Scanning Cysteine Accessibility of EmrE, an H⁺-coupled Multidrug Transporter from Escherichia coli, Reveals a Hydrophobic Pathway for Solutes*

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EmrE is a 12-kDa Escherichia coli multidrug transporter that confers resistance to a wide variety of toxic reagents by actively removing them in exchange for hydrogen ions. The three native Cys residues in EmrE are inaccessible to N-ethylmaleimide (NEM) and a series of other sulphydryls. In addition, each of the three residues can be replaced with Ser without significant loss of activity. A protein without all the three Cys residues (Cys-less) has been generated and shown to be functional. Using this Cys-less protein, we have now generated a series of 48 single Cys replacements throughout the protein. The majority of them (43) show transport activity as judged from the ability of the mutant proteins to confer resistance against toxic compounds and from in vitro analysis of their activity in proteoliposomes. Here we describe the use of these mutants to study the accessibility to NEM, a membrane permeant sulphydryl reagent. The study has been done systematically so that in one transmembrane segment (TMS2) each single residue was replaced. In each of the other three transmembrane segments, at least four residues covering one turn of the helix were replaced. The results show that although the residues in putative hydrophilic loops readily react with NEM, none of the residues in putative transmembrane domains are accessible to the reagent. The results imply very tight packing of the protein without any continuous aqueous domain. Based on the findings described in this work, we conclude that in EmrE the substrates are translocated through a hydrophobic pathway.

EmrE is an Escherichia coli multidrug transporter that confers resistance to a wide variety of toxicants by actively removing them in exchange for hydrogen ions (1–4). EmrE is a highly hydrophobic 12-kDa protein that has been purified by taking advantage of its unique solubility in organic solvents. After solubilization and purification, it retains its ability to transport hydrophobic 12-kDa protein that has been purified by taking advantage of its unique solubility in organic solvents. After solubilization and purification, it retains its ability to transport

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without all the three Cys residues (CL) has been generated and shown to be functional (19). Using this Cys-less protein, we have now generated a series of 48 single Cys replacements throughout the protein. The majority of them (43) demonstrate transport activity as judged from the ability of the mutant proteins to confer resistance against toxic compounds and from in vitro analysis of their activity in proteoliposomes. Here we describe the use of these mutants to study the accessibility to NEM, a well characterized sulfhydryl reagent. The study has been done systematically so that in one TMS (TMS2) each single residue was replaced. In each of the other three TMSs, at least four residues covering one turn of the helix were replaced. The results show that although the residues in putative hydrophilic loops readily react, none of the residues in putative transmembrane domains is accessible to NEM. The results suggest very tight packing of the protein without any continuous aqueous domain. In striking contrast with the findings for the other ion-coupled transporters, the results with EmrE suggest the existence of a hydrophobic pathway through which the substrates are translocated.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *E. coli* JM110 (20) and plasmid pKK56-CL (5, 19) are used throughout this work. In plasmid pKK56-CL, EmrE is cloned into EcoRI and HindIII sites of pKK223-3 (Amersham Pharmacia Biotech). In addition, the three cysteines (Cys19, Cys41, and Cys69) have been replaced with Ser (19).

**Mutagenesis—** Mutants were obtained by polymerase chain reaction mutagenesis using the overlap extension procedure described by Ho et al. (21). For each mutation a set of two overlapping oligonucleotide primers containing the desired mutation were constructed. The outside primers were those used for the wild type EmrE (5). The template was always CL-EmrE.

Mutagenic oligonucleotides were prepared incorporating a unique restriction site to facilitate mutant identification, and in several cases this required an additional conservative mutation. Mutated DNA was identified by the acquisition of the new restriction site and sequenced (ABI PRISM™ 377, Perkin-Elmer) to ensure that no other mutations occurred during the amplification process.

**Resistance to Toxic Compounds—** For testing resistance to toxic compounds, cells were grown overnight at 37 °C in LB-Amp medium. 5 μl of serial 10-fold dilutions of the cultures (10⁻¹–10⁻⁶) were spotted on plates containing the desired compounds as indicated for each experiment. Growth was visualized after 24 h at 37 °C. The assay was repeated at least three times for each mutant.

**Expression, Purification, and Reconstitution of EmrE—** *E. coli* JM110 transformed with the appropriate plasmid was grown in minimal medium A supplemented with glycerol (0.5%), thiamine (2.5 μM), and ampicillin (50 μg/ml), and a mixture of amino acids (MEM amino acids; Sigma). When the culture reached an A₆₀₀nm of 0.8, isopropyl-β-D-thiogalactoside was added to 0.5 mM; 2 h later, the cells were harvested by centrifugation.

EmrE was extracted essentially as described (5) from 50 mg of cells (wet weight) with 550 μl of chloroform:methanol (1:1) incubated for 20 min on ice. For phase separation 110 μl of H₂O were added, and the suspension was centrifuged. The upper phase was removed, and 4 volumes of acetone were added to the lower phase. After at least 1 h at −20 °C, EmrE was collected by centrifugation, washed with cold acetone, and dried. For analysis in SDS-polyacrylamide gel electrophoresis, the sample was resuspended in sample buffer and analyzed in 16% gels as described (22).

For reconstitution, 4–9 μg of extracted protein was mixed with *E. coli* phospholipids (18 μl of a suspension of 50 mg/ml in water diluted prior to the experiment in 180 μl of chloroform:methanol, 1-2) and 750 μl of chloroform:methanol (1:1). The suspension was dried with Argon and resuspended in a solution (60 μl) containing 0.18 M NH₄Cl, 0.015 M Tris-HCl, pH 6.9. The suspension was 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 35 μM [¹⁴C]methyl viologen (90 nCi), 140 mM KCl, 10 mM Tricine, and 5 mM MgCl₂, pH 9.5. At given times the reaction was stopped by dilution with 2 μl of the same ice cold solution, filtering through Schleicher & Schuell filters (0.2 μm), and washing with an additional 12 μl of solution. The radioactivity on the filters was estimated by liquid scintillation. In each experiment the values obtained in a control reaction with 5 μM nigericin were subtracted from all experimental points. This background was between 5 and 10% of the experimental values. The assay was done in triplicates and repeated at least three times.

**Labeling with [¹⁴C]NEM and Measurement of Expression Levels—** Cells (50 mg of wet weight) were suspended in 500 μl of a solution containing 100 mM potassium phosphate, pH 7.5, and 5 mM MgSO₄. [¹⁴C]NEM (NEN Life Science Products) was added to a final concentration of 0.5 mM (2 Ci/mmol). After 20 min at room temperature the reaction was stopped by addition of 20 mM dithiothreitol. After 10 min 1 ml of above buffer was added, and the cells were collected by centrifugation and washed once with the same buffer. EmrE was extracted from the pellet as described above and analyzed in SDS gels. The amount of EmrE in each mutant was estimated from the Coomassie-stained gels. The amount of radioactivity associated with EmrE was quantitated after exposure of the gel to a Phosphorimager FUJIX BAS 100. The assay was repeated at least three times for each mutant protein.

**RESULTS**

A Vast Majority of the Residues Can Be Replaced with Cys without Loss of Activity—The CL-EmrE protein displays significant transport activity and therefore provides a good starting point for generation of mutant proteins with single Cys replacements. In this work we report the results obtained after replacing all the amino acids (starting at position 28 and continuing up to position 57) in TMS2 and its adjacent loops (Fig. 1). In addition, at least four mutants (one helical turn) were analyzed in each of the other TMSs and one in each hydrophilic loop (Fig. 1), a total of 48 single Cys replacements.

The activity of each of the mutant proteins has been tested in vivo and in vitro. In vivo, the resistance conferred by each of the proteins was assessed by testing the ability of cells expressing them to grow under otherwise nonpermissive conditions. This was achieved in solid media containing either ethidium (200 μg/ml), acriflavine (100 μg/ml), or methyl viologen (0.1 mM) in which 5 μl of logarithmic dilutions of an overnight culture were spotted (Fig. 2, three right panels). Cells carrying the vector plasmid without any insert cannot grow in these media at any of the dilutions tested. Cells expressing either wild type EmrE or the CL-EmrE were able to grow at each of the dilutions. At the high dilutions (10⁻³–10⁻⁵), the single colonies observed displayed a similar size indicating similar growth rates. As expected (not shown) all the cells grew to a similar degree in control plates containing only ampicillin and none of the toxic compounds. This assay provides a dynamic range to qualitatively analyze activity of the mutants generated. We assume that growth at dilutions of 10⁻³–10⁻⁵ is a result of robust EmrE activity. Growth on ethidium is taken as the standard because, as will be seen below, in some mutants the specificity toward other substrates may be modified.

In TMS2 and the adjacent loops, 28 of the 30 mutants generated were capable of conferring significant resistance to ethidium. Most (25 mutants) were indistinguishable from the wild type; growth was observed also at dilutions of 10⁻⁴ or 10⁻⁵. Two mutant proteins (Y40C and F44C) conferred no measurable resistance. Three mutant proteins (V34C, C41, and I54C) seemed to be less effective than the wild type.

The results for mutants in the other TMSs are summarized in

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2 The mutants are named as follows: single amino acid replacements are named with the letter of the original amino acid, then its position in the protein, and the letter of the new amino acid. The protein in which all the cysteines were replaced is named CL. All the proteins described here with single Cys residues are in a CL background and therefore only the position of the single Cys residue is mentioned. The three mutants C39, C41, and C95 retain each of the Cys residues present in the wild type protein, whereas the other two were mutated to Ser.
In TMS1 single unique replacements were generated distal and terminal to the essential glutamyl residue at position 14 (8). Mutant proteins I11C, L12C, A13C, V15C, I16C, G17C, and T18C were tested for function (Fig. 3). Although T18C and I11C showed no activity, G17C displayed some marginal activity. The other four mutants displayed an activity indistinguishable from that of the wild type protein. Mutants in TMS3, S72C, L73C, L74C, and S75C displayed resistance to ethidium similar to wild type. Mutants in TMS4, I94C, C95, A96C, and G97C displayed wild type activity, whereas L93C showed no activity at all in this assay. Cysteine replacements in loops 4 and 5 (L83C and H110C) exhibited full activity. The levels of resistance to different substrates are being used here to obtain a very qualitative idea of the activity of the various mutants. However, no absolute quantitation can be made because the levels of expression of EmrE do differ among the various mutants. Notwithstanding this limitation, several important conclusions can be made. Five mutant proteins are incapable of conferring measurable resistance: I11C and T18C in TMS1, Y40C and F44C in TMS2, and L93C in TMS4. Except for I11C and T18C, the mutants are not expressed to detectable levels even under full induction of expression with isopropyl-β-D-thiogalactoside (not shown). These residues may play a role in folding and stability of the protein. All the other mutants tested, albeit expressed to diverse levels, are capable of conferring some degree of resistance to ethidium. We conclude that with the exception of the above mentioned residues, each one of the other 43 residues tested can be replaced with Cys and produces a mutant protein that maintains an activity significant enough to confer resistance to ethidium. The fact that such a large number of residues are so permissive has been observed with other transporters as well (12, 16, 23). It is noteworthy that even residues (such as Val 15, Arg29, Pro32, Leu47, Thr50, Pro55, Ile94, and Gly97) that are highly conserved in the family of the MiniTEXANs (1, 24) can be replaced without loss of phenotypic complementation. The conservation of these residues may be necessary for functions other than the one measured and/or for stability of the protein.

Some Replacements Modify the Specificity toward Other Substrates—EmrE is a multidrug transporter and, as such, transports a variety of toxic substrates. The specificity of all the mutants to several substrates has been compared using the phenotypic assay described above (Figs. 2 and 3). In general, growth in the presence of acriflavine yielded a pattern very similar to that displayed in ethidium with the exception of three mutants in TMS3 that displayed a decreased resistance to this toxic agent: S72C, L74C, and S75C (Fig. 3). A few strains in TMS2 (V34C, T36C, A52C, and I54C) also showed some decrease of resistance to acriflavine (Fig. 2). More dramatic differences were detected when growth was challenged with 0.1 mM methyl viologen.

**Fig. 2.** Growth phenotype of cells expressing single Cys replacements in TMS2 and its distal and proximal loops. E. coli JM109 cells transformed with pKK56-EmrE (WT), pKK56-CL (CL), pKK223–3 (vec), or the various mutants were grown overnight at 37 °C in LB-Amp medium. 5 μl of 10⁻¹–10⁻⁶ dilution of the culture were spotted on a series of LB-Amp plates with various EmrE substrates or on a plate with no addition. Growth was analyzed after overnight incubation at 37 °C. With the three toxicants used, growth of cells bearing pKK56-CL or pKK56-WT EmrE was indistinguishable. At the concentrations used in this experiment, growth of pKK223–3 bearing cells was undetectable at each of the dilutions used (see **three right panels** for each of the compounds tested). Growth in the control plates was practically identical for all mutants tested (not shown). The heights of the bars indicate the maximal dilution at which cell growth was detected. Mutants Y40C and F44C were not expressed at detectable levels.

**Fig. 3.** In TMS1 single unique replacements were generated distal and terminal to the essential glutamyl residue at position 14 (8). Mutant proteins I11C, L12C, A13C, V15C, I16C, G17C, and T18C were tested for function (Fig. 3). Although
showed robust resistance to ethidium and acriflavine but no resistance to methyl viologen. I54C showed a very significant resistance to ethidium and acriflavine but no resistance to methyl viologen. A few others are also noteworthy: V34C, T36C, C41, A42C, L47C, Q49C, and G57C displayed 2–3 orders of magnitude less resistance to methyl viologen, relative to the others (Fig. 2).

Most Mutants Are Also Functional after Purification and Reconstitution in Proteoliposomes—All the mutant proteins have been purified and assayed for ΔpH driven [14C]methyl viologen uptake activity in proteoliposomes. Most of them (34 mutants) display significant levels of transport that range between 200 and 2000 pmol/min/mg. In addition to the five mutant proteins that do not display any activity in vivo, a few others lose practically all their activity (100 pmol/min/mg protein) upon purification. Even though they display activity in vivo, no activity was detected with protein extracted from: L12C and A13C in TMS 1; P32C, P55C, T56C, and G57C in the loops either distal or proximal to TMS2; and S72C and L74C in TMS3. These residues may be important for the proper folding after the organic solvent treatment or for proper reconstitution into the proteoliposomes. Other assays, independent of the extraction with organic solvents, are now being developed to further test this phenomenon.

Cys Residues in Positions at Putative Transmembrane Domains Are Inaccessible to [14C]NEM—We have previously shown that the activity of the wild type protein is unaffected by exposure to NEM and other sulfhydryls even at high concentrations (19). We interpreted these results as suggesting that the Cys residues are not accessible to NEM. We now confirm this conclusion by directly measuring accessibility of the Cys residues to [14C]NEM and extend our studies to all the single Cys replacements generated. Each mutant protein was challenged with the radiolabel in the intact cell. It was then partially purified, and the radioactivity associated with it was measured after separation on SDS-polyacrylamide gel electrophoresis. Using this experimental protocol, neither the wild type protein nor the fully Cys-less protein (CL-EmrE) were labeled at all (Fig. 4). As expected, T28C, a mutant with a Cys residue in a putative hydrophilic loop, was fully labeled. From quantitation of the amount of radioactivity incorporated and the protein in the gel, it can be estimated that about 1 mol of [14C]NEM was incorporated per mol of T28C. To demonstrate that the lack of labeling of the wild type protein is due to a structural constraint, EmrE was denatured with a mixture of 2% SDS and 8 M urea. Under these conditions all three Cys residues are fully accessible, and about 3 mol NEM are incorporated per mol EmrE. As expected, no further increase in the labeling level was observed under these conditions in either CL or T28C. These results confirm our hypothesis that the Cys residues in the wild type protein are inaccessible to NEM and allow for a detailed study of the reactivity of Cys at each one of

FIG. 3. Growth phenotype of cells expressing single Cys replacements in various regions of EmrE. Reaction conditions are as described for Fig. 2, except that dilutions of only 10^-4–10^-6 were tested. In addition, in cases where, at the highest dilution, smaller colonies were detected after overnight incubation, the bars are open. Mutant L93C was not expressed at detectable levels.
the mutants generated.

The above experimental paradigm was used to assess the level of labeling of all the functional single Cys replacements generated in this work. The results in Fig. 5 describe the labeling of residues in TMS2 and the adjacent loops. A distinct pattern of labeling was observed in which three domains are apparent: in the first group (from Thr28 to Ser33) all the residues were labeled, although to varying degrees (between 30 and 100%). We suggest that these residues are exposed to solvent and form part of loop 2. A similar conclusion is reached with another group of residues starting at Tyr53 and continuing to Gly57. We conclude that these residues form part of loop 3. A very different behavior is observed with a large group of residues inaccessible to [14C]NEM. This group includes all the residues at positions 34–52. In several mutants (V34C, T36C, A42C, S43C, L46C, and T50C), there was no measurable radioactivity associated with the protein. In the others, with two exceptions, the level was significant but always lower than 10%. The only two mutants in this domain that are labeled to levels of about 20% are Q49C and L51C.

To assess the rates of labeling, two fully accessible mutants were compared with two that are not labeled. Although both T50C and T36C are not labeled even after 2 h incubation, T28C and G57C are fully labeled after 5–10 min (data not shown).

To show that the lack of labeling is due to structural constraints, all the proteins that are inaccessible to [14C]NEM in the intact cell were challenged with the label after purification and solubilization in the presence of 2% SDS. Most of the mutant proteins reacted with the sulfhydryl reagent under these conditions (data not shown). However, three of them, V34C, L47C, and A48C, were accessible only upon treatment with harsher denaturing conditions (2% SDS and 8 M urea).

A similar picture is uncovered when the accessibility of residues in the other three TMS is analyzed. The results of this study are summarized in Fig. 6. In each TMS, at least four contiguous residues were replaced with Cys and challenged with NEM. In TMS1, L12C, A13C, and I16C were nearly completely inaccessible. Low labeling of mutants V15C (13% of T28C) and G17C (20% of T28C) was observed (Fig. 6). In TMS3, mutants S72C, L73C, and L74C showed no reactivity whatsoever, whereas S75C was labeled to a low (23% of T28C) but significant degree. In TMS4, mutants I94C, C95, A96C, and G97C were not labeled to any measurable degree. As expected, residues in hydrophilic loops 4 (L83C) and 5 (H110C) were fully labeled.

**DISCUSSION**

Only a very small number of residues appear essential to EmrE function. In this work we describe the generation of single Cys replacements in 48 residues, about 50% of the protein, and nearly all of them (43 residues) show a measurable activity. Also, 7 of the 8 charged residues in the protein can be replaced without loss of activity. Interestingly, even residues conserved in the family can be replaced without loss of activity. As mentioned above, the conservation may be necessary for functions other than the ones we are testing. Among the inactive mutants, in three cases (Y40C, F44C, and L93C) the protein is not detectable at measurable levels and therefore little can be concluded about the role of these residues on catalytic activity. It is possible that they play a role in folding, insertion, or stability of the protein. Replacement of Ile11 and Thr18 with Cys yields practically inactive proteins. It is interesting that both Ile11 and Thr18 are located at a distance of about one helical turn from the Glu14, the only charged residue in the putative membrane domain. Glu14 cannot be replaced even with Asp, suggesting a central role in the catalytic cycle (8, 24). The role of both residues needs to be studied further by a

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3. H. Yerushalmi and S. Schuldiner, unpublished results.
more detailed characterization of the mutant proteins and by
replacements with other amino acids. In addition to Ile\textsuperscript{14},
Glu\textsuperscript{11}, and Thr\textsuperscript{18} only two other residues, Tyr\textsuperscript{28} and Trp\textsuperscript{28}
in TMS3, have been previously shown to be important for activity.
Even substitution with other aromatic amino acids caused a
complete loss of activity (8). The finding that most residues of
ion-coupled transporters can be replaced without serious im-
pairment of function has already been described and discussed
in extensive studies of the E. coli lac permease (16). In these
studies, only six positions (of 417 positions) are clearly irreplace-
able. It has been suggested that the role of most of the nonessen-
tial amino acids may be to provide a structural scaffold.

In our studies, mutagenesis of two groups of residues has
generated proteins with modified specificity to at least one of
the substrates of EmrE. Practically all replacements in TMS3
display decreased resistance to acriflavine. In TMS2, a total of
eight mutants show a significant decreased resistance to
methyl viologen. They seem to cluster on two faces of the
\alpha-helix (Fig. 7): one group at positions 36, 47, and 54 and the
other group at positions 34, 41, 48, and 52 with 42 somewhat
close to this cluster. Interestingly, the first group is in the same
face of two proteins that are not expressed (Y40C and F44C).
These mutants provide an excellent tool for further investiga-
tion of the location of the binding contacts of substrates.

The results described in this work demonstrate that all the
residues in putative membrane domains of EmrE are prac-
tically inaccessible to \textsuperscript{[14C]}NEM (Fig. 1). The reaction of male-
hydrides with sulphydryl groups involves addition of mercaptide
ions in the protein to the olefinic double bond (25). Therefore
low levels of labeling may result from either one of the follow-
ing reasons or a combination of them: steric hindrance, lack of
ionization of the sulphydryl group, or low reactivity because of
neighboring residues. NEM is a relatively hydrophobic and
small maleimide that can freely cross lipid membranes. The
latter contention is supported in this work by the fact that
residues in all the hydrophilic loops are fully accessible. In
loops 2 (residues Thr\textsuperscript{28} to Ser\textsuperscript{38}) and 3 (Tyr\textsuperscript{28} to Gly\textsuperscript{37}), all the
residues react with NEM. The lower reactivity of some of the
residues in hydrophilic loops (such as at the position of Leu\textsuperscript{28})
may reflect steric hindrance, because clearly its neighbors react
quantitatively. Residues at positions Pro\textsuperscript{32} and Ser\textsuperscript{33} and Tyr\textsuperscript{33}
to Gly\textsuperscript{36} may be at the edge of the membrane domain. In loop 4
and at the C terminus one residue was tested, and they were
shown to be fully accessible to NEM.

In contrast, none of the residues in putative transmembrane
domains reacted with NEM to a significant degree. In this
domain some of the sulphydryl residues face the lipid milieu,
and they do not seem to be able to release their proton and
therefore cannot react with NEM. As for the rest of the resi-
dues, very tight packing of the helices would prevent even
relatively small reagents such as NEM from approaching.

In the case of other proteins such as the Tn-10 encoded
tetracycline/H\textsuperscript{+} antiporter and the erythrocyte anion ex-
changer, evidence has been provided that residues in some
transmembrane domains are inaccessible to the permeant sul-
hydryl reagent NEM (12–14). It was therefore suggested that
NEM could be used to identify the membrane embedded dom-
ains. However, other studies have clearly identified reactive
residues in membrane domains (15–18). The reactive residues
are thought to delineate water-filled cavities that may repre-
sent part of the substrate translocation pathway. Also, in the
extensive studies performed with the E. coli lac permease parts
of the membrane embedded areas were found inaccessible to
NEM. Yet, many others reacted freely with NEM (16). Indeed,
both in the lac permease (16) and in UhpT (17), the NEM-
reactive residues were shown to be located in the vicinity of the
binding site of the hydrophilic substrates. Unlike in the other
transporters, in EmrE, every single TMS was shown to be
inaccessible to NEM, suggesting that the substrates are not
translocated through an hydrophilic pathway.

The results described in the present work cannot exclude the
presence of a highly selective filter that prevents molecules
other than substrates from accessing a putative aqueous path-
way in EmrE. However, this possibility is refuted by Fourier
transform infrared studies of EmrE in which we found that a
large fraction of the amide protons do not readily exchange
with solvent deuterium (6). Taken as a whole, the results
described support the model of a tightly packed four helix
antiparallel bundle in which the majority of the protein is well
embedded in the membrane and not accessible to solvent.
The boundaries of the embedded segments as estimated with this
technique are in remarkably good agreement with the second-
ary structure determined from the NMR analysis of the protein
(7).

In the case of EmrE, the substrates are quite hydrophobic,
and therefore it maybe energetically more favorable to interact
directly with the protein rather than permeate through a wa-
ter-filled pathway. Movement of substrates through a tightly
packed protein must require disruption and reorganization of
the existing structure. These types of interaction have been
observed in several cases. For example, NMR shows that the
singular structures of soluble synthetic four helical bundles
adopt a disordered array of states upon binding a hydrophobic
heme cofactor. The interaction of the heme with the polypep-
tide is quite specific and displays a relatively high affinity in
the nanomolar range (26). Tetraphenylphosphonium, a high
affinity substrate of EmrE, interacts specifically also with
BmrR, a transcription activator of the Bmr gene, a Bacillus
subtilis multidrug transporter (27). In this protein, the tetra-
phenylphosphonium binds to a hydrophobic pocket with a key
electrostatic component (Glu\textsuperscript{134}) at its bottom (28). The
entrance of tetraphenylphosphonium to the binding pocket occurs
after unfolding of a nine-residue \alpha-helix. At present, without
further structural information, we can only speculate that a
similar type of binding site may exist in EmrE as well where
only one negatively charged and essential Glu residue (Glu\textsuperscript{14})
is present in the putative transmembrane domains of the pro-
tein. The results presented above also suggest that entrance of
the ligand to the binding site may require movement of parts of
the protein.
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