The Key Residue for Substrate Transport (Glu$^{14}$) in the EmrE Dimer Is Asymmetric*§

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Transport proteins exhibiting broad substrate specificities are major determinants for the phenomenon of multidrug resistance. The Escherichia coli multidrug transporter EmrE, a 4-transmembrane, helical 12-kDa membrane protein, forms a functional dimer to transport a diverse array of aromatic, positively charged substrates in a proton/drug antiport fashion. Here, we report $^{13}$C chemical shifts of the essential residue Glu$^{14}$ within the binding pocket. To ensure a native environment, EmrE was reconstituted into E. coli lipids. Experiments were carried out using one- and two-dimensional double quantum filtered $^{13}$C solid state NMR. For an unambiguous assignment of Glu$^{14}$, an E25A mutation was introduced to create a single glutamate mutant. Glu$^{14}$ was $^{13}$C-labeled using cell-free expression. Purity, labeling, homogeneity, and functionality were probed by mass spectrometry, NMR spectroscopy, freeze fracture electron microscopy, and transport assays. For Glu$^{14}$, two distinct sets of chemical shifts were observed that indicates structural asymmetry in the binding pocket of homodimeric EmrE. Upon addition of ethidium bromide, chemical shift changes and altered line shapes were observed, demonstrating substrate coordination by both Glu$^{14}$ in the dimer.

Multidrug drug resistance, in particular bacterial resistance to clinical antibiotics, is a widely known phenomenon. Basic defense mechanisms of bacteria include permeability barriers, inactivation of antimicrobials, modification of antibiotic targets, and active drug efflux (1). Active efflux is conducted by primary and secondary active transport proteins and the latter are found in almost all transporter families (2). The molecular mechanism of the broad substrate specificity of multidrug efflux pumps is not yet fully understood. To this end structural studies are desirable, but currently only 13 three-dimensional structures of different transport proteins are known and not every transport family is represented. The only multidrug transporter with known three-dimensional structure is AcrB (4, 5). A three-dimensional structure of the ABC transporter Sav1866 has also been reported (6), which is assumed to function as a multidrug efflux pump.

Here, we are especially interested in Escherichia coli EmrE, a member of the medically relevant SMR$^{2}$ protein family (TC number 2.A.7.1 (8, 9)). Due to its small size (12 kDa), EmrE was originally proposed as ideal structure-function paradigm (10). It has attracted significant interest due to its controversial topological organization (11–15), oligomerization state (16–19), transport cycle steps (12, 20–23), and unknown three-dimensional structure (24). EmrE transports a diverse array of aromatic, positively charged substrates in exchange for protons (21) via at least one occluded transport cycle intermediate state (25). Other SMR proteins have overlapping but significantly different substrate specificities with measured affinities in the nanomolar to millimolar range (20, 26). All SMR proteins are of similar size (~11–12 kDa), have a 4-transmembrane helix topology and a highly conserved key residue Glu$^{14}$ (20, 27). It has been shown that Glu$^{14}$ is an essential residue and directly involved in drug and proton binding (28–30). It can reasonably be assumed, that Glu$^{14}$ of both protomers in a dimer form a shared binding pocket (31, 32).

Whether EmrE forms a symmetric or an asymmetric dimer should be reflected in the chemical shifts of residues such as Glu$^{14}$, which are likely to be found at the dimerization interface. We therefore $^{13}$C-labeled Glu$^{14}$ in EmrE by utilizing a cell-free expression system. To allow an unambiguous NMR analysis, the nonessential residue Glu$^{25}$ was replaced with alanine (EmrE E25A) to create a single glutamate mutant. The protein was reconstituted into E. coli lipids allowing the most native environment possible. The sample was maintained at pH 8.0. Under these conditions, without a pH gradient, EmrE, like other secondary transporters (33), is able to bind substrate and can undergo equilibrium substrate exchange reactions (20, 29, 30). Substrate exchange reactions are biologically relevant as they are only 15 times slower than active transport (30). Non-invasive solid state NMR revealed significantly different chemical shifts between both Glu$^{14}$ residues that change upon substrate exchange.

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‡ The abbreviations used are: SMR, small multidrug transporter; DDM, n-dodecyl-β-D-maltoside; bR, bacteriorhodopsin; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; DHBs, matrix 2,5-dihydroxybenzoic acid:2-hydroxy-5-methoxy benzoic acid (10:1); MAS, magic angle spinning; DQSO, double quantum single quantum; DQF, double quantum filter; DQ, double quantum; TPP$^{7}$, tetraphenylphosphonium.

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binding. Our data indicate an asymmetric homodimer for EmrE with and without substrate bound.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials were purchased from Sigma Chemical Co (St. Louis) unless indicated otherwise. Nickel-nitritolriotic acid was obtained from Qiagen (Hilden). BioBeads SM-2 were purchased from Bio-Rad (Munich). Complete Mini protease inhibitor, pyruvate kinase, and E. coli tRNA MRE 600 were purchased from Roche Diagnostics GmbH. Phosphoenolpyruvate monopotassium salt was obtained from Merck (Darmstadt, Germany). DNA for crystallography was obtained from Glycon (Lackenwalde). A 15-kDa cut-off microdialyzer was purchased from Spectra/Por (Rancho Dominguez, CA). [U-13C,15N]Glutamate was obtained from Cambridge Isotope Laboratories (Andover, MA). A protein calibration mixture was purchased from Applied Biosystems/MDS SCIEX (Darmstadt). DHBs was obtained from Bruker Daltonics (Bremen).

**Cloning**—Wild type EmrE with myc and his tag in a pT7-7 vector derivative (23) was kindly provided by S. Schuldiner, Hebrew University of Jerusalem. Wild type EmrE in a pET21a vector was kindly provided by F. Bernhard, J. W. Goethe University, Frankfurt. For this study we constructed the mutant EmrE E25A with His$_{6}$ tag in a pET21a + vector. EmrE was amplified from the pET21a + vector using a forward primer with a NdeI restriction site (cgcaatatacggtgccgaatatacgggtg) and a reverse primer with a HindIII restriction site (cataaccctattctttactagctggttctggt). The amplified fragment and the vector pET21a + vector were digested with endonucleases NdeI and HindIII, the vector was dephosphorylated with nuclease NdeI and HindIII, the vector was dephosphorylated with T4 DNA ligase. The mutation E25A was introduced using the QuikChange site-directed mutagenesis methodology with the mutagenic primer (cgcaatatacggtgccgaatatacgggtg).

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**In Vitro and in Vivo Expression**—Coupled cell free transcription/translation, as previously described (34), was used for the expression of EmrE E25A as precipitate. Expression of soluble EmrE E25A was achieved using 0.1% (w/v) DDM in the reaction mixture (35). [U-13C,15N]Glutamate was used to produce selectively glutamate-labeled EmrE E25A. The bacterial strain used for in vivo expression was E. coli TA15 (36). It was additionally transformed with the pGPI-2 plasmid encoding the T7 polymerase under a heat shock promoter AP$_{L}$ (37). Expression was performed as previously described (25).

**Protein Purification**—In vitro produced EmrE was purified by cell disruption and membrane preparation as previously described (25). In vitro (soluble and precipitate) and in vivo produced EmrE was diluted into 25 ml of solubilizing buffer (100 mM NaCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol, 10 mM imidazole, 1 pill Roche mini complete protease inhibitor, and 1% (w/v) DDM) and incubated for 1 h at room temperature. After removal of the unsolubilized components by centrifugation (184,000 $g$ for 50 min), the supernatant was mixed with nickel-nitritolriotic acid resin for 80 min at 4 °C in a beaker. The resin was washed with wash buffer (100 mM NaCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol, 30 mM imidazole, and 0.1% (w/v) DDM) until A$_{280}$ was below 0.05. The protein was eluted with the same buffer containing 300 mM imidazole and the approximate protein concentration was determined at 280 nm using the elution buffer as a blank.

**Protein Reconstitution**—EmrE was reconstituted at a protein/lipid mole ratio of 1:100 for solid state NMR in buffer (100 mM NaCl, 50 mM Tris, pH 8.0, and 1 mM dithiothreitol) and at 1:450 mole ratio for fluorescence spectroscopy in buffer (150 mM KCl, 5 mM K$_{2}$EDTA, pH 7.3) using the following method. 4 mg/ml E. coli total lipid extract was solubilized with a 1:2 mole ratio of DDM for 1 h at room temperature with sonication. Protein and lipid/detergent solutions were mixed at the required ratio and incubated for 1 h at room temperature, followed by detergent removal using pre-washed and sonicated Bio-Beads SM-2. In total 3 × 30 mg of Bio-Beads/mg of detergent were added and incubated for 1 h at room temperature and once at 4 °C overnight. Bio-Beads were removed and the liposomes pelleted by centrifugation. Incubation with 3 μM EtBr was performed for 30 min at room temperature in the buffer (100 mM NaCl, 50 mM Tris, pH 8.0, and 1 mM dithiothreitol). The EtBr concentration was chosen to theoretically provide greater than 99% occupancy at the binding site. For the fluorescence transport assay bR at a 1:280 protein/lipid mole ratio was added to the mixture and freeze/thawed, which results in an ~90% inside out orientation of bR in the vesicles (38).

**Fluorescence-based Transport Assays**—Transport assays were carried out exactly as described previously (25).

**MALDI-TOF Mass Spectrometry**—Purified EmrE, ~0.64 mg/ml, was used (20 mM NaCl, 15 mM Tris, pH 8.0, 0.1–0.2% (w/v) DDM). Matrix DHBs was prepared using acetoniitrile, 0.1% trifluoroacetic acid (1:2, v/v) as solvent. Additionally, a protein calibration mixture was added to the matrix solution as an internal standard. 1 μl of matrix solution was mixed with 1 μl of diluted EmrE (in a ratio of 1:10 with water) and dried. The protein mass determination was performed with a Voyager DE-PRO (Applied Biosystems, MDS Sciex). Spectra were recorded in the linear mode (25 kV acceleration voltage, 93% grid voltage, 350 ns delay time). Spectra were smoothed, noise-filtered, labeled, and internally calibrated (two-point calibration) using Data Explorer version 4.9 software (Applied Biosystems, MDS Sciex).

**Freeze Fracturing**—Proteoliposomes in sample holders were frozen in ethane cooled to ~180 °C by liquid nitrogen. Fracturing at ~120 °C and replication at a shadowing angle of 45° with platinum/carbon was performed with a BAF 060 freeze-fracturing unit from Bal-Tec Inc. (Principality of Lichtenstein).

**NMR Spectroscopy**—XWIN-NMR and Topspin 2.0 from Bruker (Karlsruhe, Germany) and Sparky 3 (T. D. Goddard and D. G. Kneller, University of California) were used for data acquisition and processing.
**Solid State NMR Spectroscopy**—$^{13}$C spectra at 150.90 and 100.62 MHz were recorded on 600 and 400 MHz Bruker Avance spectrometers, respectively, using 4 mm MAS DVT probeheads. Reconstituted samples containing ~1 mg of EmrE were transferred to a 4-mm Bruker style zirconia rotor (RototecSpintec, Germany), sealed by high resolution MAS inserts, and quick-frozen in liquid nitrogen. Sample measurements were performed at 200 K with MAS spinning rate of 10,000 ± 5 Hz. DQSQ correlation spectra of the INADEQUATE type (39, 40) were collected by using the SR22 pulse sequence previously described (41). One-dimensional DQF spectroscopy were also collected with the SR22 pulse sequence and similar settings. A relaxation delay of 1.5 s and 4 dummy scans were used. Cross-polarization contact time was 500 µs, $^1$H 90° pulse was 3 µs, $^{13}$C 90° pulse was 3.4 µs, and 400 µs DQ excitation and reconversion time was used. Time proportional phase incrementation acquisition was used for two-dimensional spectra (42). $^1$H decoupling during the DQ excitation and reconversion was achieved using continuous wave irradiation at ~100 kHz and during $t_1$ and $t_2$, evolution composite decoupling using SPINAL 64 (43) at 83.3 kHz was used. Experiments were recorded with 45,000 Hz sweepwidth in the direct dimension and 90,000 Hz in the indirect dimension. Exponential 150 Hz line broadening was used in both dimensions. The spectra were recorded with 128 or 96 increments in the indirect dimension and 2720 points in the direct dimension. Scan numbers varied between experiments and are indicated in the figure legends. $^1$H and $^{13}$C were referenced to 0 ppm with tetramethylsilane at 4 °C and ~2,000 Hz spinning. For chemical shift anisotropy line shape fitting in Topspin 2.0 the Solids Lineshape Analysis tool was used with mixed Gaussian/Lorentzian line shapes.

**RESULTS**

**Sample Preparation**—To investigate the electronic structure of Glu$^{14}$ in EmrE, a selectively labeled sample was required. For this, in vitro transcription and translation has to be employed as selective glutamate labeling in vivo is difficult due to isotope scrambling (44). In addition, a single glutamate mutation is necessary to avoid spectral overlap and to obtain unambiguous data. Cell-free protein expression has been carried out both as continuous and no protein aggregation was detected. A typical spectrum is shown in Fig. 1. The absence of other signals and the result of SDS-PAGE show, that EmrE E25A was pure. Mass spectrometry, in addition to SDS-PAGE, is a very sensitive tool to assess contamination with soluble proteins. Membrane proteins other than EmrE do not occur in our cell-free expression system. We have verified that no $^{13}$C-glutamate scrambling in the in vitro expression system takes place by performing a complete translation and transcription experiment without DNA template. A $^{13}$C NMR analysis of the whole reaction mixture did not show any signs of glutamate to glutamine conversion (see supplemental Fig. S1).

Purified protein solubilized in DDM was directly reconstituted into E. coli total lipids at a 1:100 mol ratio. We selected E. coli lipids to ensure a native membrane environment. Reconstitution was consistently successful, as monitored by freeze fracture microscopy. Even after many days of NMR experiments, samples were very homogeneous without any aggregates as shown in Fig. 2.

We verified the activity of cell-free-produced EmrE E25A and wild type EmrE prepared in vivo with a previously described ethidium bromide transport assay (25). In the course of the assay, bR driven H$^+$ pumping and EtBr fluorescence is initiated by light excitation at 545 nm. EmrE then uses the generated pH gradient as energy source for an antiport of EtBr. The transport can be detected by an increase of EtBr total fluorescence intensity due to binding to a transport cycle intermediate (25). The time traces in Fig. 3 show an increase of total fluorescence for EmrE produced in vivo and EmrE E25A produced cell free both as precipitate and in the presence of detergent. Traces are almost identical and transport activity ceases when adding carbonyl cyanide 3-chlorophenylhydrazone, which uncouples...
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FIGURE 3. EtBr transport activity of in vitro expressed EmrE E25A in E. coli total lipid liposomes. EmrE E25A expressed as precipitate (A), EmrE E25A expressed soluble in the presence of detergents (C), and wild type EmrE expressed in vivo (E) all show transport activity in the presence of a pH gradient as judged by the increase in EtBr fluorescence intensity (see text). Time traces B, D, and F are negative controls generated by adding to samples A, C, and E the uncoupler carbonyl cyanide 3-chlorophenylhydrazone, which collapses the proton motive force (B), the ionophore nigericin, which collapses the pH gradient (D), or the competitive but non-fluorescent substrate TPP⁺ (E). The experiments show that EmrE E25A produced in vitro as precipitate is active.

both ΔpH and Δψ (45), nigericin, which uncouples ΔpH (21), or TPP⁺ a competitive non-fluorescent substrate (20). These data verified that active protein was produced using in vitro translation/transcription and that both expression modes, as precipitate or in the presence of detergent, are possible. As a consequence of these results, all NMR experiments described below were carried out on EmrE E25A expressed as precipitate due to the significantly higher yields.

One-dimensional NMR—DQF ¹³C MAS NMR spectra of [¹³C]glutamate-labeled EmrE E25A in E. coli lipids are shown in Fig. 4. Filtering was necessary to remove the ¹³C natural abundance contributions (1.1%), from lipids and unlabeled residues, which overlap with the resonances of Glu14. The spectrum shown in Fig. 4a reveals a symmetric Cα doublet (labeled Glu I and Glu II), which becomes asymmetric upon addition of EtBr (see Fig. 4b). The Cα splitting was reproducible as verified with two independent sample preparations (see supplemental Fig. S2). The spectral contributions of natural abundance spin pairs (0.01%), which will not be suppressed by double quantum filtering, was assessed by acquiring a ¹³C DQF spectrum of E. coli lipids alone (Fig. 4c). Despite the very low abundance of naturally occurring spin pairs, a CH₂ signal can clearly be seen. This observation is not surprising considering that 100 lipids with ~28 methylene groups contribute ~30% of the magnetization to the EmrE E25A glutamate side chain signal.

Two-dimensional DQSQ Spectra—To unambiguously assign C’, Cα, Cβ, Cγ, and Cδ resonances, and to check whether all resonances are doubled, two-dimensional ¹³C DQSQ experiments were carried out. These correlation spectra contain chemical shift information for all nuclei in the direct dimension and depict bond connectivities in the indirect dimension (46). Two-dimensional spectra without substrate (Fig. 5a) and with EtBr (Fig. 5b) indicate at first inspection multiple peaks, with distinct chemical shifts, for each nucleus. INADEQUATE type assignment walks starting from both major Cα peaks shown in Fig. 5, a and b, confirm peak doublets for all Glu14 carbons. An unambiguous assignment in this type of spectra is aided by the fact that the sum of two peak frequencies in the direct dimension is equal to their frequencies in the indirect dimension. As already indicated in Fig. 4, we refer to the set of resonances connected to the less shielded Cα as Glu I (blue lines in Fig. 5)

FIGURE 4. One-dimensional ¹³C MAS NMR spectra of EmrE E25A, in E. coli total lipids, and Cα line shape fitting. a, the single glutamate mutant EmrE E25A was selectively [¹³C]glutamate labeled and reconstituted into E. coli total lipid liposomes at a 1:100 protein:lipid mol ratio. ¹³C double quantum filtering (25,000 acquisitions) was applied to suppress ¹³C natural abundance signals. A highly reproducible, symmetric peak doublet is observed for the Cα signal around 55 ppm. b, after the addition of EtBr the Cα peak doublet is retained but becomes asymmetric (70,000 acquisitions). c, DQF removes efficiently the background signal of the 1.1% naturally occurring ¹³C nuclei but does not affect the naturally occurring 0.01% spin pairs. Their spectral contribution has been probed with E. coli lipids, which show a clear signal in the DQF spectrum at the CH₂ position. This result is not surprising considering the large number of lipids per protein with 24–28 CH₂ groups per lipid. Marked with an asterisk is a spectrometer artifact occurring at 168 ppm at 150.90 MHz. d, a line shape analysis of the Cα resonances of Glu14 in EmrE E25A shows that both peaks are of similar integral intensity with a line width of 3 (Glu I) and 4 ppm (Glu II). A, upon addition of EtBr, Glu I and Glu II are shifted, become more narrow (1.8 and 2.8 ppm), and additional populations are observed. Here a minimum of four peaks is required for an adequate description of the whole line shape. The minor populations contribute 28% to the total magnetization. Protein spectra (a, b, d, and e) were acquired at 150.9 MHz and the lipid spectrum (c) at 100.62 MHz ¹³C Larmor frequency.
and the other as Glu II (red lines in Fig. 5). Chemical shifts for both Glu I and Glu II with and without EtBr are summarized in Table 1.

**Line Shape Analysis**—Multiple distinct molecular environments are suggested by the need for at least two distinct sets of chemical shifts to describe the $^{13}$C spectra of Glu. To obtain a more quantitative picture, a line shape analysis was carried out for the $\text{Ca}$ peaks that showed the most pronounced splitting. The symmetric $\text{Ca}$ doublet of EmrE E25A can be adequately described by two resonances of similar integral intensity with respective line widths of 3.0 (Glu I) and 4.0 ppm (Glu II) (Fig. 4d). The different chemical shifts of these two resonances are indicative of two inequivalent EmrE E25A populations. The line width indicates a limited amount of heterogeneity.

In contrast, four peaks are required to describe the asymmetric $\text{Ca}$ line shape that is observed in the presence of EtBr (Fig. 4e). The two main peaks, Glu I and Glu II, are of equal integral intensity. The two additional peaks contribute 28% magnetization to the spectrum. The addition of EtBr therefore causes Glu to be present in more than two distinct molecular environments. The line width of both main peaks is reduced to 1.8 (Glu I) and 2.8 ppm (Glu II) compared with EmrE E25A without substrate, suggesting a decrease in structural heterogeneity concomitant with an increase of populations.

**DISCUSSION**

**General**

In this study we set out to probe the proposed active site structure of the functional homodimeric EmrE with and without the substrate EtBr. For this investigation the single glutamate mutant EmrE E25A was selectively isotope labeled at the remaining conserved Glu. This enabled unambiguous chemical shift assignment and simplified data interpretation. The capability of *E. coli*-based cell-free transcription and translation systems to express wild type EmrE in a functional form with and without detergent was previously shown (17, 35). Furthermore, it has been reported that amino acid selective isotope labeling without dilution or scrambling is possible (34).

We performed several control experiments to verify that cell-free expressed EmrE E25A is indeed functional and correctly labeled. We were able to show that preparations as precipitate as well as in the presence of detergent produce functional protein. The precipitation method was preferred here as the protein yield was higher.

The protein was reconstituted into *E. coli* lipids under conditions that allow the protein to be functional in the exchange mode. This active protein conformation was trapped by freezing with liquid nitrogen. Non-invasive solid state NMR revealed significantly different chemical shifts between both Glu residues with and without addition of ethidium bromide. This indicates an asymmetric homodimer for EmrE with and without substrate bound. In this study the key to understanding the chemical environment of glutamate lies in the chemical shifts.
Inequivalence of Glu14 Residues in Homodimeric EmrE Is Genuine

Our data clearly show two distinct and similarly abundant populations of Glu14 (Glu I and Glu II) are observed. A number of controls were carried out to exclude that the observed Glu14 populations were caused by sample preparation problems such as impurities, incorrect 13C labeling, aggregation, or sample disintegration.

EmrE E25A preparations contain no protein contamination as judged by MALDI-TOF mass spectrometry and SDS-PAGE (Fig. 1). No isotope scrambling took place as verified by analytical NMR analysis (supplemental Fig. S1) and as judged by the spectral pattern characteristic for glutamic acid (Fig. 5).

Aggregation could be excluded by freeze fracture microscopy, which was performed after NMR measurements and detected no signs of protein aggregation (Fig. 2). Finally, sample deterioration, due to e.g. radiofrequency irradiation, can be excluded because the spectra of frozen samples did not change with time (supplemental Fig. S2). Results were reproducible as judged by additional, independent sample preparations (supplemental Fig. S2).

The Glu14 Residues of Different Protomers in the Homodimeric EmrE Binding Pocket Are Asymmetric

Possible reasons for the genuine inequivalence of Glu14 could be (a) freeze trapping of general conformational flexibility, (b) freeze trapping of conformations that are in dynamic exchange, (c) a protonation/deprotonation equilibrium, or (d) a structurally asymmetric dimer.

Freeze Trapping of Thermal Motions Causes Increased Line Widths—At least two sets of chemical shifts are found for all glutamate nuclei with chemical shift differences between +1.9 ppm (Glu I Ca versus Glu I Ca + EtBr) and −2.8 ppm (Glu II Cy versus Glu II Cy + EtBr) (see Fig. 6). Peak doubling has been observed previously by solid state NMR and structural signifi-
that the side chains within the homodimer are found in different chemical environments. They are inequivalent.

Based on this evidence we conclude that the peak splitting is genuine and caused by the asymmetry of functional EmrE E25A dimers in \textit{E. coli} lipid liposomes at pH 8.0. This conclusion is also supported by the similar intensities of both Glu I and Glu II.

\textbf{Interpretation of Glu\textsuperscript{14} Line Shape and Chemical Shifts}

The symmetric line shape of EmrE E25A \textit{C}\textit{a} could be fitted best by two peaks of similar integral intensity (Fig. 4d). The line widths are most likely caused by residual structural heterogeneity trapped by sample freezing, as discussed above. The chemical shifts belonging to these two Glu\textsuperscript{14} populations show significant differences (Table 1). The chemical shifts of both backbone carbons differ by 4.9 ppm for \textit{C}\textit{a} and 3.7 ppm for \textit{C}\textit{r}. The chemical shifts of these backbone atoms are mainly influenced by the backbone secondary structure and neighboring residues. In the EmrE homodimer, both Glu\textsuperscript{14} residues have the same preceding and proceeding amino acid, so the differences must be caused by slightly different backbone conformations.

Three liquid state NMR studies on SMR proteins (EmrE and Smr) have been reported before (57–59). All studies used non-native membrane mimicking conditions such as chloroform/methanol for monomeric EmrE (59), lysopalmitoyl phosphatidylglycerol micelles for dimeric Smr (58), or biPC (1,2-di-O-tetradecyl- and 1,2-di-O-hexyl-sn-glycero-3-phosphocholine spiked with dihexanoylphosphatidylserine) bicelles for dimeric SMR (57). Interestingly, no asymmetry was observed and the reported \textsuperscript{13}C chemical shifts of Glu\textsuperscript{14} deviate from our measurements up to 8.4 ppm (see supplemental Fig. S3). This underlines the importance of a native lipid environment and supports arguments that the dimer only assumes an asymmetric conformation in its active state within the membrane (22, 47, 54, 60).

\textbf{Interpretation of Glu\textsuperscript{14} Line Shape and Chemical Shifts upon Substrate Addition}

Upon substrate addition the \textit{C}\textit{a} line shape becomes asymmetric and had to be fitted by four peaks. The line widths of both main peaks Glu I and Glu II was considerably reduced, which is most likely caused by reduced thermal motions in the presence of the substrate EtBr. After addition of the substrate EtBr, the transporter should be in equilibrium exchange conformations. These conformations include an open state of EmrE E25A, an exchange intermediate, and one release position. Therefore, additional peaks would be expected as seen in Fig. 4b. Not all of these resonances are populated equally possibly due to the different residence times for each state and an unsaturated binding pocket. However, the EtBr concentration was chosen to theoretically provide greater than 99\% occupancy at the binding site.

It is not possible to unequivocally assign both Glu\textsuperscript{14} populations Glu I and Glu II in EmrE E25A to both main populations found in the presence of substrate. Assuming that the more shielded Glu I population is the same in both situations, changes in chemical shifts can be discussed. Only with this assignment are realistic chemical shift changes obtained.

The differences between Glu\textsuperscript{14} chemical shifts without and with EtBr are summarized in Fig. 6. Changes of up to \textasciitilde2.8 ppm are observed. Upon substrate binding both glutamate C\textit{r} chemical shifts change toward values indicative of protonation or long-range electrostatic interactions. The C\textit{\beta} and C\textit{\gamma} chemical shifts concertedly shift upfield supporting the notion that the chemical environment of both Glu\textsuperscript{14} residues within the asymmetric homodimer changes. We suggest that both glutamates coordinate EtBr giving rise to two different EtBr/glutamate interactions and therefore different chemical shifts.

\textbf{An Asymmetric Binding Pocket of EmrE in the Context of Known EmrE Data}

Asymmetry has been reported for SMR proteins before. A subgroup of EmrE-like SMR proteins (YdgE/F (61, 62), yvdr/S, ykkK/D (62), and EbrA/EbrB (63–65)) has been found to act as hetero-oligomers with a defined topology for each molecule. Such hetero-oligomers are obviously asymmetric at the glutamate residue and for EbrA/B it could be shown that the two glutamates are functionally inequivalent (63). Additionally, EmrE can be manipulated to force it into a unique and defined topology. A dimer produced this way to yield one C\textit{n}, and one C\textit{oom} protomer showed functional inequivalence of the Glu\textsuperscript{14} residues (14).

Independently, EPR spectroscopy on EmrE in solution revealed a heterogeneous conformational state at Glu\textsuperscript{14} and an asymmetric dimer was seen in the EM structure (31, 66). It was postulated that the monomers take up a unique fold as seen by liquid state NMR and CD spectroscopy but upon dimerization each protomer assumes a different conformation. Such induced asymmetry has been previously described for human immunodeficiency virus, type-1 reverse transcriptase, hot dog thromesterase Paal, and for the trimeric multidrug transporter AcrB (3, 5, 7).

Our data show directly, that both Glu\textsuperscript{14} in the EmrE dimer are in a structurally inequivalent environment and coordinate substrate binding. This structural asymmetry could be consistent with both an antiparallel and parallel dimer topology.

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