

# On Parallel and Antiparallel Topology of a Homodimeric Multidrug Transporter\*

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The recently suggested antiparallel topology of EmrE has intriguing implications for many aspects of the biology of ion-coupled transporters. However, it is at odds with biochemical data that demonstrated the same topology for all protomers in the intact cell and with extensive cross-linking studies. To examine this apparent contradiction we chemically cross-linked dimers with a rigid bifunctional maleimide using Cys replacements at positions not permissible by an antiparallel topology. A purified cross-linked dimer binds substrate and transports it in proteoliposomes with kinetic constants similar to those of the non-cross-linked dimer. The cross-linked dimers do not interact with non-cross-linked dimers as judged from the fact that inactive mutants do not affect their activity (negative dominance). The results support the contention that EmrE with parallel topology is fully functional. We show that the detergents used in crystallization increase the fraction of monomers in solution. We suggest that the antiparallel orientation observed is a result of the arrangement of the monomers in the crystal. Functionality of EmrE with the suggested antiparallel orientation of the monomers remains to be characterized.

Recent publications describing high resolution structures of transporters are changing a field that has been waiting avidly for such advancements (1–4). This welcome revolution provides fundamental insights for designing new biochemical/biophysical approaches and will deepen understanding of transport mechanisms.

However, in some cases, as for the channel-forming peptide Gramicidin, for the ABC transporter MsbA, and for EmrE, an *Escherichia coli* ion-coupled multidrug transporter, different structures have been reported for the same protein, and it is not evident that these purportedly different conformations are physiologically relevant (5–8). The question is raised whether these are proteins with multiple conformations that fulfill functions yet unknown to us or whether the reported conformations are an experimental artifact created by the different milieu the proteins face when removed from their native environments. This may turn out to be especially critical for membrane pro-

teins where we can only feebly mimic the original conditions after solubilization with detergents. A leading criterion at this stage should be whether the protein has some measurable function in the detergent-solubilized state.

Two x-ray structures of EmrE have been published, and they are very different from each other (7, 8). The presence of substrate in the second one may be responsible for the large differences between the two structures, although similar substrate-induced conformational changes were not observed in two-dimensional crystals (9, 10). The x-ray structure shows an asymmetric dimer with the protomers in an antiparallel topological orientation. This finding has obvious and exciting similarities to the internal structural repeat found in several membrane proteins such as aquaporins, CIC channel, and the neurotransmitter transporter homologue LeuT (11–13). However, it is at odds with biochemical data that demonstrated the same topology for all protomers in the intact cell and in membrane vesicles (14) and with extensive cross-linking studies (15).

Because an apparent antiparallel topology of a homodimer has many intriguing implications regarding biogenesis, insertion, and evolution of ion-coupled transporters, the topic has already attracted much attention (16–18). To investigate the apparent contradiction between our previous work and the proposed antiparallel topology we reevaluated our cross-linking studies. We chemically cross-linked dimers using Cys replacements in transmembrane 4 (TM4)<sup>2</sup> at positions not permissible by an antiparallel topology. To test whether the cross-linked proteins reflect a physiologically relevant conformation, we purified one of them and showed it is fully functional. The cross-linked dimers bind substrate and transport it in proteoliposomes with kinetic constants similar to those of the non-cross-linked dimer. The cross-linked dimers do not interact with non-cross-linked dimers as judged from the fact that inactive mutants do not affect their activity (negative dominance). In addition, the cross-linked dimers are remarkably more stable to heat treatment. The results support the contention that EmrE with a parallel topology is fully functional. We cannot rule out the existence of an antiparallel arrangement outside of the crystal world, but if it exists its functionality remains to be characterized. Our work with the detergents used for crystallization of EmrE shows that they weaken the interaction of the

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<sup>2</sup> The abbreviations used are: TM, transmembrane segment; EmrE, EmrE tagged with Myc epitope and six His residues; CAMY, a cysteine-less EmrE that was built with alanine replacements; MV<sup>2+</sup>, methyl viologen; TPP<sup>+</sup>, tetraphenylphosphonium; DDM, n-dodecyl- $\beta$ -maltooside; OG, octyl- $\beta$ -glucoside; NG, nonyl- $\beta$ -glucoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

## Parallel Topology of EmrE

monomers in the dimer. We suggest that the monomers generated are the ones that crystallize and the antiparallel arrangement in the crystal is, most likely, a crystallographic dimer.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains, Plasmids, and Mutagenesis

*E. coli* DH5 $\alpha$  (Invitrogen) and TA15 (19) strains were used throughout this work. TA15 strain was previously transformed with plasmid pGP1–2, which codes for the T7 polymerase under the inducible control of the  $\lambda$  PL promoter (20). The plasmids used for EmrE gene expression are pT7–7 (20) derivatives with a His<sub>6</sub> tag (EmrE-His, for simplicity will be called EmrE throughout this report) (21). The construction of the mutants was as previously described (22). The template used for mutagenesis was pT7–7 CAMY (a cysteine-less EmrE with alanine replacements) (23). All of the PCR-amplified products were sequenced to ensure that no other mutations occurred during the amplification process.

#### Overexpression and Purification of EmrE

TA15 cells that bear plasmids pGP1–2 and pT7–7 containing His-tagged EmrE constructs were grown and induced for EmrE expression as previously described. For specific labeling with [<sup>35</sup>S]methionine the procedure was as described (24). For purification, cells were resuspended in buffer containing 150 mM NaCl, 15 mM Tris, pH 7.5, 250 mM sucrose, 2.5 mM MgSO<sub>4</sub>, 15 mM  $\beta$ -mercaptoethanol, 15  $\mu$ g/ml DNaseI, and 1 mM phenylmethylsulfonyl fluoride (5 ml of buffer/g of wet weight cells). Cells were disrupted using a Microfluidics microfluidizer processor (M-110EHi) (25). For protein solubilization, 1% of DDM and 50 mM imidazole were added, and the mixture was incubated for 1 h at 4 °C. Insolubilized material was removed by centrifugation at 240,000  $\times$  g for 45 min at 4 °C. The solubilized protein was loaded on HiTrap<sup>TM</sup> chelating HP column (Amersham Biosciences) mounted on Akta Explorer (Amersham Biosciences) and washed with 0.08% DDM, 150 mM NaCl, 15 mM Tris-HCl, and 50 mM imidazole, pH 7.5, till A<sub>280</sub> of the flow-through decreased below 0.05. EmrE was eluted with a gradient of up to 300 mM imidazole. Major peak fractions were pooled, and the protein solution was brought to  $\sim$ 1 mg/ml EmrE (according to A<sub>280</sub>). The protein stock was aliquoted, frozen in liquid air, and stored at  $-70$  °C. In the case of the D84C mutant, after solubilization the  $\beta$ -mercaptoethanol was removed by desalting on a G50-Sephadex column equilibrated with 1% DDM, 150 mM NaCl, 15 mM Tris-HCl, and 50 mM imidazole. The cross-linking reagent o-PDM was immediately added (0.5 mM) and incubated for 1 h at 65 °C following 5 min at 75 °C.

#### o-PDM Cross-linking and Purification of the Cross-linked Protein

Protein stock stored at  $-70$  °C was rapidly thawed at 30 °C, and 1% DDM and 0.5 mM o-PDM were added to the protein solution. After 1 h at 45 °C followed by 10 min at 60 °C, the mixture was diluted 15-fold with buffer containing 50 mM Tris-Cl, pH 8.6, and 0.02% DDM and loaded on a HiTrap Q-Sepharose Fast Flow column mounted on Akta Explorer (Amersham Biosciences). The column was washed with 5 column volumes

of the above buffer, and the cross-linked protein was eluted by stepwise elevation of the NaCl concentration to 220 mM. The peak fractions were pooled, and the protein solution was brought to  $\sim$ 1 mg/ml EmrE (according to A<sub>280</sub>). The protein stock was aliquoted, frozen in liquid air, and stored at  $-70$  °C.

#### Reconstitution of EmrE

Reconstitution was performed essentially as described (26). Purified proteins were diluted with 150 mM NaCl, 15 mM Tris-Cl, pH 7.5 (sodium buffer), and 0.08% DDM to lower imidazole concentration below 20 mM (if needed) and allowed to bind to nickel-nitrilotriacetic acid for 1 h at 4 °C. The beads were washed with at least 4 ml of sodium buffer containing 1% n-octyl- $\beta$ -D-glucopyranoside (Glycon GmbH), 30 mM imidazole, and 15 mM  $\beta$ -mercaptoethanol. The protein was eluted with 500  $\mu$ l of the same buffer containing 200 mM imidazole and mixed with 375  $\mu$ l of *E. coli* phospholipid mix (10 mg of *E. coli* lipids (Avanti Polar Lipids, Alabaster, AL), 1.2% n-octyl- $\beta$ -D-glucopyranoside, 15 mM Tris-Cl, pH 7.5, and 150 mM NaCl). Eluted protein and phospholipids were sonicated together in a bath-type sonicator to clarity and diluted in buffer containing 0.15 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 15 mM Tris, pH 7.5, and 1 mM dithiothreitol. After 20 min at room temperature, samples were centrifuged at 250,000  $\times$  g for 60 min and the pellet was resuspended in 100  $\mu$ l of the same buffer, frozen in liquid air, and stored at  $-70$  °C. Prior to the transport assay, the proteoliposomes were thawed at room temperature and sonicated lightly to clarity.

To determine the protein concentration in the proteoliposomes, they were solubilized in buffer containing 15 mM Tris-Cl, pH 7.5, 6 M urea, and 2% SDS (SDS-urea buffer) for 15 min. The solubilized proteins were immobilized on nickel-nitrilotriacetic acid beads for 1 h at room temperature. The unbound material was discarded, and the His-tagged protein bound to beads was washed twice with SDS-urea buffer. The protein was eluted from the beads using a buffer containing 200 mM  $\beta$ -mercaptoethanol, 100 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.2% bromphenol blue, and 450 mM imidazole and analyzed by SDS-PAGE. The intensity of the staining was quantitated using Image Gauge 3.46 Fujifilm software. The results were calibrated to protein amount after staining of the same gels with Coomassie staining, scanning, and quantitation using the same software. Each experiment was performed at least three times.

#### [<sup>3</sup>H]TPP<sup>+</sup> Binding and [<sup>14</sup>C] Methyl Viologen Uptake Assays

Tetraphenylphosphonium (TPP<sup>+</sup>) binding was assayed essentially as described (21). Uptake of [<sup>14</sup>C]methyl viologen (MV<sup>2+</sup>) into proteoliposomes was assayed at 25 °C by dilution of 2  $\mu$ l of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> containing proteoliposomes into 200  $\mu$ l of an ammonium-free solution (26, 27). The latter contained 20  $\mu$ M [<sup>14</sup>C]MV<sup>2+</sup> (11.9 mCi/mmol; Sigma), 140 mM K<sub>2</sub>SO<sub>4</sub>, 10 mM Tricine, 5 mM MgCl<sub>2</sub>, and 10 mM Tris, pH 8.5. Each experiment was performed at least twice.

#### Monomer-swapping Experiments

**Heat Treatment**—Protein at a concentration of  $\sim$ 0.5  $\mu$ g/ml was mixed with increasing amounts of purified EmrE E14C protein in 300  $\mu$ l of 0.08% DDM-sodium buffer. After 10 min at

80 °C the samples were allowed to cool down and subjected to pulse centrifugation.

**Octyl Glucoside Treatment**—EmrE mutants E14C and CAMY were solubilized in buffer containing 150 mM NaCl, 15 mM Tris-HCl, and either OG 2% or DDM 1% (membrane concentration was adjusted to the equivalent of 30 and 3.8 ng of EmrE/ $\mu$ l) and incubated for 30 min at 30 °C on a shaker. After centrifugation for 10 min at 14K rpm, increasing amounts of E14C mutants were added to 50 ng of CAMY protein. DDM (1%) was added to the OG-treated samples, and they were incubated for 20 min at 30 °C.

### Heat Denaturation

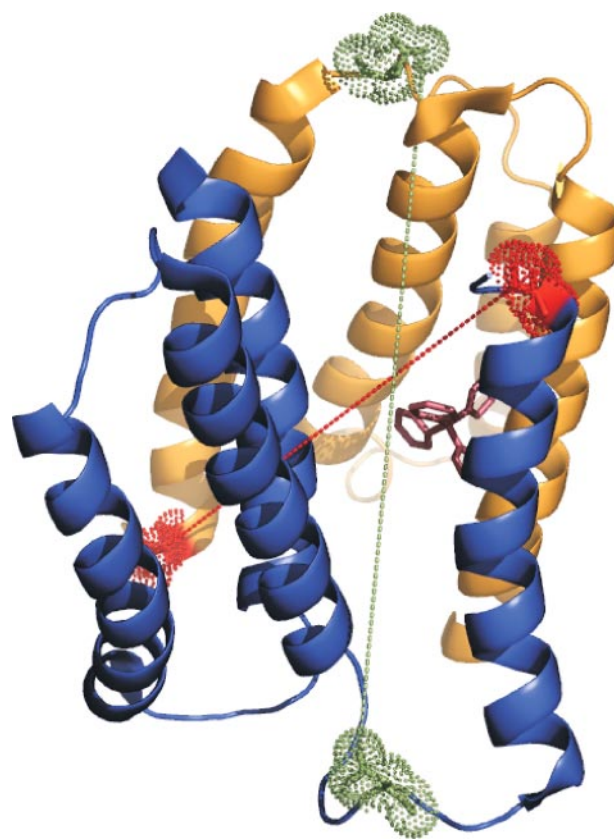
Purified protein was diluted to  $\sim$ 0.1  $\mu$ g/ml in 900  $\mu$ l of 0.08% DDM-sodium buffer and incubated at 80 °C for the indicated time periods. After incubation, protein solutions were allowed to cool down and subjected to pulse centrifugation.

## RESULTS

**Single Cysteine Replacements Chosen to Probe Relative Arrangement of the EmrE TM4 Segments within the Dimer**—EmrE contains four transmembrane helices (TM), connected by relatively short loops. The relative arrangement of the TM4 segments varies drastically in the published x-ray structures. To reevaluate our data obtained with the use of various cross-linking reagents we focused on two single cysteine replacements at the N and C termini of the TM4, D84C and T108C. Existing EmrE x-ray structures do not permit homo-cross-linking at these positions (Fig. 1). Both D84C and T108C mutations are tolerated by EmrE without any loss of function, reside outside the hydrophobic protein core, and react readily with maleimide reagents.

**Preparation of Cross-linked EmrE Homodimers**—Both T108C and D84C single cysteine replacements of EmrE have shown high ( $\sim$  60–70%) extent of intermonomer cross-linking when treated with o-PDM, a bifunctional rigid maleimide reagent (Fig. 2A). Such high yields suggest that cross-linked pairs correspond to highly populated conformations of EmrE. To eliminate the possibility that these cross-linked species derive from misfolded or inactive protein, we purified one of them (T108C) in quantities sufficient for functional characterization. Scaling up of the protein concentration decreased the cross-linking yield; therefore, optimization of the reaction conditions and purification of the cross-linked species were required. The T108C cross-linked species was enriched up to  $\sim$ 90% purity by anion exchange chromatography on a HiTrap Q-Sepharose column as described under “Experimental Procedures” (Fig. 2, B and C). o-PDM-treated D84C with  $\sim$ 40% cross-linked content was used throughout this work because we were not able to purify it further. The T108C cross-linked and the untreated proteins behave identically in size exclusion chromatography (Fig. 2D), showing that large complexes are not generated by multiple cross-linking between dimers.

**TPP<sup>+</sup> Binding Activity of the Cross-linked Homodimers**—The cross-linked and purified T108C displays high binding affinity of TPP<sup>+</sup> almost identical to that of the untreated protein.  $K_D$  and  $B_{max}$  of the T108C and T108C cross-linked protein were measured using radiolabeled TPP<sup>+</sup>. The  $K_D$ s were quite similar



**FIGURE 1. Ribbon representation of the EmrE antiparallel dimer structure in complex with TPP<sup>+</sup>, Protein Data Bank accession code 2F2M.** The monomers are colored differently to facilitate identification. The amino acids that were substituted with unique cysteines are labeled green (Asp-84) and red (Thr-108). The inter-cysteine distances, predicted by the structure, are  $\sim$ 48 Å for D84C to D84C pair and  $\sim$ 35 Å for T108C to T108C pair.

(3.7 and 6.4 nm, respectively), and the  $B_{max}$  was even slightly better for the cross-linked protein (0.25 and 0.42 mol TPP<sup>+</sup>/mol EmrE, respectively), most likely because of the higher purity of the latter achieved by the ion exchange chromatography. TPP<sup>+</sup> binding activity of the D84C mutant slightly increases after cross-linking with o-PDM (data not shown). However, because of the relatively lower content of the cross-linked species ( $\sim$ 40%) we cannot reach definitive conclusions about its activity with this assay.

**MV<sup>2+</sup> Uptake Activity of the Cross-linked EmrE T108C**—To further test the full functionality of the cross-linked T108C we reconstituted it into proteoliposomes and assayed its ability to catalyze proton gradient-driven uptake of MV<sup>2+</sup>. Upon generation of a pH gradient, the rates of uptake and the level of accumulation of MV<sup>2+</sup> detected in proteoliposomes reconstituted with either untreated or cross-linked T108C were very similar (Fig. 3). These findings allow us to conclude that EmrE cross-linked at position 108 is a fully functional transporter. This result also suggests that no extensive relative movements of the EmrE C terminus take place during MV<sup>2+</sup> transport, because the covalent link introduced between cysteines at 108 position has no negative impact on the uptake rate.

**Probing Interdimeric Interaction by “Monomer Swapping” with E14C-inactive EmrE Mutant**—EmrE dimers solubilized with DDM may be reversibly dissociated by a short heat treatment. If a mixture of two mutant proteins is subjected to this

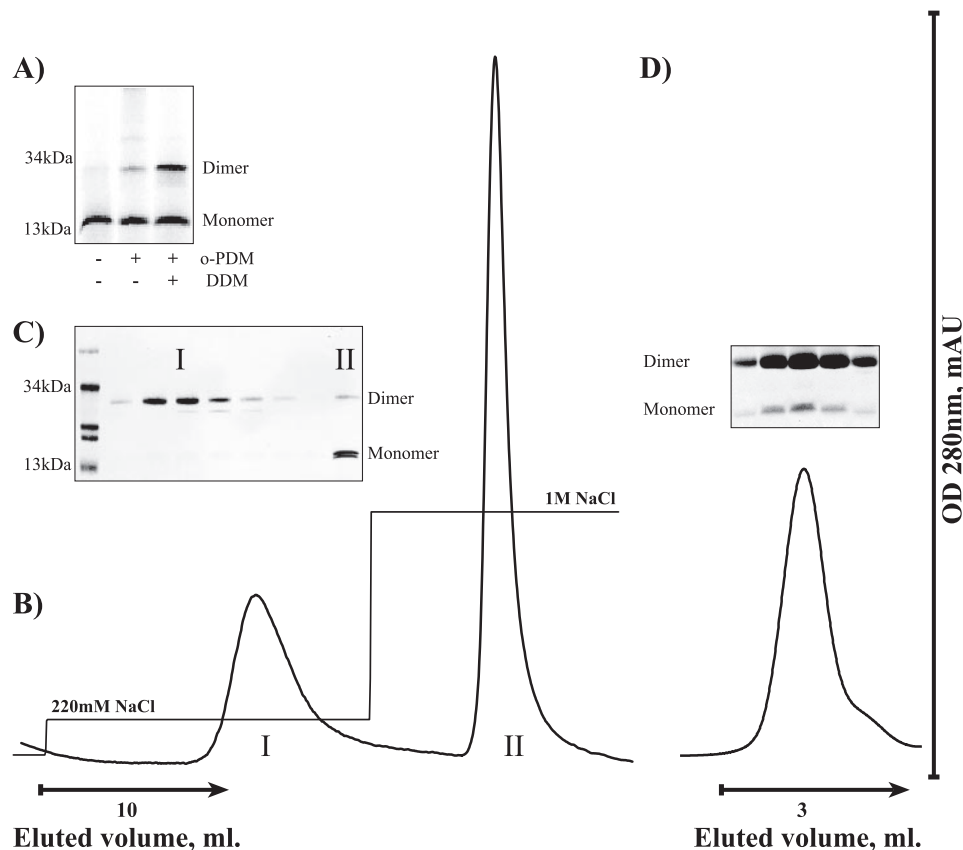


FIGURE 2. Purification of EmrE cross-linked at position T108C with o-PDM. A, EmrE T108C (left lane) radiolabeled with [<sup>35</sup>S]methionine (15) was solubilized and cross-linked with o-PDM (right lane) as described under "Experimental Procedures." The proteins were analyzed using SDS-PAGE, visualized with a Fujifilm LAS-1000 imaging system, and digitally analyzed with Image Gauge 3.46 Fujifilm software. B, elution profile of o-PDM-treated EmrE T108C on a 5-ml Q FF-Sepharose column. For details see "Experimental Procedures." C, the protein content of the eluted fractions was analyzed using SDS-PAGE and visualized by Coomassie stain. In the pooled peak I, eluted from the column at ~220 mM NaCl, ~90% of the protein was cross-linked. In various preparations, homogeneity ranged between 80–95%. D, o-PDM-treated EmrE T108C co-elutes with untreated mutant. The column used was a 20 ml Superdex 200 HR column. Bottom, optical density trace; top, the protein content of the eluted fractions was analyzed using SDS-PAGE and visualized by Coomassie stain.

procedure, mixed dimers are formed upon cooling (monomer swapping) (24). As was shown previously, EmrE E14C does not bind TPP<sup>+</sup> but may take part in the formation of mixed dimers with other EmrE mutants. These mixed dimers display ~20-fold lower affinity toward TPP<sup>+</sup>, a phenomenon defined as a negative dominant effect of the inactive mutant on the functional one (24, 28). We used this experimental paradigm to test whether the activity of the homo-cross-linked dimers T108C and D84C is derived from interdimeric interaction. Increasing amounts of EmrE E14C were added to cross-linked and untreated D84C and T108C proteins; the mixtures were subjected to the monomer-swapping procedure and assayed for TPP<sup>+</sup> binding activity. At the TPP<sup>+</sup> concentration used in the assay (2.5 nM) the contribution of mixed oligomers containing E14C is insignificant due to their lower affinity, and therefore inhibition of binding reflects the formation of mixed dimers (24). After monomer swapping with a 10-fold excess of the E14C mutant, the binding activity of the protein that was not treated with cross-linker was inhibited >80% (Fig. 4A, open circles). Under the same conditions, binding activity of cross-linked T108C is only barely

affected (~15%; Fig. 4A, closed circles). In a parallel experiment with the cross-linked D84C, activity decreased by ~40% (Fig. 4B). The degree of inhibition of activity of both cross-linked mutants is in line with the percent of the cross-linked species in the assayed reaction (~90 and 40%, respectively, for T108C and D84C). The results support the conclusion that in both mutants cross-linking prevents monomer swapping and the activity remaining after addition of an excess of the inactive EmrE E14C reflects the percent of cross-linked fully functional dimer.

**Comparing Heat Stability of the Untreated and Cross-linked EmrE T108C**—EmrE displays rather remarkable heat stability in the solubilized state (24). TPP<sup>+</sup> binding activity is irreversibly lost after incubation periods at high temperatures (80 °C) longer than 30 min (Fig. 5). Assuming that the first stage of the process that leads to the denatured protein is dimer dissociation, we compared the stability of the untreated and cross-linked T108C against the heat denaturation. Cross-linked and untreated 108C were diluted to ~0.1 μg/ml to encourage dimer dissociation and incubated for increasing time periods at 80 °C. As seen in Fig. 5 the cross-linked dimer (closed circles) lost practically no activity (~90%) even after 6 h of incubation at

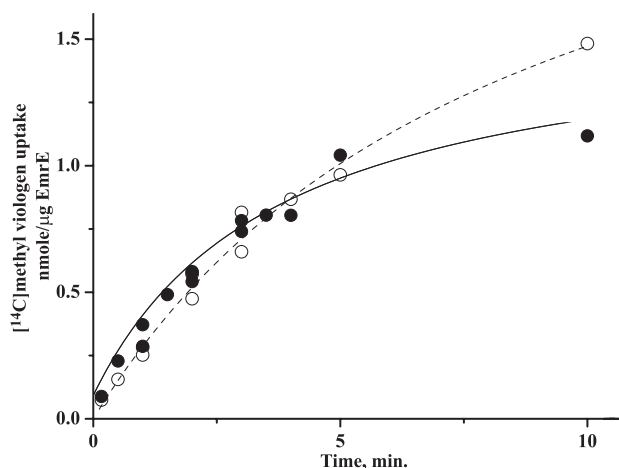


FIGURE 3. Proton-driven methyl viologen transport activity of cross-linked T108C. Ammonium-loaded proteoliposomes reconstituted with cross-linked purified T108C (filled circles) or with control untreated T108C (open circles) were diluted into an ammonium-free medium containing 20 μM [<sup>14</sup>C]methyl viologen, pH 8.5, and radioactivity incorporated at various times was measured as described under "Experimental Procedures."

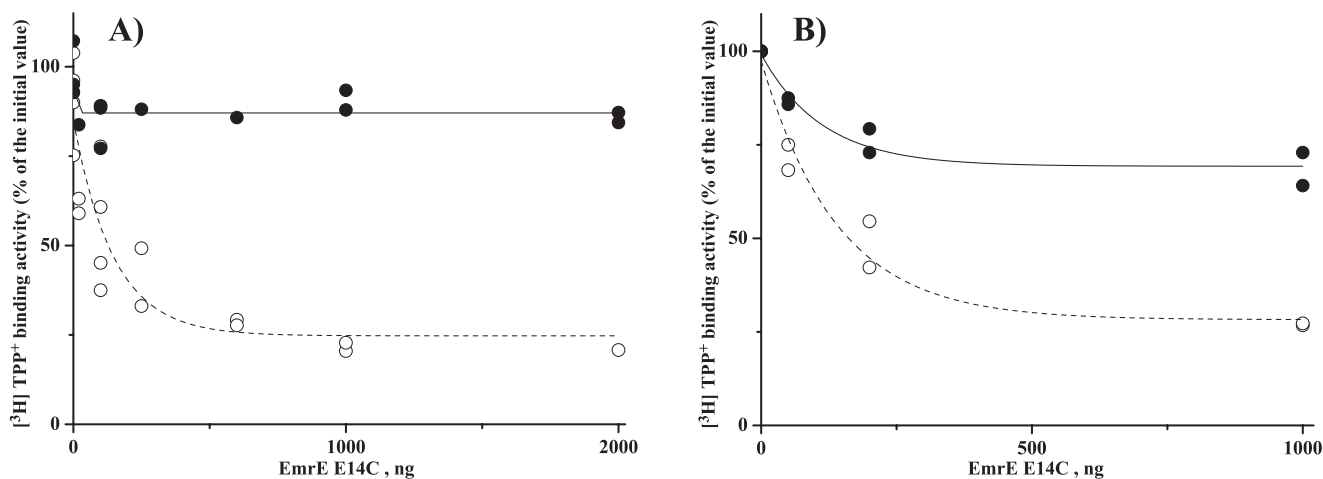


FIGURE 4. **Negative dominance does not occur when the functional dimer is cross-linked.** Increasing amounts of the inactive EmrE mutant E14C were added to 60 ng of the untreated (*open circles*) and cross-linked (*filled circles*) T108C (A) or to 100 ng of the corresponding D84C (B) proteins. After incubation at 80 °C (10 min), the mixture was transferred to 4 °C and assayed for [<sup>3</sup>H]TPP<sup>+</sup> binding as described under "Experimental Procedures."

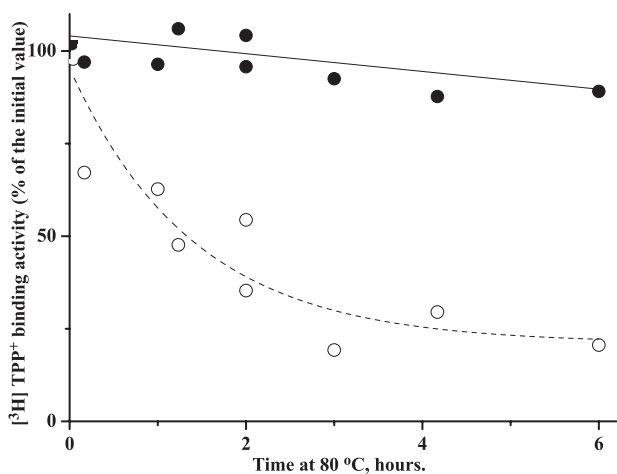


FIGURE 5. **Time course of the thermal denaturation of cross-linked T108C.** Purified proteins were diluted to ~0.1 μg/ml and subjected to incubation at 80 °C. At the indicated times, they were cooled to 4 °C and ligand binding was measured with 2.5 nM [<sup>3</sup>H]TPP<sup>+</sup> as described under "Experimental Procedures." Cross-linked T108C (*filled circles*); untreated T108C (*open circles*).

80 °C, whereas the untreated protein was already inhibited by 50% after 1 h. Overnight incubations at 80 °C inhibited practically all the activity of both proteins (not shown). The dramatic stability of the cross-linked protein to heat supports the contention that the first step in the denaturation involves dimer dissociation. The results also suggest that the functional unit is within the parallel cross-linked dimer.

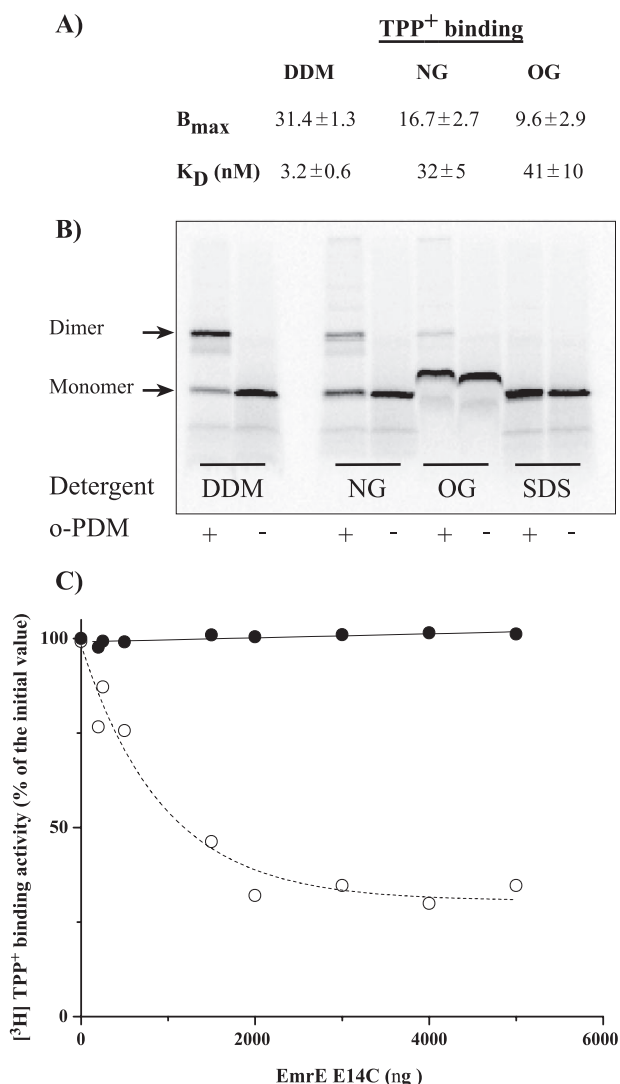
**Detergents That Inhibit Binding Activity Also Inhibit *o*-PDM-mediated Cross-linking**—Because our results demonstrate the functionality of an EmrE dimer with the protomers arranged in a parallel configuration, we searched for possible factors that could explain the generation of antiparallel dimers in the crystal structures published. We explored whether some of the conditions used to prepare the protein for crystallization could somehow affect activity and/or the oligomeric state. Because one of the most important factors determining the oligomeric state of a membrane protein is the nature of the detergent used for solubilization, we tested the effect of detergents used for crystallization on TPP<sup>+</sup> binding activity, a function that requires an intact dimer. When EmrE was

extracted with NG or OG the binding capacity of the protein was significantly diminished (16.7 and 9.6 pmol/μg EmrE, respectively, and Fig. 6A) as compared with the maximum measured with protein extracted with DDM (31.4 pmol/μg EmrE). The results suggest that in the presence of NG or OG only a fraction (53 and 30%, respectively) of the protein binds TPP<sup>+</sup>. In addition, the remaining functional fraction bound with an affinity 10-fold lower than that detected in the DDM protein, 32 and 41 nM for NG and OG, respectively, as compared with 3.2 nM in DDM.

To test whether the above inhibition was due to an effect on the oligomeric state of the protein we tested *o*-PDM cross-linking of EmrE with a single Cys at position 108 after solubilization with either one of the detergents. The results in Fig. 6B show that although a significant fraction of the protein (72%) was cross-linked when the detergent used was DDM, much lower cross-linking was observed when the detergents used were NG or OG (50 and 17%, respectively). As expected, SDS treatment completely inhibited cross-linking.

**Octyl Glucoside Influences Dimer-Monomer Equilibrium**—The results are consistent with the suggestion that treatment with either NG or OG affects and weakens the interaction of the monomers in the functional EmrE dimer. To test the possibility that a significant fraction of the protein is in the monomeric state we took advantage again of the fact that that hetero-oligomers with one inactive subunit display a lower affinity to TPP<sup>+</sup> (24) (Fig. 4). Mixing of DDM-solubilized EmrE with a large excess of the inactive mutant E14C did not impair the function of EmrE (Fig. 6C, *closed circles*) unless the dimer was dissociated by a short heat treatment (Fig. 4). We used this assay to assess whether a fraction of the OG-treated protein is dissociated. We solubilized EmrE and EmrE E14C with OG and added increasing amounts of the inactive protein to a constant amount of EmrE. After a short incubation DDM was added to a final concentration of 1%. The inhibition of activity caused by OG was fully reversible by the addition of DDM (Fig. 6C, no E14C added). Increasing amounts of E14C markedly inhibited the TPP<sup>+</sup> binding activity in a pattern practically identical to that observed using the heat treatment.

## Parallel Topology of EmrE



**FIGURE 6. Octyl and nonyl glucosides influence dimer-monomer equilibrium.** *A*, octyl and nonyl glucosides inhibit TPP<sup>+</sup> binding. [<sup>3</sup>H]TPP<sup>+</sup> binding was measured as described under "Experimental Procedures" at concentrations between 0.5 and 200 nM. The kinetic constants were calculated with Origin 7.0 software (OriginLab, Northampton, MA). *B*, octyl and nonyl glucosides inhibit o-PDM mediated cross-linking. EmrE T108C labeled with [<sup>35</sup>S]methionine (15) was extracted with either one of the detergents indicated at a concentration of 1% DDM, NG, and SDS and 2% OG. In the presence of OG, EmrE reproducibly displays an anomalous apparent higher *M<sub>r</sub>*. Cross-linking was performed essentially as described under "Experimental Procedures." The proteins were analyzed using SDS-PAGE, visualized with a Fujifilm LAS-1000 imaging system, and digitally analyzed with Image Gauge 3.46 Fujifilm software. *C*, octyl glucoside increases the fraction of monomeric EmrE. Increasing amounts of the inactive EmrE mutant E14C were added to 50 ng of CAMY. The membranes were solubilized in DDM (filled circles) or in OG (open circles). After solubilization DDM was added to the OG-treated samples and [<sup>3</sup>H]TPP<sup>+</sup> binding was measured as described under "Experimental Procedures."

## DISCUSSION

The recently suggested antiparallel topology of EmrE, a homodimeric H<sup>+</sup>-coupled multidrug transporter, has many intriguing implications regarding biogenesis, insertion, and evolution of ion-coupled transporters. In the present work we characterized a protein cross-linked via Cys residues at positions incompatible with an antiparallel topology. Experimental constraints provided by cross-linking experiments may be limited by the fact that they provide us with a snapshot of only one

of the possible conformations of the proteins and maybe even one that is not necessarily physiologically relevant. In addition, chemical modification of the protein required for cross-linking may result in misfolding or denaturation of the protein. Therefore, results obtained from cross-linking experiments have to be evaluated with caution. In this work, we used a most stringent criterion: we tested whether the protein was fully functional after cross-linking. We used o-PDM, a bifunctional rigid maleimide cross-linking reagent, to introduce stable covalent bridges between thiol groups separated by a distance that may vary within the 7–11 Å range (29). The choice of the mutants to use was based on our previous experiments that led us to suggest that intermonomer interface within the EmrE dimer is formed by TM1 and TM4 segments. The results also showed that residues at both the N- and C-terminal ends of TM4 are at similar distance from the neighbor TM4 and suggest a parallel arrangement. In addition, D84C and T108C, the two single cysteine replacements used in this work, are well tolerated by EmrE and fully functional according to phenotype, ligand binding, and transport assays (Ref. 26 and data not shown) and therefore should represent functionally folded protein.

Both published x-ray structures do not permit intermonomer homo-cross-linking mediated by o-PDM at either one of the above positions. The reaction at the positions we chose resulted in cross-linking of a large fraction of the protein but, as in most other documented cases, did not go to completion. Therefore, the cross-linked species were purified by ion exchange chromatography, and although we were able to purify the species cross-linked at position 108 to >90% homogeneity, we did not succeed in purifying the one cross-linked at position 84 over 40% purity. The purified protein cross-linked at position 108 displayed substrate binding and transport with kinetic properties similar to that of the non-cross-linked species. The affinity to TPP<sup>+</sup> and the number of binding sites detected (0.42 mol ligand/mol EmrE) suggest that practically all the protein is functional. After reconstitution, the T108C cross-linked protein displayed H<sup>+</sup>-driven transport of methyl viologen at rates and to levels practically identical to those displayed by the non-cross-linked species.

The cross-linked protein is fully functional, and practically every dimer has been shown to bind ligand as suggested by the fact that it can bind one mol TPP<sup>+</sup>/2.4 mol EmrE, only slightly lower than the previously determined stoichiometry. In any case, we challenged the contention that the functional unit may be formed by the interaction of two cross-linked dimers. To test this point we used two different approaches: first, taking advantage of the previously developed monomer-swapping method to probe the participation of the cross-linked proteins in oligomeric equilibrium and second, testing the impact of the cross-linking on the stability of EmrE toward heat denaturation. At both 84 and 108 positions, o-PDM cross-linking prevented EmrE from entering into oligomerization equilibrium. Both untreated T108C and D84C mutant proteins lost ~80% of the substrate binding activity while subjected to the monomer-swapping procedure with an excess of the inactive mutant EmrE E14C. Cross-linked T108C kept ~85% of its activity under the same conditions, whereas the cross-linked D84C

mutant lost 40% of the activity in accordance with the degree of cross-linking.

Detergent-solubilized wild-type EmrE is remarkably stable to heat denaturation. Still, incubation times at 80 °C longer than 30 min result in irreversible loss of the substrate binding activity. Because it is most likely that the first stage of the thermal denaturation of EmrE is dimer dissociation, it was logical to suggest that stabilization of functionally relevant intermonomer contacts will result in overall stabilization of the EmrE. Indeed, cross-linked T108C was found to be dramatically more stable toward the heat denaturation when compared with the untreated protein.

Cross-linking between dimers rather than within the dimer was also ruled out in our experiments. If it occurred once for a dimer-dimer interaction the result would be that half of the protein remains monomeric. If the cross-linking occurred more than once it would generate tetramers or even higher entities. This is not the case in our preparations where the cross-linked dimer is >90% pure. As mentioned above, the cross-linked and the untreated protein behave identically in size exclusion chromatography, showing that large complexes are not generated by multiple cross-linking between dimers.

Although it is clear from our results that the dimer with parallel topology is fully functional, we cannot rule out the existence of oligomers with antiparallel arrangement of the protomers. Several proteins in the SMR (small multidrug resistance) family have been suggested to function as hetero-oligomers, based on the finding that some pairs confer resistance phenotype only when co-expressed together. A bioinformatic analysis of this group of proteins suggests that the distribution of positive charges is different in a way that would predict a topology of opposite direction for each of the protomers, *i.e.* antiparallel (18). Based on this result and on a global analysis of the topology of all the *E. coli* membrane proteins, it was suggested that the hetero-oligomeric small multidrug resistance proteins may be in an antiparallel topology (18, 30). Further mutational studies showed that, as expected, the topology of EmrE in the membrane is sensitive to the distribution of positive charges in the protein in agreement with the well established positive inside rule for the topology of membrane proteins. For EmrE, the topological studies in intact cells in our laboratory suggest that all the protein is in a C-in configuration (14). The studies using PhoA and green fluorescent protein fusions suggest that all the fused "wild-type" protein is in a C-out configuration, *i.e.* high PhoA activity and practically nil green fluorescent protein fluorescence (18). The results with the mutated proteins may represent a mixture of configurations of dimers (some with C-out, some with C-in) but not necessarily anti-parallel protomers in the dimer. The discrepancy in the topology of the wild type between the two laboratories may stem from the fact that the constructs and the strains being used are different in what may be quite a significant way. Whereas our laboratory has added a Myc epitope before a His<sub>6</sub> tag at the C terminus (21), others have added a His<sub>6</sub> tag and protease cleavage sites at the N terminus (7, 8), and yet others have added large reporters such as PhoA and green fluorescent protein at the C terminus (18). This topic awaits a detailed

study, but in any case, none of the results thus far reported support an antiparallel topology of wild-type EmrE.

We present here a possible explanation for the antiparallel topology observed in the published structures. The dimeric structure of EmrE is sensitive to the nature of the detergent used. Whereas DDM-solubilized protein dissociates only under extreme conditions, some detergents, such as OG and NG, increase the fraction of the monomeric form of the protein. Monomers may form crystal contacts very different from those in the functional protein. Indeed, many monomeric proteins have been shown to form antiparallel crystallographic dimers (for examples, see Refs. 1, 31, 32). In the antiparallel dimer of EmrE observed in the crystal structure, the protomers are asymmetric and TPP<sup>+</sup> is bound to one of them (7). TPP<sup>+</sup> has indeed been shown to bind to EmrE with nanomolar affinity (21), but it has also been shown to bind to SDS-treated protein with ~four orders of magnitude lower affinity (33). In addition, TPP<sup>+</sup> and other similar substrates have been previously shown to bind nonspecifically to unrelated proteins and to membranes, most likely due to a mixed hydrophobic and electrostatic interaction (see, for example, Refs. 34 and 35, respectively). Because the crystallization was performed in the presence of millimolar concentrations of substrate and protein (7), TPP<sup>+</sup> may be binding non-specifically to EmrE rather than reflecting its location in the functional dimer.

In addition to supporting the conclusion that the parallel dimer is functional, our results contribute to our understanding of some functional aspects of EmrE. The protein can be fixed at either one of the two termini of TM4 and it can still function in substrate binding and, at least in the case of T108C, also in H<sup>+</sup>-coupled accumulation of methyl viologen. Therefore, the results support the conclusion that no sweeping relative movements of TM4 are necessary for catalysis.

We may speculate that the evolutionary challenge of recognition and transport of the wide spectra of the substrates may have selected for small multidrug resistance proteins permissive for both parallel and antiparallel orientation of the monomers within the dimer. In such a way one protein with the same sequence may extend the range of the substrate specificity in the very spirit of multidrug resistance. It appears, however, that most of the biochemical data obtained in our laboratory are relevant to the EmrE with the parallel orientation of the monomers within the dimer. Functionality of EmrE or close homologues with an antiparallel orientation of the monomers remains to be characterized.

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## REFERENCES

1. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) *Science* **301**, 610–615
2. Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) *Science* **301**, 616–620
3. Gouaux, E., and Mackinnon, R. (2005) *Science* **310**, 1461–1465
4. Locher, K. P., Bass, R. B., and Rees, D. C. (2003) *Science* **301**, 603–604

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- Andersen, O. S., Apell, H. J., Bamberg, E., Busath, D. D., Koeppe, R. E., II, Sigworth, F. J., Szabo, G., Urry, D. W., and Woolley, A. (1999) *Nat. Struct. Biol.* **6**, 609
- Davidson, A. L., and Chen, J. (2005) *Science* **308**, 963–965
- Pornillos, O., Chen, Y. J., Chen, A. P., and Chang, G. (2005) *Science* **310**, 1950–1953
- Ma, C., and Chang, G. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2852–2857
- Tate, C. G., Ubarretxena-Belandia, I., and Baldwin, J. M. (2003) *J. Mol. Biol.* **332**, 229–242
- Ubarretxena-Belandia, I., Baldwin, J. M., Schuldiner, S., and Tate, C. G. (2003) *EMBO J.* **22**, 6175–6181
- Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J. B., Engel, A., and Fujiyoshi, Y. (2000) *Nature* **407**, 599–605
- Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) *Nature* **415**, 287–294
- Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) *Nature* **437**, 215–223
- Ninio, S., Elbaz, Y., and Schuldiner, S. (2004) *FEBS Lett.* **562**, 193–196
- Soskine, M., Steiner-Mordoch, S., and Schuldiner, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12043–12048
- Pornillos, O., and Chang, G. (2006) *FEBS Lett.* **580**, 358–362
- Bowie, J. U. (2006) *Nat. Struct. Mol. Biol.* **13**, 94–96
- Rapp, M., Granseth, E., Seppala, S., and von Heijne, G. (2006) *Nat. Struct. Mol. Biol.* **13**, 112–116
- 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
- Tabor, S., and Richardson, C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1074–1078
- Muth, T. R., and Schuldiner, S. (2000) *EMBO J.* **19**, 234–240
- Steiner Mordoch, S., Granot, D., Lebendiker, M., and Schuldiner, S. (1999) *J. Biol. Chem.* **274**, 19480–19486
- Gutman, N., Steiner-Mordoch, S., and Schuldiner, S. (2003) *J. Biol. Chem.* **278**, 16082–16087
- Rotem, D., Sal-man, N., and Schuldiner, S. (2001) *J. Biol. Chem.* **276**, 48243–48249
- Elbaz, Y., Tayer, N., Steinfeld, E., Steiner-Mordoch, S., and Schuldiner, S. (2005) *Biochemistry* **44**, 7369–7377
- Yerushalmi, H., Mordoch, S. S., and Schuldiner, S. (2001) *J. Biol. Chem.* **276**, 12744–12748
- Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1995) *J. Biol. Chem.* **270**, 6856–6863
- Elbaz, Y., Steiner-Mordoch, S., Danieli, T., and Schuldiner, S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 1519–1524
- Green, N. S., Reisler, E., and Houk, K. N. (2001) *Protein Sci.* **10**, 1293–1304
- Daley, D. O., Rapp, M., Granseth, E., Melen, K., Drew, D., and von Heijne, G. (2005) *Science* **308**, 1321–1323
- Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N., and Spudich, J. L. (2001) *Science* **293**, 1499–1503
- Chang, G. (2003) *J. Mol. Biol.* **330**, 419–430
- Sikora, C. W., and Turner, R. J. (2005) *Biophys. J.* **88**, 475–482
- Molteni, V., Greenwald, J., Rhodes, D., Hwang, Y., Kwiatkowski, W., Bushman, F. D., Siegel, J. S., and Choe, S. (2001) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **57**, 536–544
- Lolkema, J. S., Abbing, A., Hellingwerf, K. J., and Konings, W. N. (1983) *Eur. J. Biochem.* **130**, 287–292