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van Geest, M; Lolkema, JS

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Transmembrane Segment (TMS) VIII of the \(\text{Na}^+/\text{Citrate}\) Transporter CitS Requires Downstream TMS IX for Insertion in the \(\text{Escherichia coli}\) Membrane*

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Marileen van Geest and Juke S. Lolkema‡

From the Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands

The amino acid sequence of the sodium ion-dependent citrate transporter CitS of \(K.\ pneumoniae\) contains 12 hydrophobic stretches that could form membrane-spanning segments. A previous analysis of the membrane topology in \(E.\ coli\) using the PhoA gene fusion technique indicated that only nine of these hydrophobic segments span the membrane, while three segments, Vb, VIII and IX, were predicted to have a periplasmic location (Van Geest, M., and Lolkema, J. S. (1996) \textit{J. Biol. Chem.} 271, 25582–25589). A topology study of C-termi-
nally truncated CitS molecules in dog pancreas microsomes revealed that the protein traverses the endoplas-
mic reticulum membrane 11 times. In agreement with the PhoA fusion data, segment Vb was predicted to have a periplasmic location, but, in contrast, segments VIII and IX were found to be membrane-spanning (Van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) \textit{J. Biol. Chem.} 274, 2816–2823).

In the present study, using site-directed Cys labeling, the topology of segments VIII and IX in the full-length CitS protein was determined in the \(E.\ coli\) membrane. Engineered cysteine residues in the loop between the two segments were accessible to a membrane-imperme-
able thiol reagent exclusively from the cytoplasmic side of the membrane, demonstrating that transmembrane
segments (TMSs) VIII and IX are both membrane-span-
nig. It follows that the folding of CitS in the \(E.\ coli\) and endoplasmic reticulum membrane is the same. Cysteine accessibility studies of CitS-PhoA fusion molecules demon-
strated that in the \(E.\ coli\) membrane segment VIII is exported to the periplasm in the absence of the C-termi-
nal CitS sequences, thus explaining why the PhoA fu-
sions do not correctly predict the topology. An engi-
neered cysteine residue downstream of TMS VIII moved from a periplasmic to a cytoplasmic location when the fusion protein containing TMSs I–VIII was extended with segment IX. Thus, downstream segment IX is both essential and sufficient for the insertion of segment VIII of CitS in the \(E.\ coli\) membrane.

In the endoplasmic reticulum (ER),¹ insertion of integral membrane proteins into the membrane is mediated by the same machinery that is responsible for the translocation of preproteins to the lumen. Preprotein translocation and mem-
bane protein insertion are inherent functions of the Sec ma-
achinery (1–3). Evidence is accumulating that also in bacteria the secretion machinery is involved in the integration of mem-
brane proteins in the cytoplasmic membrane (4–7), although there are differences between the systems. In the ER, translo-
cation and insertion proceed cotranslationally and are driven by the synthesis of the nascent chain on the ribosome. In \(E.\ coli\), translocation is post-translational and is driven by ATP hydrolysis catalyzed by the SecA subunit, a component not present in the eukaryotic system, and by the proton motive force across the membrane (8–10).

We have analyzed the membrane topology of the \(\text{Na}^+\)-de-
pendent citrate transporter CitS of \(Klebsiella pneumoniae\) upon insertion in both the ER membrane and the \(E.\ coli\) cyto-
plasmic membrane. The resulting models showed remarkable similarities as well as remarkable differences. The amino acid sequence of CitS contains 12 hydrophobic segments that are long and hydrophobic enough to span the membrane in \(\alpha\)-helical form, suggesting a membrane topology with 12 putative transmembrane segments (TMSs) (11). Analysis of a series of C-terminally deleted CitS molecules fused to the re-
porter molecule alkaline phosphatase (PhoA fusions) expressed in \(E.\ coli\) indicated that only nine of the 12 hydrophobic seg-
ments were transmembrane, while the three remaining seg-
ments, Vb, VIII, and IX, were in the periplasm (Fig. 1A). The cytoplasmic and periplasmic localization of the \(\text{NH}_2\) and \(\text{COOH}\)
terminus, respectively, was confirmed by tagging of the termini of CitS with the biotin acceptor domain of the oxaloacetate decarboxylase of \(K. pneumoniae\) (12). The same series of C-
terminally truncated CitS molecules was expressed in ER mi-
crosomes, using leader peptidase as the insertion vehicle and the leader peptidase P2 domain as the topological reporter (13). Similar to the bacterial studies, it was found that segment Vb was not transmembrane but was translocated to the lumen. However, in contrast to what was observed in the bacterial system, segments VIII and IX were found to be transmem-
brane, resulting in a membrane topology with 11 TMS in the ER membrane (Fig. 1B). The difference in the two models arises from fusion constructs with the reporter fused to sites in between hydrophobic segments VIII and IX. In the microsomes, the reporter remained in the cytoplasm, while in \(E.\ coli\) the reporter was located in the periplasm, even when the CitS moiety contained 23 residues of the positively charged, hydro-
philic loop between the two segments.

The two folding models obtained for CitS may be explained in either of two ways: (i) both models are correct, meaning that the bacterial and eukaryotic insertion machineries fold the same polypeptide in two different ways; or (ii) the folding of

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‡ To whom correspondence should be addressed: Dept. of Microbiol-
ogy, University of Groningen, Kerklaan 30, 9751 NN Haren, The Neth-
erlands. Tel.: 31-50-3632155; Fax: 31-50-3632154; E-mail: j.s.lolkema@
biol.rug.nl.
¹ The abbreviations used are: ER, endoplasmic reticulum; AmdSiS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; MBP, 3-[(4-n-
ma-leimidopropynyl)biocytin; PCR, polymerase chain reaction; ISO, inside-
out; PAGE, polyacrylamide gel electrophoresis.

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CitS in the two systems is the same, but not all truncated fragments have the same membrane topology as in the full-length protein. More concretely, the hybrid protein with the fusion site between segments VIII and IX would wrongly indicate the localization of the fusion site in the full-length protein either in the bacterial or microsomal system.

In the present study, we used site-directed cysteine labeling to determine the localization of segments VIII and IX in the full-length CitS protein in the *E. coli* membrane. The results demonstrated that both segments are transmembrane, showing that the folding of the CitS molecule is the same in the ER and *E. coli* membrane. It is concluded that CitS fragments of the fusion proteins with fusion sites between segment VIII and IX do not fold correctly in the *E. coli* membrane in the absence of the downstream CitS sequences. To understand the misfolding of the CitS fragment, we subsequently studied the folding of a number of CitS-PhoA fusion proteins by examining the accessibility of native and introduced cysteine residues in the CitS moiety. It followed that segment VIII has a periplasmic location in the truncated CitS molecule lacking the three C-terminal transmembrane segments, while the addition of downstream TMS IX was found to be both essential and sufficient for insertion of TMS VIII in the *E. coli* membrane. The accessibility of the Cys residues in the different truncated CitS fragments showed the interaction between successive transmembrane and loop domains as the protein is inserted into the membrane. The results are discussed in the context of the insertion mechanism of CitS in the *E. coli* membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ni²⁺-nitrilotriacetic acid resin was obtained from QiAGEN, and monoclonal antibodies against alkaline phosphatase were from Chemicon International, Inc. (Temecula, CA). 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AmdiS) and 3-(N-maleimidomaleimidylpropyl)biocytin (MBP) were purchased from Molecular Probes, Inc. (Eugene, OR). Oligonucleotides were obtained from Eurosequence (Groningen, The Netherlands). Immunopure streptavidin was obtained from Pierce.

**Bacterial Strains and Growth Conditions**—*E. coli* strains BL21(DE3) and MC1061 were routinely grown in Luria broth medium at 37 °C. *E. coli* derivatives cloned in the vector pBluescript II SK (Stratagene, La Jolla, CA) were expressed in *E. coli* BL21(DE3), and derivatives were cloned in pBluescript II KS in *E. coli* MC1061. Expression was obtained without induction. Carbenicillin was added at a final concentration of 100 μg/ml. Citrate transport activity in the recombinant strains was detected as blue halos around colonies on Simmons citrate agar plates (Difco). Alkaline phosphatase activity was detected by blue colonies on Luria broth agar plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; XP) at a concentration of 40 μg/ml.

**Genetic Manipulations**—Standard recombinant DNA procedures were used essentially as described by Sambrook et al. (14). All of the fragments obtained by polymerase chain reaction (PCR) were sequenced after subcloning using an automated sequence.

**Construction of pBlHisCitS**—The *citS* gene with a *Nco*I site in the start codon was isolated from pSN1 (12) by digestion with *Nco*I and *Xba*I and was ligated into plasmid pKShis digested with the same two enzymes, resulting in pBlHisCitS. The host vector pKShis, previously described by Gaillard et al. (15), is a modified pBluescript II KS phagemid carrying a linker that codes for six histidine residues flanked at the 3′-end by a *Nco*I site in the start codon of LacZ. In plasmid pBlHisCitS, the *citS* gene follows the His tag coding sequence.

**Construction of pG315C and pS333C**—Single cysteine substitutions were made in the *citS* gene at position 315 (G315C) and at position 333 (S333C) by oligonucleotide-directed site-specific mutagenesis using a two-step PCR method. Both positions are on a DNA fragment flanked by unique *Cel*II (around residue 240) and *Pst*I (around residue 596) restriction sites in the *citS* gene. In the first step, to construct G315C, two PCR products were obtained using pBlHisCitS as template DNA and the following combinations of primers (Table I): (i) the *Cel*II forward primer and the mutagenic G315C reverse primer and (ii) the mutagenic G315C forward primer and the PhoA reverse primer. The two partially overlapping PCR products were purified from an agarose gel and mixed.

The mixture was used as template DNA in a second PCR step using the *Cel*II forward primer with the PhoA reverse primer. The resulting fragment was digested with the *Cel*II and PhoA restriction enzymes and exchanged with the equivalent fragment of pHisCitS. The pg333C mutant was made in a similar way.

**Construction of pSA328C315 and pSA360C315**—Plasmid pg315C, carrying the *citS* gene with the G315C mutation, was used to substitute the three wild-type cysteine residues Cys⁷⁷⁰, Cys⁷⁸⁷, and Cys⁸⁴⁷ for serine residues by a three-step PCR method. The three positions are on the same *Cel*II/PhoA fragment described above. In the first step, two PCR fragments (A and B) were obtained using pg315C as template DNA and the following combinations of primers (Table I): (i) the *Cel*II forward primer and the mutagenic C278S reverse primer (fragment A), (ii) the mutagenic C278S forward primer and the mutagenic C347S reverse primer (fragment B). The two partially overlapping fragments were purified from an agarose gel, mixed, and used as template DNA in the second PCR step using the *Cel*II forward primer and the C347S reverse primer, resulting in fragment AB. Following the same procedure, a fragment CD was obtained by combining in the first step the following two sets of primers: (i) the C317S forward primer with the C347S reverse primer (fragment C) and (ii) the C347S forward primer and the PhoA reverse primer (fragment D) and in the second step the C317S forward primer with the PhoA reverse primer (fragment CD).

The partially overlapping products AB and CD were purified from an agarose gel, mixed, and used in a third PCR step as template DNA and the *Cel*II forward primer and the PhoA reverse primer. The resulting PCR product (ABCD) was digested with the *Cel*II and PhoA restriction enzymes and exchanged with the equivalent fragment of pg315C. The resulting plasmid, encoding CitS with the mutations C278S, C317S, C347S, and G315C, was used as template DNA in a PCR to amplify CitS fragments containing residues 174–328 and 174–390 by using the forward *Nco*I-
was washed with 2 ml of buffer and with 1 ml of buffer containing 30 mM imidazole, incubated for 2.5 h at 4 °C under continuous agitation. Undissolved material was removed by ultracentrifugation. Membranes were treated with 250 μl MBP by incubating for 10 min at room temperature and, subsequently, with N3-nitrotriacetic acid resin (100 μl/10 mg of protein), equilibrated in solubilization buffer containing 20 μl imidazole, incubated for 2.5 h at 4 °C under continuous shaking, and, subsequently, poured into a column. The column was washed with 2 ml of buffer and with 1 ml of buffer containing 30 mM imidazole. The protein was eluted with 2 × 250 μl of buffer containing 200 μM imidazole. A 12.5-μl aliquot of the eluate was mixed with 5 μl of 5 mg/ml streptavidin, and a control sample received 5 μl of buffer. After a 10-min incubation at room temperature, 4 μl of a low SDS loading buffer (final SDS concentration: 0.4%) was added, and the samples were incubated for another 10 min at room temperature, after which 5-μl samples were loaded onto SDS-polyacrylamide gels containing 10% polyacrylamide. Proteins were stained with silver.

In case of the CitS-PhoA fusions, the membranes were resuspended in 50 mM Tris-HCl, pH 6.5. Equal aliquots of 12.5 μl were mixed with streptavidin and buffer as above, followed by SDS-PAGE. After running the gels, the proteins were transferred to Immobilon-P membranes (Millipore Corp.) by semidyselectrophoretic blotting. PhoA fusion proteins were detected with monoclonal antibodies directed against PhoA used at a dilution of 1:5000. Antibodies were visualized using the Western-light™ chemiluminescence detection kit with CSPD™ as a substrate as recommended by the manufacturer (Tropix).

**RESULTS**

Membrane Topology of Segments VIII and IX in Full-length *CitS* in the *E. coli* Membrane—*CitS* tagged with six histidine residues (His tag) at the N terminus is fully active (16) and could be partially purified in a single step by Ni2+-nitrilotriacetic acid affinity chromatography (Fig. 2). Wild-type *CitS* contains five cysteine residues, all of which are located in the C-terminal half of the protein (see Fig. 1). None of the native cysteine residues of *CitS*, in solubilized membranes or partially purified form, could be labeled with MBP, a thiol reagent containing a biotin moiety. Labeling can be visualized by binding of streptavidin, which results in a stable complex, even in SDS. The complex is evident from a mobility shift on SDS-PAGE (17). No such shift was observed when partially purified *CitS* was treated with MBP (Fig. 2A, left panel). In contrast, a shift was detected when a cysteine residue was engineered at position 315 in mutant G315C or position 333 in mutant S333C, both located in the loop between segments VIII and IX (Fig. 2A), showing that residues 315 and 333 are accessible for MBP modification. Both mutants formed blue halos on Simmons agar plates of similar size as observed with the wild-type transporter, indicating similar activities.

ISO membranes of cells expressing G315C or S333C were preincubated with and without AmidS, a membrane-impermeable thiol reagent, followed by labeling with MBP. Subsequently, MBP labeling was visualized by exposing the purified proteins to streptavidin prior to SDS-PAGE analysis. Pretreatment with AmidS completely prevented labeling with MBP, demonstrating that residues 315 and 333 are accessible to the membrane-impermeable AmidS from the cytoplasmic side of

### Table I

<table>
<thead>
<tr>
<th>Description</th>
<th>DNA sequence</th>
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<tbody>
<tr>
<td>Cell II forward</td>
<td>5'-CACACCTGGCTAGCGCGGAAGGG-3'</td>
</tr>
<tr>
<td>Pst I reverse</td>
<td>5'-GGGCCATACACGGCTGCATGAT-3'</td>
</tr>
<tr>
<td>Mutagenic G315C forward</td>
<td>5'-ACCGGCTCTTCGCTGCTACCCGGAATC-3'</td>
</tr>
<tr>
<td>Mutagenic G315C reverse</td>
<td>5'-TGAGGCACGCGAGGGCGTTCAG3'</td>
</tr>
<tr>
<td>Mutagenic S333C forward</td>
<td>5'-GTCGCCGTCTCTCTACCCGCTGCAG3'</td>
</tr>
<tr>
<td>Mutagenic S333C reverse</td>
<td>5'-GTCGGGTAGAGGACCGCGACCGCATC-3'</td>
</tr>
</tbody>
</table>

*a CellII restriction site in boldface type.
*b PstI restriction site in boldface type.
*c Cysteine substitutions in boldface type.
*d Serine substitutions in boldface type.

174 primer with the reverse NcoI and reverse NcoI-360 primers, respectively (Table I). The fragments were digested with NcoI and ligated into the NcoI site of pSA174, encoding a CitS-PhoA fusion protein with PhoA fused to CitS residue 174 using the NcoI site around codon 174 (12). Transformants with the correct orientation of the inserted NcoI fragment were selected on alkaline phosphatase indicator plates as blue colonies, followed by sequencing of the insert. In the resulting constructs, PhoA is fused at CitS residues 328 (SA328C315) and 360 (SA360C315), the three native cysteine residues in the CitS moiety are replaced by serine residues, and a single cysteine residue in the CitS moiety is present at position 315.

Preparation of Inside-out (ISO) Membranes—Cells were grown to an A600 of 0.5, harvested, and washed once with 50 mM potassium phosphate, pH 7, 100 mM NaCl. ISO membranes were prepared by resuspending the cells in the same buffer containing 1 mM EDTA, 1 mM MgSO4, and a trace amount of deoxyribonuclease. Cells were broken by one passage through a French press cell operated at 10,000 p.s.i. at 4 °C and immediately mixed with a phenylmethylsulfonyl fluoride solution yielding a final concentration of 1 mM. Unbroken cells and debris were removed by centrifugation at 14,000 × g for 10 min at 4 °C, and the ISO membranes were collected from the supernatant by ultracentrifugation at 100,000 × g for 30 min. Membranes were washed once in 50 mM potassium phosphate, pH 7, and stored in liquid nitrogen.

**Cysteine Labeling Studies—**MBP was dissolved in dimethyl sulfoxide (50 μM) and AmidS was dissolved in H2O (50 μM) prior to usage. To block periplasmic cysteine residues with the membrane-impermeable AmidS into whole cells expressing His-tagged CitS derivatives or CitS-PhoA fusion proteins were harvested at an A600 of 0.5; washed with 50 mM potassium phosphate, pH 7, 100 mM KCl; and resuspended in the same buffer to an A600 of 60. AmidS was added, yielding a final concentration of 1 mM. After incubation for 30 min at 30 °C, the cells were washed three times with excess buffer and converted into ISO membranes as described above. To block cytoplasmic cysteine residues with AmidS, ISO membranes (5 mg/ml) were treated with 1 mM AmidS for 30 min at room temperature. Diluting the membranes 3 times stopped the reaction, after which the membranes were collected by centrifugation using a Beckman Airfuge, followed by resuspension of the membranes in buffer without AmidS. Cysteine residues were labeled with the membrane-permeable MBP by incubating ISO membranes (5 mg/ml) with 250 μl MBP for 10 min at room temperature. The reaction was stopped by adding 10 mM dithiothreitol from a 1 × stock solution, followed by washing once with buffer without dithiothreitol.

In the case of the His-tagged CitS derivatives, the ISO membranes were solubilized in 50 mM potassium phosphate, pH 8, 400 mM NaCl, 10% glycerol, and 1% Triton X-100 and left on ice for 1 h with intermittent agitation. Undissolved material was removed by ultracentrifugation for 20 min at 100,000 × g. When indicated, solubilized membranes were treated with 250 μl MBP by incubating for 10 min at room temperature and, subsequently, mixed with N3-nitrotriacetic acid resin (100 μl/10 mg of protein), equilibrated in solubilization buffer containing 20 μl imidazole, incubated for 2.5 h at 4 °C under continuous shaking, and, subsequently, poured into a column. The column was washed with 2 ml of buffer and with 1 ml of buffer containing 30 μl imidazole. The protein was eluted with 2 × 250 μl of buffer containing 200 μM imidazole. A 12.5-μl aliquot of the eluate was mixed with 5 μl of 5 mg/ml streptavidin, and a control sample received 5 μl of buffer. After a 10-min incubation at room temperature, 4 μl of a low SDS loading buffer (final SDS concentration: 0.4%) was added, and the samples were incubated for another 10 min at room temperature, after which 5-μl samples were loaded onto SDS-polyacrylamide gels containing 10% polyacrylamide. Proteins were stained with silver.

**ISO membranes of cells expressing G315C or S333C were preincubated with and without AmidS, a membrane-impermeable thiol reagent, followed by labeling with MBP. Subsequently, MBP labeling was visualized by exposing the purified proteins to streptavidin prior to SDS-PAGE analysis. Pretreatment with AmidS completely prevented labeling with MBP, demonstrating that residues 315 and 333 are accessible to the membrane-impermeable AmidS from the cytoplasmic side of the membrane.**

**DNA sequences of the oligonucleotides used in this study**

- Mutagenic C347S forward
- Mutagenic C347S reverse
- Mutagenic C278S forward
- Mutagenic C278S reverse
- Mutagenic G315C forward
- Mutagenic G315C reverse
- Mutagenic S333C forward
- Mutagenic S333C reverse
- Mutagenic C317S forward
- Mutagenic C317S reverse
- Mutagenic S333C forward
- Mutagenic S333C reverse
- Mutagenic C347S reverse
the membrane (Fig. 2B). In agreement, in the complementary experiment, when whole cells expressing G315C or S333C were treated with membrane-impermeable AmdiS, the subsequent addition of MBP to the solubilized proteins resulted for both mutants in labeling of the proteins (Fig. 2C). The cytoplasmic location of residue 315 and 333 indicates that segment VIII and IX are both transmembrane, thus falsifying the PhoA fusion model in this region. It is concluded that the CitS protein traverses the E. coli membrane 11 times, similar to what was observed in the ER membrane (see Fig. 1B).

Cys Labeling of CitS-PhoA Fusion Proteins—Alkaline phosphatase fusions to sites in the cytoplasmic loop between TMS VIII and IX resulted in a periplasmic location of the reporter molecule (12), indicating misfolding of the CitS moiety. To provide insight into the misfolding of the CitS moiety in these particular fusion proteins, we have determined the membrane topology of the CitS-PhoA fusion proteins by specific labeling of native and introduced cysteine residues in the CitS moiety.

CitS-PhoA fusion protein SA46 contains the first 46 amino acids of CitS, none of which is a Cys residue. The protein contains the first transmembrane segment TMS I of CitS and showed a high alkaline phosphatase activity, indicating that the PhoA moiety is efficiently exported to the periplasm (12).
Fig. 4. Cysteine labeling of SA290. Cells expressing CitS-PhoA fusion protein SA290 were treated according to protocols 2 and 3 (see Fig. 3A), as indicated. The solubilized membranes were mixed with (+) or without (−) streptavidin, prior to gel analysis and immunoblotting using antibodies directed against PhoA. The arrow indicates the full-length fusion protein. Top, topology model of SA290 indicating the position of Cys278 (C) and alkaline phosphatase (AP). A circled Cys residue indicates accessibility for MBP.

Fig. 5. Labeling of SA328C315 and SA328. Cells expressing the CitS-PhoA fusion proteins SA328C315 and SA328 were treated with MBP according to protocols 2 and 3 (see Fig. 3A), as indicated. The resulting membranes were processed as described in the legends to Figs. 3 and 4. Top, topology model of SA328. The native and accessible cysteine residues (Cys278 and Cys317) are indicated with a circled C. The position of the mutation G315C is indicated.

thiol(s) of PhoA located in the lumen of the ISO membranes.

In fusion protein SA455, alkaline phosphatase is fused to the COOH terminus of CitS, and all of the five native cysteine residues of CitS are present. According to the 11-TMS model, Cys278 and Cys347 are located in transmembrane segments VII and IX, respectively. The remaining cysteines are located in cytoplasmic loops, Cys317 in the loop between TMSs VIII and IX and Cys347 in the loop between TMSs X and XI (see Fig. 1). Whole cells expressing SA455 were incubated with the hydrophilic AmdIS reagent to block the cysteine residue(s) in the PhoA moiety. Subsequently, the cells were converted into ISO membranes and labeled with MBP (protocol 2). Exposure to streptavidin did not reduce the intensity of the band corresponding to the full-length fusion protein, indicating that the Cys residues of the CitS moiety are not accessible to the MBP reagent (Fig. 3B) as was observed above (Fig. 2). The bands at higher apparent molecular weight correspond to multimers of the CitS protein.

Fusion Protein SA290—CitS-PhoA fusion protein SA290, containing the first seven transmembrane segments of CitS, showed a high alkaline phosphatase activity, indicating a periplasmic PhoA moiety (12). The CitS part of the fusion protein contains a single cysteine residue, Cys278, in the periplasmic half of TMS VII. Whole cells expressing SA290 were blocked with AmdIS and converted into ISO membranes followed by treatment with MBP. Exposure to streptavidin prior to SDS-PAGE analysis resulted in a marked decrease in intensity of the bands corresponding to the full-length fusion protein (Fig. 4, protocol 2), indicating that the Cys278 residue is accessible to MBP and not to AmdIS added at the periplasmic side of the membrane. Treatment of the same ISO membranes with AmdIS prior to labeling with MBP still resulted in labeling of Cys278 (Fig. 4, protocol 3), indicating that Cys278 was also not accessible to AmdIS added at the cytoplasmic side of the membrane. The inaccessibility of Cys278 to the hydrophilic reagent AmdIS from either side of the membrane is consistent with the location of Cys278 in the membrane. The labeling of Cys278 in the truncated protein contrasts with the situation in the complete CitS protein, where no labeling occurs.

Fusion Protein SA328—High alkaline phosphatase activity of CitS-PhoA fusion protein SA328, containing the first eight transmembrane segments of CitS, suggested that the PhoA moiety is in the periplasm (12). Since the above experiments demonstrated that the fusion point in the complete CitS molecule is in the cytoplasm, this implies that the CitS part in SA328 is not correctly folded. SA328 contains two cysteine residues, Cys278 in TMS VII and Cys317 in the hydrophilic region just in front of the fusion site. To verify the periplasmic location of PhoA in SA328, the two cysteine residues were replaced by Ser, and a Cys residue was engineered at position 315, resulting in the mutant fusion protein SA328C315. Position 315 is in the fusion site region in front of Cys317 in the wild-type protein and was shown above to be accessible in the complete CitS molecule to both MBP and AmdIS, the latter when added at the cytoplasmic side of the membrane. Expression of SA328C315 in E. coli resulted in blue colonies on XPlates, indicative of high PhoA activity. Labeling of Cys315 and the PhoA cysteine(s) of SA328C315 by MBP was effectively blocked by prior treatment of the cells with AmdIS, demonstrating that the PhoA moiety and Cys315 are in the periplasm (Fig. 5, protocol 2).

The membrane topology of the CitS moiety in fusion protein SA328 was examined through the accessibility of C278 in TMS VII of SA328. Cells expressing SA328 were treated with AmdIS to block the PhoA cysteine(s) and, if accessible, the periplasmically located Cys317 of the CitS moiety. MBP treatment of ISO membranes resulted in labeling of the fusion protein (Fig. 5, protocol 2), showing that AmdIS did not block Cys278 added at the periplasmic side of the membrane. Treatment of the same ISO membranes with AmdIS prior to MBP labeling similarly
Insertion of CitS in the E. coli Membrane

The hydrophathy profile of the amino acid sequence of CitS reveals 12 stretches of amino acids that are hydrophobic and long enough to span the membrane (13). A study in E. coli with C-terminally truncated CitS molecules fused in front of the mature part of alkaline phosphatase (PhoA fusions) indicated a membrane topology model with nine membrane-spanning segments with the remaining three hydrophobic segments (Vb, VIII, and IX) in the periplasm (Fig. 1B) (12). A topology study in the ER membrane using the same truncated CitS molecules confirmed the exclusion of segment Vb from the membrane, but in contrast to the E. coli results, segments VIII and IX were found to be membrane-spanning. In the present study, the apparent contradiction between both models was resolved by examining the topology of segments VIII and IX in the E. coli membrane in the context of the complete and functional CitS molecule. Two cysteine residues introduced in the hydrophilic domain between segments VIII and IX were mapped in the cytoplasm by their accessibility to membrane-permeable and -impermeable thiol reagents. The results demonstrate that segments VIII and IX span the E. coli membrane in the full-length CitS molecule and, therefore, that the membrane topology of CitS is the same in the ER and E. coli membrane showing 11 TMS.

The exclusion of segments VIII and IX from the membrane in the PhoA model was based upon a series of CitS-PhoA fusion proteins with the fusion sites at different positions in between segments VIII and IX. The fusion proteins all resulted in high alkaline phosphatase activity, demonstrating a periplasmic location of the PhoA molecule and suggesting a periplasmic location of transmembrane segment VIII in these fusion proteins (12). We studied the folding of the CitS moiety in several CitS-PhoA fusion proteins in order to obtain information about the insertion process of CitS in the E. coli membrane. A transmembrane disposition of segment VII in CitS-PhoA fusion proteins containing TMSs I–VII and I–VIII (SA290 and SA328, respectively) was consistent with the labeling characteristics of Cys278, located in the C-terminal half of TMS VII. In both fusion proteins, Cys278 reacted with the membrane-permeable sulphydryl reagent MBP and could not be blocked by the impermeable reagent AmdSDS added at either side of the membrane, indicating that Cys278 in TMS VII was in the hydrophobic core of the membrane. Together with the periplasmic location of the introduced cysteine residue at position 315, downstream of TMS VIII (fusion protein SA328C315), it follows that the high PhoA activity of SA328 is indeed caused by misfolding of segment VIII. TMS VIII does not fold back across the membrane when the CitS molecule is truncated between segments VIII and IX. Remarkably, elongation of the CitS moiety in fusion protein SA328 with the next segment, TMS IX, moved the Cys278 residue from a periplasmic to a cytoplasmic location (Figs. 5 and 6). Apparently, the presence of TMS IX moves Cys278 from a periplasmic location in SA328C315 to a cytoplasmic location in SA360C315 (Figs. 5 and 6).

SA360 that contains the wild-type cysteine residues behaved like SA360C315; i.e. after treatment of the cells with AmdSDS, the fusion protein could be labeled with MBP (Fig. 2, protocol 2), and the labeling could be blocked with AmdSDS added at the cytoplasmic side of the membrane (not shown). Since Cys315 is the only cysteine residue in the cytoplasm, it is concluded that in contrast to what is observed in the complete CitS molecule, this residue is accessible in SA360. The lack of labeling after blocking with AmdSDS at both sides of the membrane indicates that the two cysteine residues Cys278 and Cys347 are protected by the conformation of the protein as observed in the complete CitS molecule, in contrast to what is observed in fusion proteins SA328 and SA290 in the case of Cys278.

**DISCUSSION**

SA360 contains the wild-type cysteine residues behaved like SA360C315; i.e. after treatment of the cells with AmdSDS, the fusion protein could be labeled with MBP (Fig. 2, protocol 2), and the labeling could be blocked with AmdSDS added at the cytoplasmic side of the membrane (not shown). Since Cys315 is the only cysteine residue in the cytoplasm, it is concluded that in contrast to what is observed in the complete CitS molecule, this residue is accessible in SA360. The lack of labeling after blocking with AmdSDS at both sides of the membrane indicates that the two cysteine residues Cys278 and Cys347 are protected by the conformation of the protein as observed in the complete CitS molecule, in contrast to what is observed in fusion proteins SA328 and SA290 in the case of Cys278.
segment VIII was transmembrane in the absence of the downstream CitS sequences (13). This difference suggests differences in the bacterial and ER insertion mechanisms or is related to different conditions during insertion, e.g. the presence or absence of a membrane potential, differences in experimental system (e.g. in vivo versus in vitro), or differences in the reporter system (e.g. alkaline phosphatase versus the P2 domain of leader peptidase).

The process of membrane protein insertion into the E. coli membrane is still poorly understood. Evidence is increasing that insertion of integral membrane proteins in the cytoplasmic membrane of bacteria proceeds via the preprotein translocation machinery, the Sec system, in a similar, but not identical, manner as observed in the ER membrane, which is much better understood (4–7). It may be anticipated that also in bacteria the insertion and ultimate folding of the protein is determined both by topological signals in the native chain and by the insertion machinery (23–25). The unexpected folding of the truncated protein consisting of the first eight TMSs of CitS may be a manifestation of specific interactions between the nascent chain and the Sec system. CitS contains five TMSs with an average hydrophobicity of 0.75 on the scale of Eisenberg (13, 28). There does not seem to be much reason why TMS VIII would not be transmembrane unless the insertion machinery “tells” it to wait for TMS IX. The high hydrophobicity of TMS VIII makes it difficult to become protected against labeling, suggesting an interaction between the cytoplasmic domain between TMSs VIII and IX and downstream CitS sequences, possibly the loop between TMSs X and XI. The remaining two Cys residues, Cys398 and Cys414, in the latter cytoplasmic loop are also inaccessible in the complete protein.

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REFERENCES