}$NPYLYGLGAILAEVGTTLM$^{21}$ was used to calculate the percentage modification of Glu-14. No measurable difference in the ionization efficiency of the unmodified and modified peptides was detected. Each point represents the average of two independent experiments. Maximum peak height in the absence of modification was $\sim 4 \times 10^5$ counts. B, concentration dependence of TPP$^+$ effect on DiPC modification of Glu-14. Purified EmrE (∼40 μM) in DDM at pH 6.5 was incubated for 10 min at 30 °C with 2% dimethyl sulfoxide (Me$_2$SO), 10 mM DiPC, or 10 mM DiPC in the presence of TPP$^+$ (0.1 or 1 mM). The total ion current from peptide $^{2}$NPYLYGLGAILAEVGTTLM$^{21}$ modified by DiPC divided by the total ion current from unmodified and modified $^{2}$NPYLYGLGAILAEVGTTLM$^{21}$ was used to calculate the percentage modification of Glu-14. No measurable difference in the ionization efficiency of the unmodified and modified peptides was detected. Each point represents the average of two independent experiments. Maximum peak height in the absence of modification was $\sim 4 \times 10^5$ counts.
conformational heterodimer, the Glu-14 residues are in a different environment, one solvent 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 (Fig. 5, Model I). Critically, such a model indicates that there are two equally sized subpopulations of Glu-14 carboxyls, each residing in a significantly different environment, most likely with different pKₐ and accessibility to DiPC, EDAC, TPP⁺. However, the results of this study indicated that ~80% of Glu-14 are modified by DiPC and, similarly, TPP⁺ protects against ~80% of this modification, suggesting that the Glu-14 residues in the functional unit of EmrE are functionally equivalent, playing a direct role in substrate binding.

Finally, previous studies demonstrate that practically all (>80%) the EmrE monomers release a proton from the Glu-14 carboxyl upon substrate binding (26), implying that both Glu-14s in the EmrE dimer are involved in proton binding and release. Furthermore, an proton-substrate antiport studied in proteoliposomes was found to be electroneutral for doubly charged substrates and electrogenic for singly charged substrates (28). These findings led to the conclusion that two protons are exchanged with one substrate molecule in each transport cycle. Taken together, the results support a model (Fig. 5, Model II) where the basic functional unit is a dimer with both monomers participating in a single binding site shared by protons and substrate.

REFERENCES


FIG. 5. Two alternative mechanisms of action for oligomeric EmrE. Transmembrane domain 1 from each EmrE monomer is shown with Glu-14 marked. In both models the stoichiometry of H⁺ to TPP⁺ is 2:1. In Model I, the delocalized positive charge on the cationic substrate (TPP⁺) is neutralized by two Glu-14. Two other Glu-14s bind H⁺, causing a conformational change leading to TPP⁺ extrusion. In Model II, both Glu-14s are involved in neutralizing the positive charge on TPP⁺. Protonation at the same Glu-14 leads to the conformational change causing TPP⁺ extrusion.