Negative Dominance Studies Demonstrate the Oligomeric Structure of EmrE, a Multidrug Antiporter from Escherichia coli*

(Received for publication, August 2, 1996, and in revised form, September 23, 1996)

Hagit Yerushalmi, Mario Lebendiker, and Shimon Schuldiner‡

From the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

---

EmrE, the smallest known ion-coupled transporter, is an Escherichia coli 12-kDa protein 80% helical and soluble in organic solvents. EmrE is a polypeptide antiporter that exchanges hydrogen ions with aromatic toxic cations such as methyl viologen. Since it is many times smaller than the classical consensus 12 transmembrane segment transporters, it was particularly interesting to determine its oligomeric state. For this purpose, a series of nonfunctional mutations has been generated and characterized to test their effect on the activity of the wild-type protein upon mixing. As opposed to the wild-type, these mutants do not confer resistance to methyl viologen, ethidium bromide, or a series of other toxicants. Co-expression of each of the nonfunctional mutants with the wild-type protein results in a reduction in the ability of the functional transporter to confer resistance to several toxicants. To perform mixing experiments in vitro, all the mutants have been purified by extraction with organic solvents, reconstituted in proteoliposomes, and found to be inactive. When co-reconstituted with wild-type protein, they inhibit the activity of the latter in a dose-dependent form up to full inhibition. We assume that this inhibition is due to the formation of mixed oligomers in which the presence of one nonfunctional subunit causes full inactivation. A binomial analysis of the results based on the latter assumptions do not provide statistically significant answers but suggests that the oligomer is composed of three subunits. The results described provide the first in vitro demonstration of the functional oligomeric structure of an ion-coupled transporter.

A great diversity of multidrug transporters are known to us today. They actively remove a wide variety of toxicants in an energy-dependent process and thereby decrease the concentration of the offending compounds near their target. We can group these transporters into several different families based on structure similarities (1–5). A unique family (Smr or MiniTEXANS) is represented by very small proteins, about 100 amino acids long, that render bacteria resistant to a variety of toxic cations (6, 7).

Two MiniTEXANs, Smr (8, 9) and EmrE (10) have been characterized, purified, and reconstituted in a functional form. Both proteins catalyze H+/cation antiport in proteoliposomes reconstituted with purified transporter, and they are capable of recognizing a wide range of inhibitors and substrates. In addition, EmrE has been shown to display unique properties of solubility in organic solvents such as a mixture of chloroform and methanol (10). After solubilization in the above solvent, the protein retains its ability to transport, as judged from the fact that it can be reconstituted in a functional form. Using transmission FTIR and oriented attenuated total reflection-FTIR spectra, the protein was found to be a 4-membered transmembrane antiparallel helical bundle. The helices in EmrE are oriented perpendicular to the lipid bilayer with a tilt angle of 27° with respect to the bilayer normal (11). Residues in the substrate translocation pathway have been identified with thiol reactive substrates (12).

Although it is evident that EmrE is an ion-coupled transporter, it is three to eight times smaller than the classical 12 transmembrane segments (TMS)

consensus. Thus, the question whether this small protein can function as a monomer was raised and is addressed in this work. A successful approach, used with channel proteins such as Shaker (13), MiniK (14), and others, is based on mixing experiments in which inactive subunits or subunits with modified properties were mixed with the wild-type protein at various ratios. The results suggested the formation of oligomers and allowed for an estimate of its size.

In this work, evidence is presented that EmrE functions as a homooligomer. The effect of several inactive mutants on the activity of the wild-type protein was tested in a mixing approach both in vitro and in vivo. In the in vivo experiments, various mutants were coexpressed with the wild-type protein and shown to significantly decrease the ability of the latter to confer resistance to various toxicants. To further substantiate this finding, the mutant proteins were partially purified and co-reconstituted with wild-type EmrE. The nonfunctional protein has a negative dominant effect over the functional one manifested by a full inhibition of transport activity. The results demonstrate that EmrE functions as a homooligomer.

---

**Experimental Procedures**

**Bacterial Strains and Plasmids—Escherichia coli JM109 (15) and TA15 (16)** are used throughout this work. The plasmids used are pKK56 (10), pT7-32 (10), pSN1,2 and pGP1 (17). In plasmid pKK56, EmrE is cloned into EcoRI and HindIII sites of pKK223-3 (Pharmacia Biotech Inc.), and in plasmid pT7-32, EmrE is cloned into NdeI and HindIII sites of pT7-7. pSN1 is a derivative of pACYC184 in which the BamHI-HindIII insert of pKK56 was transferred into the corresponding sites of the original vector. pSN1 and pKK56 share the same promoter, but they are compatible so that they can be used to co-express two different proteins in the same cell.

**Mutagenesis—** Mutants were obtained by polymerase chain reaction mutagenesis using the overlap extension procedure described by Ho et al. (18). For most of the mutations, a set of two overlapping oligonucleotide primers containing the desired mutation were constructed. The outside primers were those used for the wild-type EmrE (10). In one of the mutations (E14C), only two outside primers were used, and one of them contained the desired mutation. Mutagenic oligonucleotides were prepared incorporating a unique

---

*This research was supported by a grant from the Israel Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 972-2-6585992; Fax: 972-2-634625; Email: shimon@leonardo.ls.huji.ac.il.

1 The abbreviation used is: TMS, transmembrane segments.
2 S. Ninio, unpublished data.
restriction site to facilitate mutant identification, and in several cases, this was an additional conservative mutation that did not affect EmrE expression. Mutated DNA was identified by the acquisition of the new restriction site and sequenced to ensure that no other mutations occurred during the amplification process (T7 Sequencing™ Kit, Pharmacia).

The primer 5'-CGGAATTCATATGAACCCTTATATTTATCT TGGT-GTGGCATATCTTGCATGTGCTATTGG was used to obtain the E14C mutant. In this case, the unique MstI site in the wild type is abolished. 5'-GCTATGCTATCTTGCAGATTGG with the unique MboII site yielded W63F mutant; 5'-CAGGGATTGCAATCGCTATGCTGC, with the unique BsmI site and a conservative change in Ala-59, was used to give Y60F mutant. In all the mutations, wild-type DNA was used as a template.

**Resistance to Toxic Compounds**—For testing resistance to toxic compounds, cells were grown at 37 °C in LB medium with different concentrations of the compound, and cell density was estimated from absorbance at 600 nm (A600).

**Overexpression and Specific Labeling of EmrE**—pT7-32, which contains the T7 polymerase promoter phi10 and the translation start site for the T7 gene 10 protein, was used for labeling EmrE with [35S]methionine essentially as described previously (10). pT7-32 was transformed into TA15 carrying GPPl-2 (17). Transformants were grown at 30 °C in minimal medium supplemented with thiamin (2.5 μg/ml), ampicillin and kanamycin (50 μg/ml), and 0.5% glucose to a cell density of 0.6 A600. The temperature was then increased to 42 °C to induce the T7 polymerase; 15 min later, rifampicin (200 μg/ml) was added and incubation continued for an additional 10 min. Then the culture was shifted back to 30 °C for 60 min. [35S]methionine (specific activity of 1350 Ci/mmol) was added to the cell suspension (10 μCi/ml) and incubation continued for an additional 40 min. Cells were collected by centrifugation, washed with a solution containing 20 mM Tris-Cl, pH 7.5, and 150 mM NaCl, and sonicated 6 times for 10 s using a probe-type sonicator. Undissociated cells were removed by centrifugation, and the membranes were then collected by further centrifugation at 435,000 × g for 15 min. The membrane pellet was resuspended in the above buffer, frozen in liquid air, and stored at −70 °C.

For overexpression, _E. coli_ JM109/pKK56 was grown in minimal medium A with 0.5% glycerol, thiamin, and ampicillin as above. When cells reached an A600 = 1.0, isopropyl-1-thio-β-p-galactoside was added to 0.5 mM; 2 h later, the cells were harvested by centrifugation. After washing, cells were resuspended in buffer containing 10 mM Tris-Cl, pH 7.5, and 150 mM NaCl, and sonicated 23 times for 10 s using a probe-type sonicator. Undissociated cells were removed by centrifugation, and the membranes were subsequently treated with 23 mM dithiothreitol, 2.5 mM MgSO4, and 150 μg/ml DNase I and sonicated 7 times for 10 s using a probe-type sonicator. The supernatant was removed by centrifugation, and the membranes were then collected by further centrifugation at 435,000 × g for 15 min. The membrane pellet was resuspended in the above buffer, frozen in liquid air, and stored at −70 °C.

**Purification and Reconstitution of EmrE**—EmrE was extracted essentially as described (10) from 1 ml of membrane (10 mg of protein) with 23 ml of chloroform:methanol, 1:1. After an incubation of 20 min on ice, 4.6 ml of water were added for phase separation, and the suspension was centrifuged. The upper phase and the interphase were removed, and the lower phase with the enriched EmrE was stored at −70 °C, for analysis of SDS-polyacrylamide gel electrophoresis, a sample was dried, resuspended in sample buffer, and analyzed in 16% tricine gels as described (19).

For reconstitution, protein (0.6–7 μg) in the organic solvent was mixed with 18 μl of _E. coli_ phospholipids (50 μg/ml) and 180 μl chloroform:methanol, 1:2. The suspension was dried under argon and resuspended in a solution (60 μl) containing 0.19 mM NH₂Cl, 0.015 mM Tris-HCl, pH 6.9. The suspension was frozen and kept at −70 °C. Before the assay, the proteoliposome suspension was thawed and sonicated in a bath-type sonicator for a few seconds until clear.

**Transport Assay**—Transport of [14C]methyl viologen into proteoliposomes was assayed by dilution of 3 μl of the ammonium chloride containing proteoliposomes into 200 μl of an ammonium-free medium containing 25 μM [14C]methyl viologen (60 nCi/assay), 140 μM KC1, 10 mM tricine, and 5 mM MgCl₂, pH 8.5. At given times, the reaction was stopped by dilution with 2 ml of the same ice-cold solution without the radioactive substrate. After filtering through Schleicher & Schuell filters (0.2 μm), and washing with an additional 2 ml of solution. The radioactivity on the filters was estimated by liquid scintillation. In each experiment, the background value was obtained in a control reaction with 5 μM nigericin.

In _Vivo_ Co-expression—pKK56 plasmids that contained mutated EmrE were transformed into JM109 cells carrying pSN1, a derivative of the pACYC184 vector with wild-type EmrE. Resistance of the transformants to toxic compounds was tested as described above.

**In Vitro Co-reconstitution**—Proteoliposomes were prepared from wild-type and mutated EmrE using different protein ratios as indicated in the specific experiments. The amount of EmrE is quantitated by visualization in Coomassie Blue-stained SDS gels in which known amounts of pure EmrE are used for calibration. Concentration of pure EmrE is determined by a modification of Peterson (20). Transport of [14C]methyl viologen into the proteoliposomes was assayed as described before.

**RESULTS**

**Generation of Inactive Mutants of EmrE**—To generate inactive mutants, residues conserved in all the MiniTEXANS were mutagenized. In addition, the residues chosen were previously shown to play an important role in the homologous Smr protein, as judged from the loss of activity upon mutagenesis (8). Two types of residues were chosen: Glu 14, a charged residue in TMS 1; and Tyr 60 and Trp 63, two aromatics in TMS 3. As expected, the three mutants are inactive. Function was tested in intact cells as the ability to confer resistance to methyl viologen (0.2 mM) in solid media (not shown) and ethidium (600 μM) in liquid media. Cells transformed with wild-type EmrE are capable of growing under the above conditions (Fig. 1, A and C) at rates comparable with those in the absence of toxicants (Fig. 1A). However, none of the mutant proteins can confer resistance to either of the toxicants mentioned above at the given concentrations (Fig. 1, B and C). To test whether the mutant proteins are synthesized to similar levels, they were extracted with organic solvents and visualized with Coomassie Blue after SDS-polyacrylamide gel electrophoresis. E14C and Y60F are expressed to levels similar to those of the wild type, as judged from this criterion. The ex-
none of the mutants displayed any activity (Fig. 2).

In addition, none of the mutants could catalyze downhill efflux of [14C]methyl viologen from preloaded liposomes (data not shown). These experiments demonstrate that the inhibition of wild-type protein by the mutant proteins, reconstituted into proteoliposomes, is caused by the active transport catalyzed by the wild-type subunits, rather than the generation of some nonspecific leak across the membrane.

**Negative Dominance in Vitro**—Because the conclusions from in vivo experiments may be ambiguous and difficult to quantitate, EmrE and the various mutant proteins were purified and mixed in vitro as well. The proteins were overexpressed and extracted with organic solvents from membrane preparations of the corresponding strains. The purified proteins were then mixed and reconstituted as described previously (12), and transport of [14C]methyl viologen into the proteoliposomes was then assayed. To minimize possible artifacts the mixing was done in two different ways. In the first one, given amounts of inactive mutant protein were added to fixed amounts of wild type (Fig. 3). In another, the total amount of protein was kept constant, and the ratio between the wild type and the mutants was modified (Fig. 4).

The results of experiments in which the concentration of wild-type protein in the reconstituted proteoliposomes was always constant (30 ng/μl) are described in Fig. 3. The effect of the addition of increasing amounts of each of the three inactive mutant proteins is shown in panels A for mutant W63F, B for E14C, and C for Y60F. In the experiments shown in Fig. 3, A-C, three different concentrations of the mutant proteins were added (8, 30, and 120 ng/ml) so that their fractional concentration (mutant protein over total amount of protein) is 0.2, 0.5, and 0.8, respectively. Practically no inhibition was observed at the low mutant concentration. Inhibition was maximal at the highest concentration used and intermediate when the fraction of mutant protein was 0.5. It is concluded that each of the mutants has a deleterious effect on the transport catalyzed by the wild type, and the dose dependence of this inhibition is, in essence, quite similar.

The inactive proteoliposomes, reconstituted with 0.8 fractional concentration of the mutants, were also found incapable of catalyzing downhill efflux in experiments in which they were previously preloaded with [14C]methyl viologen (data not shown). These experiments demonstrate that the inhibition of transport is not due to the generation of some nonspecific leak across the membrane.

The simplest interpretation of the results described in this work is that the mixing brings about the formation of composite oligomers between the wild type and the mutant proteins. The results can be analyzed quantitatively assuming that the pres-
DISCUSSION

In this work, the oligomeric nature of EmrE has been demonstrated. EmrE is the smallest ion-coupled transporter known with only four putative transmembrane α-helical segments. Since most ion-coupled transporters are 12 TMS proteins, it has been claimed that this must be their minimal functional size, and it was therefore especially interesting to study the oligomeric nature of a 4 TMS protein.

One particularly difficult problem to resolve with hydrophobic membrane transport proteins is their functional oligomeric state. Because of their nature and the need to use detergents, biophysical and biochemical techniques do not necessarily provide us with information on the functional size of the transporter. Therefore, a genetic approach was used here.

In the in vivo studies, a clear and reproducible negative dominance effect was observed. However, these studies are limited because they may also reflect effects on plasmid copy number and protein expression and/or targeting to the membrane and because of the difficulty in manipulating the dose of each of the proteins. Since we have previously shown that a decrease of even 80% in the activity of some mutants is not necessarily reflected in the phenotype (12), it became necessary to develop an in vitro approach as well.

The in vitro studies are facilitated by the ease of purification and reconstitution of EmrE. They provide the advantage of a simple system that is easy to quantitate and control. To minimize possible artifacts due to competition of insertion into the proteoliposome, the experiments were performed in two modes. In one mode, inactive protein was added to fixed amounts of active EmrE, and a dose-dependent effect on activity was observed. These experiments were possible because of the linearity of the rate of transport observed under a wide range of protein concentrations (only part of the range was shown in Fig. 4B). This linearity was observed even in the very low protein range with a large excess of lipids over protein (1000:1, w/w) where it would have been possible, in theory, to observe an exponential relationship if more than a single monomer is required for active accumulation. The fact that an exponential relationship was not observed, however, can be due to the fact that the oligomers are maintained in the solvent system used or formed prior to reconstitution. Since the experiments described in this work demonstrate subunit mixing, we speculate...
that the oligomers form while concentrating the protein in the organic solvent, just prior to reconstitution.

In the other mode of the in vitro experiments, the total amount of protein was kept low and constant, and only the ratio between active and nonactive transporters was modified. In both modes, similar results were obtained.

An important aspect in the findings described is the fact that the negative dominance was observed with three independent mutants in which quite different replacements were made. In one, a negative charge in a putative TMS was replaced with an uncharged residue. In the others, two aromatic residues, tryptophan and tyrosine, were replaced. This fact increases the generality of the phenomenon and reduces the possibility of some nonspecific indirect inhibition. In this context, it should also be stressed that the inhibition of uptake is not due to the generation of some nonspecific leak across the membrane but also be due to the inhibition of uptake is not due to the presence of any of the possible mutant pairs either in vitro or in vivo. This finding may suggest that all the mutations are in the same part of the catalytic cycle and cannot supplant for each other. It may also hint that three copies of the appropriate residues must be present in order to have a functional oligomer.

The quantitative analysis to determine the size of the oligomer is confronted with two main problems. The technical one is due to the innate variability of the reconstitution assay of about 15%, which does not allow us to make a clear choice between two alternatives in which predicted activities do not differ by much more in most of the range. In addition, the theoretical analysis is based on the assumption that an oligomer containing one negative subunit is fully inactive. Support for this contention can be assumed from the following facts: the experimental points fit reasonably well to the theoretical curves, full inhibition of activity is obtained when enough mutant protein is added, and no positive complementation has been observed. Although the quantitative analysis does not enable to make a strict distinction between the dimer and the trimer model, a reasonable bias points to the latter. This propensity to choose the trimer model is due to the fact that, in the range in which the activity is reasonably high, the fit is best to the trimer model (Fig. 3D). The deviation is more pronounced in the range of low transport activity tested (high mutant concentration). Our bias rests also on the overwhelming consensus that a vast majority of the transporters known contain 12 putative TMS. The MiniTEXANs are a notable exception to the rule with a few others being 10 or 14 TMS (1, 2). Another interesting exception to the 12 TMS consensus is the 6 TMS adenine nucleotide translocator from mitochondria, which has been proposed to function as a dimer (21). Since the 12 TMS proteins are the vast majority and it would seem that this is the basic functional unit, it is interesting to speculate that at some time in evolution and under given conditions there must have been some kind of advantage to the larger proteins as opposed to the small and more rare ones. If so, it may be interesting to find out why some of the small ones survived evolutionary pressures. It is possible to speculate that one advantage of forming oligomers as compared with a larger monomer is the feasibility of mixing various subunits with slightly different properties that may very much broaden the range of functional possibilities, as is seen in the case of receptors for glutamate and other neurotransmitters in the nervous system of higher organisms (for example, see Ref. 22). In the same vein, we should raise some caution about the generality of the theory that all 12 TMS transporters must have evolved from a gene duplication of two halves (6 + 6 model (1)). This generalization may now require some further evaluation in light of the presence of 4 TMS transporters.

A genetic approach to study the oligomeric structure of ion-coupled transporters has been used in several cases. In the case of the β-galactoside transporter from E. coli, despite intensive studies and initial results that suggested dominance of some mutations (23), no genetic complementation has been found between point mutants (24). Complementation has been observed only between two transporters in which large deletions were engineered in different domains of the protein, as has been reported (25). This phenomenon has been interpreted as demonstrating that complementation can occur only in molecules in which the deletion leaves a gap in the structure that can be filled with another molecule containing the deleted segment (26). In the tetracycline resistance protein (TetB) two intracistronic complementation groups have been demonstrated corresponding to the N- and C-terminal halves of the transporter. The results have been interpreted as suggesting that the Tet proteins occur as oligomers (27). However, to the best of our knowledge, EmrE is the first ion-coupled transporter that has been shown to function as an oligomer using in vivo and in vitro approaches.

Acknowledgment—We thank Tal Almagor for performing some of the experiments.

REFERENCES

1. Marger, M., and Saier, M. (1993) Trends Biochem. Sci. 18, 13–20
2. Griffith, J., Baker, M., Rouch, D., Page, M., Skurray, R., Paulsen, I., Chater, K., Baldwin, S., and Henderson, P. (1993) Curr. Opin. Cell Biol. 4, 684–695
3. Doige, C. A., and Ames, G. F. L. (1993) Annu. Rev. Microbiol. 47, 291–319
4. Higgins, C. (1992) Annu. Rev. Cell Biol. 8, 67–113
5. Schuldiner, S., Shirvan, A., and Linial, M. (1995) Physiol. Rev. 75, 369–392
6. Grinius, L., Dregueine, G., Goldberg, E. B., Lian, C. H., and Projan, S. J. (1992) Plasmid 27, 119–129
7. Paulsen, I., Skurray, R., Tam, R., Saier, M., Turner, R., Weiner, J., Goldberg, E., and Grinius, L. (1996) Mol. Microbiol. 19, 1167–1175
8. Grinius, L. L., and Goldberg, E. B. (1994) J. Biol. Chem. 269, 29998–30004
9. Paulsen, I. T., Brown, M. H., Dunstan, S. J., and Skurray, R. A. (1995) J. Bacteriol. 177, 2827–2833
10. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1995) J. Biol. Chem. 270, 6586–6583
11. Arkin, L., Russ, W., Lebendiker, M., and Schuldiner, S. (1996) Biochemistry 35, 7233–7238
12. Lebendiker, M., and Schuldiner, S. (1996) J. Biol. Chem. 271, 21193–21199
13. MacKinnon, R. (1991) Nature 350, 232–235
14. Wang, K., and Goldstein, S. (1995) Neuron 14, 1303–1309
15. Yanish-Perron, C., Viera, J., and Messing, J. (1985) Gene 33, 103–199
16. Goldberg, E. B., Arbel, T., Chen, J., Karpel, R., Mackie, G. A., Schuldiner, S., and Padan, E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2615–2619
17. Tabor, S., and Richardson, C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
18. Ho, S. F., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 84, 35–46
19. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
20. Peterson, G. (1977) Anal. Biochem. 83, 346–356
21. Klugershen, M. (1999) J. Bioenerg. Biomembr. 25, 447–457
22. Hollmann, M., and Heineman, S. (1994) Annu. Rev. Neurosci. 17, 31–108
23. Mieschendahl, M., Buchel, D., Bocklage, H., and Muller-Hill, B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7652–7656
24. Wright, J. K., Weigel, U., Lustig, A., Bocklage, H., Mieschendahl, M., Muller-Hill, B., and Overath, P. (1983) FEMS Lett. 162, 11–15
25. Billing, E., and Kaback, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1524–1528
26. Kaback, H. (1995) Biochim. Biophys. Acta 1101, 210–213
27. Curiale, M. S., McMurry, L. M., and Levy, S. B. (1984) J. Bacteriol. 157, 211–217