In vitro synthesis of fully functional EmrE, a multidrug transporter, and study of its oligomeric state

Yael Elbaz*, Sonia Steiner-Mordoch*, Tsafi Danieli†, and Shimon Schuldiner*‡

1Protein Expression Facility, Wolfson Foundation Center for Applied Structural Biology, and *Alexander Silverman Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel

Edited by H. Ronald Kaback, University of California, Los Angeles, CA, and approved December 11, 2003 (received for review October 9, 2003)

EmrE is a small multidrug transporter from Escherichia coli that provides a unique model for the study of polytopic membrane proteins. Here, we show its synthesis in a cell-free system in a fully functional form. The detergent-solubilized protein binds substrates with high affinity and, when reconstituted into proteoliposomes, transports substrate in a ΔμH+ -dependent fashion. Here, we used the cell-free system to study the oligomeric properties of EmrE. EmrE functions as an oligomer, but the size of the functional oligomer has not been established unequivocally. Coexpression of two plasmids in the cell-free system allowed demonstration of functional complementation and pull-down experiments confirmed that the basic functional unit is the dimer. An additional interaction between dimers has been detected by using crosslinking between unique Cys residues. This finding implies the existence of a dimer of dimers.

Experimental Procedures

Mutagenesis. The construction and characterization of the mutants used was as described; we used E14C-His in wild-type background (7), K22C in wild-type background (22), and CLA-His (23). The mutants are named as follows: single amino acid replacements are named with the letter of the original amino acid, followed by its position in the protein and the letter of the new amino acid.

In Vitro Protein Synthesis. Protein was synthesized by using the rapid-translation system 100 E. coli HY kit (Roche Diagnostics), according to the manufacturer’s instructions except that the volume of each reaction was 20 μL. For specific experiments (reconstitution and size-exclusion chromatography), the synthesis was carried out by using the rapid-translation system 500 E. coli HY kit. Radiolabeling of EmrE was achieved by addition of 1–3 μCi (>1,000 Ci/mmol; 1 Ci = 37 GBq) of [35S]methionine (Amersham Biosciences).

Methyl Viologen Uptake and Tetraphenylphosphonium (TPP+) -Binding Assays. Uptake of [14C]methyl viologen (11.9 mM/mmol; Sigma) into proteoliposomes was assayed as described (3) by using 200

© 2004 by The National Academy of Sciences of the USA

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DDM, N-dodecyl-β-maltoside; TPP+, tetraphenylphosphonium; Ni-NTA, Ni2+-nitrilotriacetic acid; o-PDM, o-phenylene dimaleimide.

†To whom correspondence should be addressed. E-mail: shimon.schuldiner@huji.ac.il.

www.pnas.org/cgi/doi/10.1073/pnas.0306533101

© 2004 by The National Academy of Sciences of the USA
ng of EmrE-His per assay. TPP⁺ binding was assayed as described (4). After the in vitro synthesis reaction was completed, the reaction mixture (20 μl) was diluted in 200 μl of 0.08% DDM/Na buffer and the His-tagged protein was immobilized on Ni²⁺-nitritotriacetic acid (Ni-NTA) beads (Qiagen, Hilden, Germany). The beads were washed with 0.08% DDM/Na buffer containing 25 mM imidazole, suspended in 200 μl of 0.08% DDM/Na buffer containing 5 mM [³H]TPP⁺ (27 Ci/mmol; Amersham Biosciences), and incubated for 15 min at 4°C. In each experiment, the values obtained in a control reaction with 25 μM unlabeled TPP⁺ were subtracted. All binding reactions were performed in duplicate.

When inhibition of binding was tested, TPP⁺ binding was done in the absence and presence of the following substrates: ethidium bromide (50 μM), acriflavine (50 μM), and benzalkonium (0.5 μM).

Pull-Down Experiments. A 20-μl sample of the reaction volume, supplemented with [³⁵S]methionine, was diluted in the same buffer to a final volume of 200 μl, added to Ni-NTA beads washed as described above, and incubated overnight at 4°C. The protein that bound to beads was washed three times in 0.08% DDM/Na buffer containing 25 mM imidazole. The bead fraction was then incubated for 15 min at room temperature with sample buffer (100 mM Tris·Cl, pH 6.8/4% SDS/0.2% bromophenol blue/20% glycerol/200 mM 2-mercaptoethanol) plus 450 mM imidazole to release the bound protein from the beads. The radioactive protein was detected as described above and analyzed digitally with IMAGEGAUGE 3.46 software (Fujifilm).

Crosslinking with o-Phenylene Dimaleimide (o-PDM). When the protein synthesis reaction, supplemented with [³⁵S]methionine, was completed, the reaction was dia lyzed against 0.08% DDM/Na buffer overnight. o-PDM was then added to a final concentration of 400 μM, and the reaction was stopped with 15 mM β-mercaptoethanol after 1 h at room temperature. The reaction mixture was then loaded on washed Ni-NTA beads and incubated for 1 h at 4°C. Bound protein was eluted from beads, separated by SDS/PAGE, and visualized as described above. In control experiments, o-PDM (400 μM) was added to the protein already bound to the beads or after it was eluted with 0.08% DDM/Na buffer containing 150 mM imidazole.

Results

Localization of in Vitro-Synthesized EmrE and the Effect of Detergents. EmrE, a polytopic membrane protein, was synthesized in vitro by using a transcription–translation system. The obtained amounts are easily detectable in SDS/PAGE (Fig. 1A, lanes 3 and 4) and are similar to the amounts of the control reaction of GFP synthesis (Fig. 1A, lanes 1 and 2). We estimated the amount of EmrE synthesized to be ~1–2 μg in the miniscule reaction (20-μl reaction volume). In a larger reactor system of 1 ml (rapid-translation system 500 E. coli HY kit), 2–3 mg of EmrE were synthesized (data not shown). Surprisingly, EmrE was expressed at similar amounts with or without the addition of detergent (Fig. 1B, lanes 1 and 2). However, the addition of detergent affected the localization of the protein. When EmrE was synthesized in the presence of detergent (0.08% DDM), it was localized exclusively in the soluble fraction (Fig. 1B, lane 3), whereas when synthesized without any addition to the reaction mixture provided by manufacturer, it was found within the insoluble fraction only (Fig. 1B, lanes 4 and 5). These results lead us to the conclusion that in vitro-synthesized EmrE is inserted into a particulate fraction, present in the crude lysate, from which it can be removed by addition of detergent. We suggest that this is a membrane fraction because analysis by SDS/PAGE and Coomassie blue staining of this fraction reveals a large number of proteins associated with it that are solubilized with the low concentrations of mild detergents that solubilized EmrE (data not shown).

Screening of the effect of different detergent concentrations showed no significant effect on expression of EmrE, at detergent concentrations up to 0.08% DDM and 0.4% N-octyl-β-D-glucopyranoside. In the presence of higher detergent concentrations, as high as 2% DDM and 1% N-octyl-β-d-glucopyranoside, expression was significantly lower. In all cases, regardless of the presence or absence of detergent, EmrE was functional, as revealed by the assays described below.

Expression of in Vitro-Synthesized Homologous Membrane Proteins. To check whether other membrane proteins could be synthesized in vitro by using the same system, we tested expression of four other transporters that are homologues of EmrE. TBsmr, BPsmr, Psmr, and Hsmr were previously purified and characterized (20, 21). As shown in Fig. 1C, the three bacterial transporters (TBsmr, BPsmr, and Psmr) were expressed successfully, whereas
Hsmr, an archaeal multidrug transporter that was expressed successfully in *E. coli* BL21 cells, was not expressed to detectable levels in this *in vitro* system. Relative evaluation of *in vivo* versus *in vitro* expression levels of these three proteins shows that PsMr seems to express better *in vitro* than *in vivo*, whereas BPsMr demonstrated the opposite pattern. TBsMr showed high expression levels when expressed in either system.

**The Synthesized Protein Is Fully Functional.** To test whether the *in vitro* reaction yielded a functional protein, we assayed its ability to bind TPP⁺, a high-affinity substrate of EmrE (4). *In vitro*-synthesized EmrE binds [³H]TPP⁺ as well as EmrE obtained from *E. coli* membranes, with an affinity (Kₐ = 2.3 nM) very similar to that of the wild-type protein (23). In addition, [³H]TPP⁺-binding assays in the absence and presence of different substrates showed that *in vitro*-synthesized EmrE has the same substrate specificity range as EmrE obtained from *E. coli* membranes. Thus, ethidium, acriflavine, and benzalkonium inhibit TPP⁺ binding to native and *in vitro* EmrE at the same concentration range (Fig. 2A). When synthesized either in the presence or absence of any additional detergent, EmrE proved to be functional and showed, in any case, the ability to bind TPP⁺.

To test the ability of the protein to transport substrate across a membrane, synthesized EmrE was reconstituted into proteoliposomes and methyl viologen uptake was measured. As shown in Fig. 2B, the *in vitro*-synthesized protein catalyzes accumulation of methyl viologen against its concentration gradient. Transport depended on the H⁺ gradient generated across the proteoliposomes because nigericin, an ionophore that collapses the pH gradient, fully inhibited accumulation (data not shown). The rate of uptake was 7.5 nmol/min per µg of EmrE, very similar to the rates reported for the native protein (20).

We conclude that the protein synthesized in this system is fully active because it binds and transports with kinetic constants practically identical to those of the native one. In addition, the detergent-solubilized *in vitro*-synthesized EmrE was analyzed in a size-exclusion column (Superdex 200, Amersham Biosciences) and eluted as a single peak with an apparent molecular mass (150 kDa in a 0.08% DDM solution) identical to that of the native EmrE (data not shown). Therefore, the *in vitro* system provides an experimental paradigm to study various properties of EmrE.

**Coexpression of Plasmids and Pull-Down Experiments.** We used two plasmids, coding for His-tagged and -untagged proteins (Fig. 3A, lanes 1 and 2, respectively), as template in the same reaction to express both proteins simultaneously (lane 3). To test whether both proteins interact to form an oligomer, tagged and untagged proteins were coexpressed and then immobilized on Ni-NTA beads. Because only His-tagged monomers bind to the Ni-NTA beads (Fig. 3B, lane 1 compared with lane 2), only the untagged monomers that interact with tagged ones were pulled down and detected after separation by SDS/PAGE (lane 3).

Negative-dominance studies have suggested that the oligomer is the functional unit (7). More recent studies have strongly supported this suggestion, by showing that inactive mutant subunits are functionally complemented when mixed with wild-type subunits (6). This finding provided us an assay to examine whether the physical interactions between EmrE subunits described above have functional consequences also. We, therefore, coexpressed tagged E14C-His and untagged EmrE *in vitro*. The product of the reaction was immobilized on Ni-NTA beads and washed so that only His-tagged protein, or protein associated with it, would remain bound to the beads. As shown (4), the tagged E14C-His (Fig. 4, A) is inactive and does not display any TPP⁺-binding activity. However, when coexpressed with untagged wild-type protein, TPP⁺-binding activity is restored (Fig. 4, B). We conclude that the two monomers interact with each
other to form heterooligomers in which wild-type subunits functionally complement the inactive mutant. This in vitro heterooligomer showed affinity to TPP$^+$ ($K_d = 31$ nM) lower than that of the wild-type protein ($K_d = 2$ nM). This affinity is very similar to that of the heterooligomer that was generated by monomer swapping in previous studies (6).

Establishing that monomers interact to form oligomers during in vitro synthesis provided a new tool to examine the oligomeric state of EmrE further. A clear advantage of this system is that the ratios of both species can be manipulated easily and accurately. We coexpressed in vitro EmrE-His and EmrE at different DNA ratios and determined two factors for each reaction: the ratio of total EmrE: EmrE-His synthesized observed immediately at the end of the reaction and the same ratio observed after the pull-down assay (Fig. 5A). Increase in the amount of DNA coding for untagged EmrE brought about a corresponding increase in the amount of EmrE synthesized (Fig. 5A). Excess in EmrE DNA has a deleterious effect on the synthesis of EmrE-His in the same reaction, probably because of competition and maximum capacity of the system. Pull down of untagged EmrE was fully dependent on the presence of EmrE-His. The amounts of pulled-down EmrE decreased with the increase in the amount of DNA coding for EmrE because of the decrease in the amount of EmrE-His (Fig. 5A). The ratio of pulled-down EmrE to EmrE-His was quantitated as a function of the total amount synthesized of both species, and the results are shown in Fig. 5B. Our results confirm that EmrE forms an oligomer. The EmrE/EmrE-His ratio after pull down increases with the total EmrE/EmrE-His ratio (Fig. 5B). The solid line in Fig. 5B is the theoretical ratio to be expected from a randomly forming dimer. As seen in the initial part of the curve, the ratio tends to be lower than expected, suggesting a certain preference for homodimer formation. Furthermore, at the higher EmrE/EmrE-His ratios, the pulled-down product seems in most cases to have more untagged protein than expected from a dimer. Although there is some variability, the majority of the experimental points are above the expected ratio. We suggest that there is a high-affinity interaction to form a dimer that withstands extensive washes of the bead-bound protein and a weaker interaction that cannot be fully detected because of the technical limitations in the design of our experiments.

The findings described above led us to consider the possibility of two oligomer populations that exist in equilibrium: the basic dimer unit and a higher-degree oligomer formed, possibly, by two dimers. Efforts were made to detect the population with a lower affinity by lowering the stringency of the washes (smaller volumes of buffer and lower imidazole concentration) to no avail. Therefore, to test the above contention, we designed an experimental protocol by using irreversible crosslinking between monomers. We have shown (9) that o-PDM, which is a dimaleimide crosslinker, reacts with single Cys residues engineered in the EmrE monomer and generates dimers. Among the residues that crosslinked, we chose to focus here on K22C, a replacement in a hydrophilic, relatively long, and exposed loop. In the following experiments, the tagged EmrE is without Cys and, therefore, does not crosslink with o-PDM (9). The untagged protein contains a single Cys residue and can crosslink with another untagged protein in a different dimer (Fig. 6B). We coexpressed in vitro untagged K22C (in background without Cys) and tagged CLA-His, in a reaction in which K22C was in excess to optimize the formation of heterodimers of CLA-His:K22C (Fig. 6A, lane 1). When in vitro protein synthesis was completed,
the reaction was damped to stop it and to remove reducing agents, and then it was subjected to α-PDM crosslinking before immobilization on beads. As seen after SDS/PAGE analysis, when comparing samples treated with α-PDM (Fig. 6, lane 2) to a control sample (Fig. 6, lane 3), the amount of tagged CLA-His does not change. The amount of untagged K22C monomer, in a control sample (Fig. 6, lane 3), the amount of tagged CLA-His in the purified (Fig. 6, lane 4) preparation, demonstrating the high specificity of the reaction. In this context, it is noteworthy also that, under all of the conditions tested, the crosslinking reaction was inhibited when the reaction was performed in the presence of a denaturant, such as SDS (data not shown). Therefore, we conclude that the crosslinking reported here reflects a specific interaction between dimers.

Discussion

In this article, we present the in vitro synthesis of a fully functional polytopic membrane protein, EmrE, a well characterized multidrug transporter from E. coli. The amounts synthesized are large enough for biochemical and structural studies. EmrE is synthesized in the presence or absence of detergents. In the latter case, it inserts into a membrane-like fraction from which it can be solubilized at low concentrations of detergents, such as DDM. In vitro-synthesized EmrE shows TPP⁺-binding properties similar to those of the in vivo EmrE, and it has the same substrate specificity. In addition, the in vitro-synthesized EmrE was reconstituted in proteoliposomes, and it shows ΔμH⁺-dependent accumulation of substrate.

The ability to synthesize a functional protein in vitro provides a tool to numerous options for further research, including specific labeling of amino acids and incorporation of amino acids homologues. Here, we describe one of its applications, namely, the study of oligomerization of membrane proteins.

Negative-dominance studies, crosslinking, ligand binding, and heterooligomer formation all confirm that EmrE functions as an oligomer (4, 6, 7, 9, 25). A projection structure of EmrE determined by cryo-electron microscopy of two-dimensional crystals showed that the repetitive unit in the crystal was composed of eight α-helices arranged in an asymmetric manner, indicating that the minimal functional unit for substrate binding is a dimer (8, 25). Available data do not preclude the existence or functionality of higher oligomers. Our original ligand-binding measurements indicated a stoichiometry of about three monomers per bound substrate (4). These data were challenged, and it was suggested that in our measurements performed at very low EmrE concentrations, part of the population is in the monomeric form and, thereby, inactive in the binding assay (25). In our findings, however, the stoichiometry is three monomers per TPP⁺ bound as reported and does not change with the EmrE concentration (M. Soskine and S.S., unpublished data). The simplest interpretation is that the protein functions as a trimer. However, this interpretation does not agree with the structural data mentioned above (8, 25). One possible explanation for the ligand stoichiometry that we find is the equilibrium between a dimer and a tetramer, the latter form binding only one TPP⁺ at a time. However, the functional implications of tetramer formation still remain to be elucidated.
The functional complementation demonstrated in this work implies also that the presence of two Glu-14 residues per dimer is not an essential requisite for full activity. The formed hetterooligomer binds TPP⁺ with lower affinity in a pH-dependent mode (i.e., binds and releases protons with a PK in a similar range). This finding suggests that all Glu-14 residues in the oligomer are equivalent and that the whole set improves the affinity.

The EmrE dimers are very stable in the detergent-solubilized preparations, and dissociation was achieved only after exposure to high temperature or strong denaturing agents (20). This strong affinity of EmrE monomers turns out to be a major advantage in this system that allows the use of the pull-down assay. Very extensive washes of the heterooligomers do not lead to high temperature or strong denaturing agents (20). This strong affinity.

A fully functional polytopic membrane protein. This system