Determining the Secondary Structure and Orientation of EmrE, a Multi-Drug Transporter, Indicates a Transmembrane Four-Helix Bundle†

Isaiah T. Arkin,‡,§ William P. Russ,§ Mario Lebendiker,|| and Shimon Schuldiner*,||

Howard Hughes Medical Institute and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, and Institute of Life Sciences, Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904 Israel

Received January 16, 1996; Revised Manuscript Received April 1, 1996⊗

ABSTRACT: EmrE is a member of a newly emerging family of MiniTEXANS, a family of multi-drug antiporters from bacteria characterized by their small size of roughly 100 amino acids. In this report we have obtained transmission FTIR spectra of EmrE in $CHCl_3$:MeOH, DMPC vesicles, and *Escherichia coli* lipid vesicles. Secondary structure analysis has shown that both in DMPC vesicles and in $CHCl_3$:MeOH the protein adopts a highly helical secondary structure that correlates remarkably well with that predicted by hydropathy analysis. The protein was shown to be resistant to amide proton H/D exchange, providing evidence that most of the protein is embedded in the lipid bilayer. Polarized ATR-FTIR spectra of the protein in DMPC vesicles have shown that the helices are oriented with an average tilt angle of 27° from the bilayer normal. The protein was found to be less oriented in *E. coli* lipid vesicles, most likely as a result of the poor orientation of the bilayer lipids themselves. Thus, the protein is identified as a transmembrane four-helix bundle providing valuable structural data for this family of multi-drug transporters. The results set the stage for further studies aimed at deriving a detailed model for this protein.

The process of extrusion of toxicants from microorganisms has attracted wide interest (Griffith et al., 1992; Paulsen et al., 1993; Nikaido et al., 1994; Schuldiner et al., 1995; Marger et al., 1993; Lewis et al., 1994). Living organisms are constantly faced with harmful chemicals whose active elimination is imperative for survival. These chemicals are presented to the organism from a variety of sources such as metabolic waste products and environmental hazards as well as antibiotics secreted from other organisms. Removal of the toxicants is done at the cellular level in all organisms, while more evolved organisms contain in addition specialized organs and tissues devoted to these processes (e.g., the kidney and the liver).

Attention has also been focused on the ability of microorganisms to acquire resistance to antibiotics. Often, the mechanism of resistance to antibiotics is based on active extrusion of the toxicant from within the organism (Griffith et al., 1992; Paulsen et al., 1993; Nikaido et al., 1994; Schuldiner et al., 1995; Marger et al., 1993; Lewis et al., 1994).

Proteins that participate in the extrusion of toxicants from cells are usually large membrane proteins containing twelve putative transmembrane segments and are consequently difficult to characterize structurally. Recently a unique family of small (about 100 amino acids) multi-drug transporters from bacteria was detected (Grinius et al., 1992). Four members of this family, termed Smr (Grinius et al., 1992) or MiniTEXANs (Yerushalmi et al., 1995), have been identified: EmrE (also known as Ebr and MvrC) (Purewal et al., 1991; Morimyo et al., 1992) and SugE (Greener et al., 1993) in *Escherichia coli*, Smr (also known as QacC) from *Staphylococcus aureus* (Littlejohn et al., 1991; Grinius et al., 1992; Sasatsu et al., 1989), and QacE from *Klebsiella aerogenes* (Paulsen et al., 1993). Hydrophobicity analysis of the sequences of the above proteins predicts the occurrence of four putative transmembrane segments.

Smr (Paulsen et al., 1995; Grinius et al., 1994) and EmrE (Yerushalmi et al., 1995) have been characterized, purified, and reconstituted in a functional form. Both proteins catalyze H^+/cation antiport in proteoliposomes reconstituted with purified transporter and behave as multi-drug transporters capable of recognizing a wide range of inhibitors and substrates (Grinius et al., 1994; Yerushalmi et al., 1995). In addition, EmrE has been shown to display unique properties of solubility in organic solvents such as a mixture of chloroform and methanol (Yerushalmi et al., 1995). After solubilization in the above solvents, the protein retains its ability to transport as judged from the fact that it can be reconstituted in a functional mode.

In this report we have obtained transmission FTIR and oriented ATR-FTIR spectra aimed at determining the secondary structure content and orientation of the secondary structure elements of EmrE. The protein was found to be highly helical (80%) in $CHCl_3$:MeOH and in phospholipid bilayers composed of DMPC. Polarized ATR-FTIR spectra (in DMPC) have revealed that the helices in EmrE are oriented perpendicular to the lipid bilayer with a tilt angle of 27° with respect to the bilayer normal. EmrE in *E. coli* lipids was found to posses poor orientation as a consequence of the poor orientation of the *E. coli* lipids.

† This research was supported by a grant from the Israel Science Foundation to S.S. and grants from the National Institute of Health and National Foundation for Cancer Research to D.M. Engelman.

* To whom correspondence should be addressed. Tel: 972-2-6585992. FAX 972-2-634625. E-mail: shimons@vms.huji.ac.il.

‡ Howard Hughes Medical Institute, Yale University.

§ Department of Molecular Biophysics and Biochemistry, Yale University.

|| Hebrew University of Jerusalem.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

S0006-2960(96)00094-3 CCC: $12.00

MATERIALS AND METHODS

Expression, Purification, and Reconstitution of EmrE. Expression and purification of EmrE were done essentially as described previously (Yerushalmi et al., 1995). For labeling EmrE with [^{35}S]methionine, pT7-32, which contains the T7 polymerase promoter θ10 and the translation start site for the T7 gene 10 protein, was used (Pinner et al., 1992). pT7-32 was transformed into TA15 carrying pGP1-2 (Tabor et al., 1985). Transformants were grown at 30 °C in minimal medium supplemented with thiamine (2.5 μg/mL), ampicillin and kanamycin (50 μg/mL), and 0.5% glucose, to a cell density of 0.6A_{600}. 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 40 min. [^{35}S]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 and washed with a solution containing 20 mM Tris·HCl, pH 7.5, and 150 mM NaCl and sonicated three times for 10 s using a probe type sonicator. Undisrupted cells were removed by centrifugation, and the membranes were then collected by further centrifugation at 213 500*g* for 20 min. The membrane pellet was resuspended in the above buffer and 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 and thiamine and ampicillin as above. When the culture reached an $A_{600} = 0.5$, isopropyl thiogalactoside was added to 0.5 mM; 2 h later, the cells were chilled on ice and harvested by centrifugation. Membranes were prepared by disrupting the cells using a French Pressure procedure (Rosen et al., 1986), except that the buffer used was 10 mM Tris·HCl, pH 7.5, 250 mM sucrose, 150 mM choline chloride, 0.5 mM DTT, 2.5 mM $MgSO_4$, and 15 μg of DNAaseI/mL. After ultracentrifugation membranes were resuspended at 10 mg protein/mL, frozen in liquid air, and stored at −70 °C.

In order to follow EmrE during purification, membranes containing [^{35}S]methionine-labeled protein and overexpressed protein were routinely mixed to yield approximately 1200 cpm/μg of membrane protein. For extraction, membranes in a volume of 15 mL (150 mg of membrane protein) were mixed with 250 mL of a mixture of chloroform:methanol (1:1) and incubated for 20 min on ice. For phase separation, 50 mL of water was added and the suspension separated. The upper phase was removed, and the lower phase was used for further purification as will be described elsewhere (M. Lebendiker and S. Schuldiner in preparation). For analysis in SDS−PAGE, the sample was dried and resuspended in sample buffer. SDS−PAGE analysis was in 16% gels as described (Schagger et al., 1987).

Reconstitution of EmrE into lipid vesicles was achieved by adding to the dissolved protein a solution of lipid in $CHCl_3$. Dimyristoylphosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL) while *E. coli* lipids were prepared as described (Viitanen et al., 1986). The mixture was then dried and rehydrated in a solution of 0.1 mM $NaPO_4$, pH 6.8. Protein to lipid ratios ranged from 1:10 to 1:20 w/w protein:lipid.

FTIR Spectroscopy. FTIR spectra were collected on a Nicolet Magna 550 spectrometer purged with N_2 (Madison, WI) and equipped with an MCT/A detector. 1000 interferograms recorded at a spectral resolution of 4 cm^{-1} were averaged for each sample. Interferograms were processed using 1-point zero filling and Happ−Genzel apodization, followed by automatic base line correction when needed. Peak integration was performed on Fourier self-deconvoluted spectra (Kauppinen et al., 1981) using a bandwidth of 13 cm^{-1} and an enhancement factor of 2.4 (Byler & Susi, 1986). For transmission spectra 50 μL of sample (protein concentration of ca. 80 μM) was dried on Ge windows (Grasbey Specac, Kent, U.K.). For orientation studies the sample (300 μL, ca. 80 μM) was dried on a Ge internal reflection element and placed in a 25-reflection variable angle ATR accessory (Grasbey Specac, Kent, U.K.).

Secondary Structure Content Analysis. Secondary structure content estimation was done according the method of Venyaminov and Kalnin (1990a,b) and Kalnin et al. (1990). FSD peak integrations of the α-helical segment were weighed against the entire amide I region, taking into account the extinction coefficients for the different secondary structure elements, as well as explicit side chain modes, absorbing above and below the α-helical portion of the amide I region (1600−1700 cm^{-1}). By solving the three related equations relating the intensities of each of these three regions to its defined components (side chain modes obtained from the protein sequence and peptide bond modes of the different secondary structure elements) it is possible to obtain the relative secondary structure element content. Errors are obtained by using the limits of the extinction coefficients of the side chain modes as well as that of the peptide bonds.

Analysis of Orientation from ATR-FTIR Dichroism. Order parameters for the protein were determined as described previously (Arkin et al., 1995). Briefly, the measured dichroic ratio, R^{ATR}, defined as the ratio between the absorption of light polarized parallel and perpendicular to the surface of the internal reflection element, was used to calculate an order parameter using the following equation:

$$S = \frac{\epsilon_x^2 - R^{ATR}\epsilon_y^2 + \epsilon_z^2}{\epsilon_x^2 - R^{ATR}\epsilon_y^2 - 2\epsilon_z^2} \div \frac{3\cos^2\alpha - 1}{2} \qquad (1)$$

whereby ϵ_x, ϵ_y, and ϵ_z are the integrated absorption coefficients given by Harrick (1967) and α is the angle between the principal transition dipole moment and the molecular director. $\alpha = 39°$ in the case of the amide I mode, and $\alpha = 75°$ in the case of the amide II mode (Tsuboi 1962). The order parameter S is related to the tilt angle β (from the normal of the internal reflection element by the following equation:

$$S \equiv \frac{3\langle\cos^2\beta\rangle - 1}{2} \qquad (2)$$

Order parameters for the lipids are obtained by setting $\alpha = 90°$.

RESULTS

Secondary Structure Content Estimation. The correlation between the frequency of the amide I vibrational mode and the nature of the secondary structure has been well established in the literature (Braiman & Rothschild, 1988). Frequencies in the regions of 1650−1660 cm^{-1} correspond to α-helical segments while modes resonating in the regions of 1630−1640 and 1670−1685 cm^{-1} correspond to β-sheet

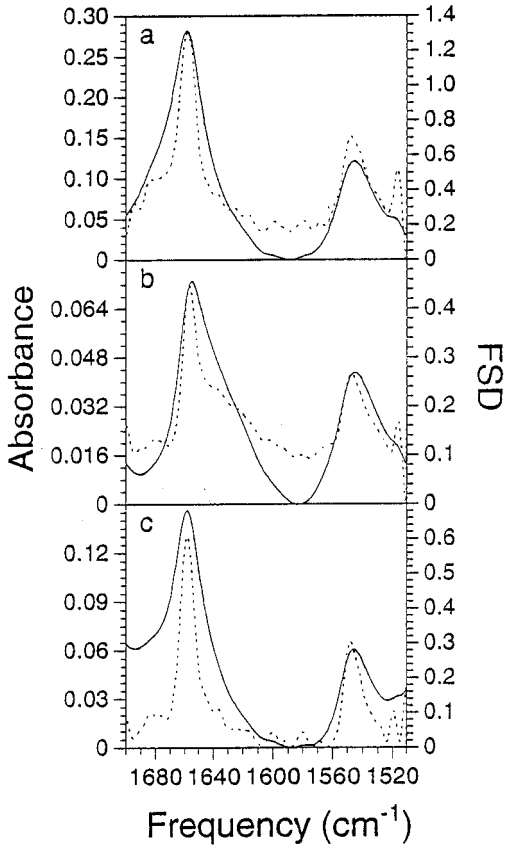

FIGURE 1: Amide I vibrational modes of EmrE. The proteins were dried from either (a) $CHCl_3$:MeOH, (b) *E. coli*, or (c) DMPC phospholipid vesicles. Spectra are presented as the solid line, while Fourier self-deconvolutions (see text for details) are presented as the dotted line.

Table 1: Secondary Structure Estimations for EmrE in DMPC and $CHCl_3$:MeOH[a]

environment	α-helix	β-sheet	random-coil
DMPC	80% ± 5%, 88/110	10% ± 1.1%, 11/110	10% ± 4%, 11/110
$CHCl_3$:MeOH	78% ± 5%, 86/110	12% ± 1.1%, 11/110	10% ± 4%, 11/110

[a] See Materials and Methods for calculation method.

elements. As seen in Figure 1 the amide I modes of EmrE dried down from $CHCl_3$:MeOH and DMPC are very similar, in that both contain a highly symmetrical band centered at 1655 cm^{-1} with no significant shoulders, indicative of a high helical content. Comparison of the ratio of the helical band to that of the entire amide I mode of FSD spectra taking into account the different extinction coefficients of the secondary structure elements and side chain modes (see Materials and Methods) yields a quantitative estimation of α-helical content. These calculations yield α-helical estimates of 78% ± 5% and 80% ± 5% for proteins dried down from $CHCl_3$:MeOH and DMPC, respectively, as listed in Table 1.

The amide I mode of EmrE in *E. coli* phospholipid vesicles is once again centered at 1655 cm^{-1} yet contains a significant shoulder at lower frequencies, as seen in Figure 1b. The nature of this asymmetry arises from additional vibrational modes (data not shown) of unsaturated bonds in the *E. coli* phospholipids occurring around that region. Thus, although it is clear that the protein is mostly α-helical, quantitative estimation of helical content would not be reliable.

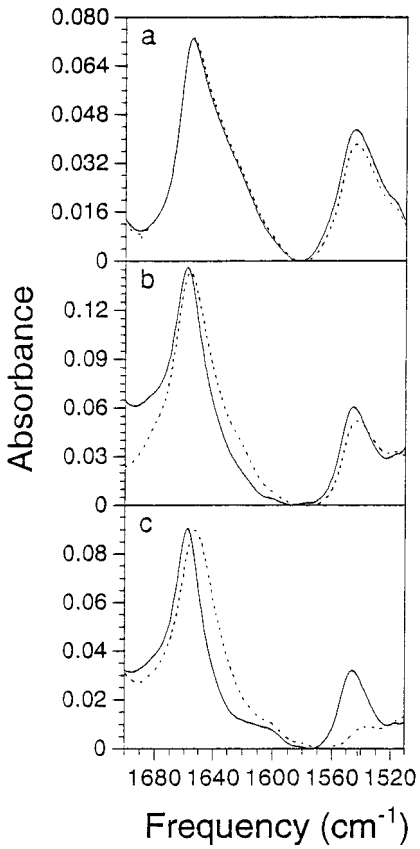

FIGURE 2: H/D exchange of EmrE in phospholipid bilayers. Amide I and amide II vibrational modes of EmrE dried down from either H_2O (solid line) or D_2O (dotted line) in (a) DMPC, (b) *E. coli* phospholipid vesicles, or (c) $CHCl_3$:MeOD.

Membrane Incorporation. Amide proton H/D exchange occurs exceedingly slowly in the hydrophobic environment of the bilayer (Braiman & Rothschild, 1988). This most likely results from the fact that this exchange involves significant charge separation during the reaction, a highly unfavorable intermediate in the low dielectric environment of the lipid bilayer. Thus, measurements of the extent of such exchange by observing the reduction in the amide II mode (N−H bond) are a reliable estimation for membrane incorporation.

As seen in Figure 2, proteins reconstituted in DMPC (panel a) or *E. coli* phospholipid vesicles (panel b) are mostly resistant to amide proton H/D exchange as seen by the negligable reduction of the amide II band at 1543 cm^{-1}. The fraction of exchanging residues most likely arises from extramembraneous groups. On the other hand H/D exchange in EmrE dissolved in $CHCl_3$:MeOD was quantitative (panel c).

Orientation of EmrE. After the high helical content of EmrE is determined and it is established that the protein is embedded in the lipid bilayer, it is possible to determine the orientation of the helices with respect to the lipid bilayer. The orientation of the bilayer itself can be established by determining the dichroic ratio of the methylene asymmetric and symmetric stretching modes at 2924 and 2852 cm^{-1}, respectively. The measured dichroic ratios and calculated order parameters are listed in Table 2. It is not surprising that the bilayer composed of *E. coli* phospholipids would orient poorly ($R^{ATR} = 1.6$, $S = 0.26 \Rightarrow \beta = 45°$) as compared to DMPC ($R^{ATR} = 1.1$, $S = 0.7 \Rightarrow \beta = 26°$) since the *E.*

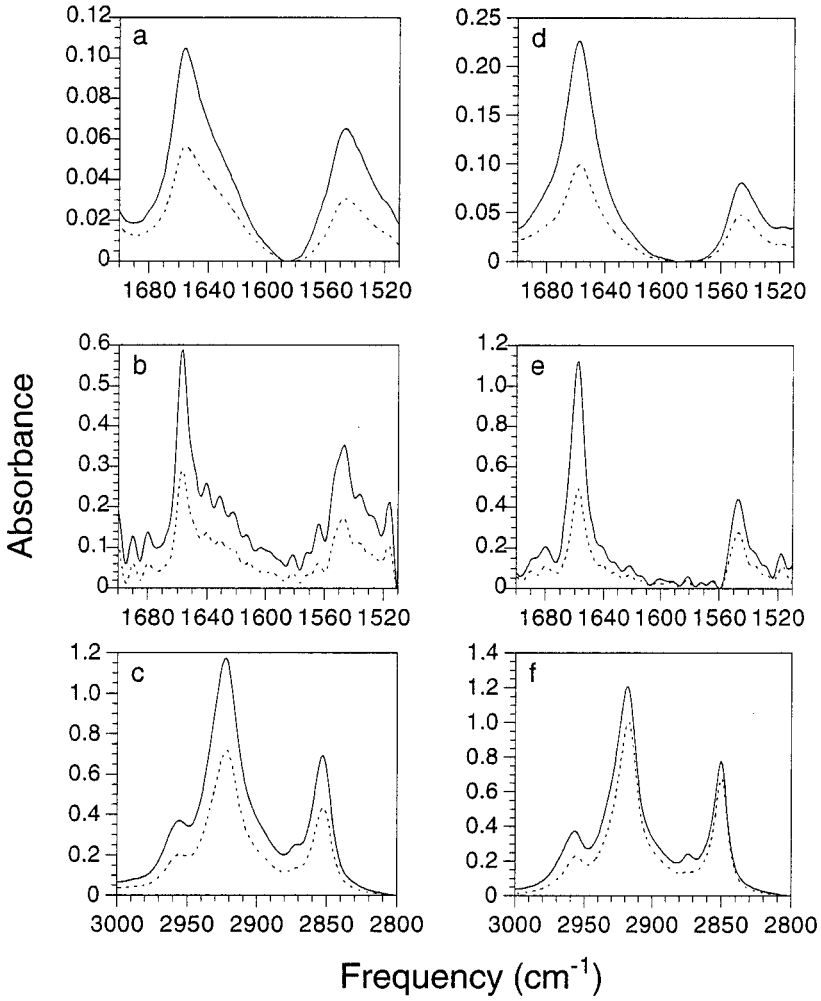

FIGURE 3: ATR-FTIR dichroism spectra of EmrE. Parallel (solid line) and perpendicular (dotted line) polarized light ATR-FTIR spectra of EmrE. (a) Amide I region of EmrE dried in *E. coli* lipids. (b) Fourier self deconvolution of a. (c) Methyl and methylene stretching modes region of *E. coli* lipids. (d) Amide I region of EmrE dried in DMPC phospholipids. (e) Fourier self-deconvolution of d. (f) Methyl and methylene stretching modes region of DMPC phospholipids.

coli lipids are in the liquid crystal phase due to the prevalence of unsaturated bonds.

In order to determine the order parameters of the α-helices in EmrE we measured the dichroic ratios of the amide I and amide II vibrational modes. Normally there is no reason to measure the dichroism of the amide II band as the information is redundant with that obtained from the amide I mode. However, since there were overlapping bands in the amide I region originating from *E. coli* lipid unsaturated bonds, amide II dichroism would yield unique orientation estimates, as listed in Table 2.

As evident the protein is only ordered in DMPC bilayers ($R^{ATR}_{amideI} = 2.8$, $S = 0.5 \Rightarrow \beta = 37°$), as expected since the *E. coli* lipid bilayer is not ordered on its own ($R^{ATR}_{amideII} = 2.2$, $S = 0.1 \Rightarrow \beta = 50°$). The derived tilt angles are calculated assuming no bilayer disorder and therefore should be regarded as the maximal tilt angle.

Table 2: Measured Dichroic Ratios (R^{ATR}), Calculated Order Parameters (S), and Derived Tilt Angles (β) for EmrE in DMPC and in *E. coli* Lipids[a]

vibration	*E. coli* lipids	DMPC
lipid acyl chain	$R^{ATR} = 1.6$, $S = 0.28 \Rightarrow \beta = 45°$	$R^{ATR} = 1.1$, $S = 0.7 \Rightarrow \beta = 26°$
amide I	$R^{ATR} = 2.1$, $S = 0.07 \Rightarrow \beta = 52°$	$R^{ATR} = 2.8$, $S = 0.5 \Rightarrow \beta = 37°$
amide II	$R^{ATR} = 2.2$, $S = 0.1 \Rightarrow \beta = 50°$	$R^{ATR} = 1.5$, $S = 0.4 \Rightarrow \beta = 39°$

[a] Lipid acyl chain (methylene asymmetric stretch), amide I, and amide II modes were centered at 2924, 1655, and 1543 cm^{-1}, respectively. Tilt angles were calculated assuming no bilayer disorder. See text for corrected protein order parameter.

DISCUSSION

Secondary Structure. Hydrophobicity analysis of the sequence of EmrE yielded four putative transmembrane domains of similar sizes: TM1, Y4−M21; TM2, L30−I54; TM3, I58−G80, and TM4, L85−S105. All together the transmembrane domains account for 86 amino acids, or 78% of the total residues of the protein. Extramembranous residues are predicted to partake in short connecting loops and therefore not to be in a helical configuration. Thus, one can equate the percentage of membrane embedded residues to helical content. Results from the transmission FTIR measurements agree remarkably well with this notion and yielded α-helical estimates of 78% and 80% for EmrE in $CHCl_3$:MeOH and DMPC, respectively. The fact that the protein retains its secondary structure in a solution of $CHCl_3$:MeOH should not be surprising as several membrane proteins have been documented to retain secondary structure in this solvent mixture (Fraga et al., 1994).

The very high helical content of EmrE precludes any other sort of secondary structures, while the length of the sequence of EmrE (110 amino acids) can discount any number of transmembrane α-helices greater than four. Furthermore, the fact that most of the amide groups in the protein do not undergo amide proton H/D exchange implies that most (ca. 80%) of the residues are embedded in the bilayer. These obeservations are only consistent with four transmembrane helices.

Membrane Orientation. The fact that the α-helices are transmembrane and not peripheral to the bilayer can be directy verified using ATR-FTIR. The derived tilt angle for EmrE in DMPC vesicles from the bilayer normal is 37°, and as previously noted the calculation of the tilt angle does not take into account any bilayer disorder and must therefore be considered as the maximal tilt angle.

Estimating bilayer disorder can be done by comparing the inherent order parameter of a model membrane ($S = 0.95$) to the order parameter measured in this study for the lipid acyl chains ($S = 0.7$). The ratio between the two ($S = 0.73$) represents the bilayer disorder. Thus dividing the calculated order parameter for EmrE in DMPC ($S_{\text{amideI}} = 0.5$) by the bilayer order parameter yields the corrected protein order parameter of $S = 0.68$ and subsequent tilt angle of $\beta = 27°$. As FTIR measures properties of the entire system as a whole, the tilt angle calculated above should be considered as the average tilt per helix.

General Structure. So far the results indicate that EmrE is a bundle of four transmembrane α-helices each roughly 20−25 amino acids in length, with an average tilt with respect to the bilayer normal of $\beta = 27°$. The tilt angle of the helices in EmrE is similar to that measured for bacteriorhodopsin (26°) by Rothschild and Clark (1979) agreeing with the structure of bacteriorhodopsin solved by cryo-electron crystallography by Henderson et al. (1990). It is instructive to compare the results of our study to high-resolution structures of soluble proteins possessing this motif.

The most-studied proteins sharing the motif of an anti-parallel four-helical bundle are Rop (Banner et al., 1987), cytochrome *b*562 (Lederer et al., 1981), cytochrome *c*′ (Finzel et al., 1985), and myohemerythrin (Sheriff et al., 1987). All of these proteins are of similar size (Rop being the exception since it is a dimer, each protamer contributing two helices) with similar average helix lengths. Interestingly, the length of the helices in EmrE, a parameter influenced by the dimensions of the bilayer, is similar that found in the soluble proteins.

What distinguishes EmrE from the soluble proteins is the relative tilt of the helices from the long axis of the complex. In the soluble proteins this angle is less than 15° while in EmrE the measured tilt angle is 27°. The tilt angle between the helices is slightly increased in the cofactor binding proteins (heme in the case of both cytochromes or iron in the case of myohemerythrin) as opossed to that found in Rop. This phenomenon may be due to the cavity formed upon splaying of the helices that generates a pocket for the cofactor. One would then assume that this sort of arrangemnt of splaying helices would be more similar to that found in EmrE accomodating space for its substrate.

The basis for the different tilt angles may reside in the different function of EmrE as opposed to the function of the soluble proteins. Both cytochromes partake in electron transfer that most likely does not involve significant conformational changes during the enzymatic cycle, and the function of Rop is that of binding RNA. EmrE on the other hand is a H^+/cationic toxin antiporter that most likely undergoes significant changes during the enzymatic cycle.

CONCLUSION

In this study we provide the most detailed structural data as of yet for a multi-drug transporter. The protein was shown to be a four-membered transmembrane anti-parallel helical bundle. The α-helical content determined by transmission FTIR was found to be nearly identical to that predicted by hydropathy analysis. Polarized ATR-FTIR has shown the tilt angle of the helices from the bilayer normal to be 27°. Taken together the data provided in this report set the stage for detailed studies aimed at determining a model for this toxin-extruding H^+ antiporter.

ACKNOWLEDGMENT

We thank D. M. Engelman (Department of Molecular Biophysics and Biochemistry, Yale University) for continuous support and helpful discussions.

REFERENCES

Arkin, I. T., Rothman, M., Ludlam, C. F., Aimoto, S., Engelman, D. M., Rothschild, K. J., & Smith, S. O. (1995) *J. Mol. Biol. 248*, 824−834.
Banner, D. W., Cesareni, G., & Tsernoglou, D. J. (1987) *Mol. Biol. 196*, 657−675.
Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541−570.
Byler, D. M., & Susi, H. (1986) *Biopolymers 25*, 469−487.
Finzel, B. C., Weber, P. C., Hardman, K. D., & Salemme, F. R. (1985) *J. Mol. Biol. 186*, 627−643.
Fraga, D., Hermolin, J., & Fillingame, R. H. (1994) *J. Biol. Chem. 28*, 2562−2567.
Greener, T., Govezensky, D., & Zamir, A. (1993) *EMBO J. 12*, 889−896.
Griffith, J., Baker, M., Rouch, D., Page, M., Skurray, R., Paulsen, I., Chater, K., Baldwin, S., & Henderson, P. (1992) *Curr. Opin. Cell Biol. 4*, 684−695.
Grinius, L., & Goldberg, E. (1994) *J. Biol. Chem. 269*, 29998−30004.
Grinius, L., Dreguniene, G., Goldberg, E. B., Liao, C. H., & Projan, S. J. (1992) *Plasmid 27*, 119−129.
Harrick, N. J. (1967) *Internal Reflection Spectroscopy*, Interscience Publishers, New York.
Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899−929.
Kalnin, N. N., Baikalov, I. A., & Venyaminov, S. Y. (1990) *Biopolymers 30*, 1273−1280.
Kauppinen, J. K., Moffatt, D. J., Mantsch, H. H., & Cameron, D. G. (1995) *Appl. Spectrosc. 35*, 271−276.
Lederer, F., Glatigny, A., Bethge, P. H., Bellamy, H. D., & Mathews, F. S. (1981) *J. Mol. Biol. 148*, 427−448.
Littlejohn, T., DiBerardino, D., Messerotti, L., Spiers, S., & Skurray, R. (1991) *Gene 101*, 59−66.
Lewis, K. (1994) *Trends Biochem. Sci. 19*, 119−123.
Marger, M., & Saier, M. (1993) *Trends Biochem. Sci. 18*, 13−6.
Morimyo, M., Hongo, E., Hama-Inaba, H., & Machida, I. (1992) *Nucleic Acids Res. 20*, 3159−3165.
Nikaido, H. (1994) *Science 264*, 382−388
Paulsen, I., & Skurray, R. (1993) *Gene 124*, 1−11.
Paulsen, I., Littlejohn, T., Radstrom, P., Sundstrom, L., Skold, O., Swedberg, G., & Skurray, R. (1993) *Antimicrob. Agents Chemother. 37*, 761−768.
Paulsen, I., Brown, M., Dunstan, S., & Skurray, R. (1995) *J. Bacteriol. 177*, 2827−2833.
Pinner, E., Padan, E., & Schuldiner, S. (1992) *J. Biol. Chem. 267*, 11064−11068.
Purewal, A. S. (1991) *FEMS Microbiol. Lett. 82*, 229−232.

Rosen, B. (1986) *Methods Enzymol. 125*, 328−336.
Rothschild, K. J., & Clark, N. A. (1979) *Biophys. J. 25*, 473−488.
Sasatsu, M., Shima, K., Shibata, Y., & Kono, M. (1989) *Nucleic Acids Res. 17*, 10103.
Sasatsu, M., Shibata, Y., Noguchi, N., & Kono, M. (1992) *FEMS Microbiol. Lett. 72*, 109−113.
Schagger, H., & Jagow, G. v. (1987) *Anal. Biochem. 166*, 368−379.
Schuldiner, S., Shirvan, A., & Linial, M. (1995) *Physiol. Rev. 75*, 369−392.
Sheriff. S., Hendrickson, W. A., & Smith, J. L. (1987) *J. Mol. Biol. 197*, 273−296.
Tabor, S., & Richardson, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
Tsuboi, M. J. (1962) *Polymer Sci. 59*, 139−153.
Venyaminov, S. Y., & Kalnin, N. N. (1990a) *Biopolymers 30*, 1243−1257.
Venyaminov, S. Y., & Kalnin, N. N. (1990b) *Biopolymers 30*, 1259−1271.
Viitanen, P., Newman, M. J., Foster, L., Wilson, T. H., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 429−452.
Yerushalmi, H., Lebendiker, M., & Schuldiner, S. (1995) *J. Biol. Chem. 270*, 6856−6863.

BI960094I