Role of a Small Cytoplasmic Domain in the Establishment of Serine Chemoreceptor Membrane Topology

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The *Escherichia coli* serine chemoreceptor takes on a simple membrane topology with two transmembrane segments separating cytoplasmically disposed N and C termini from a central periplasmic domain. We investigated the role of the small N-terminal cytoplasmic domain in membrane insertion using alkaline phosphatase gene fusions. Mutations eliminating the positive charge of the domain altered insertion dramatically, with reciprocal effects on hybrids with periplasmic and C-terminal cytoplasmic fusion junctions. Efficient export of the normally cytoplasmic C-terminal domain required that, in addition to the N-terminal changes, a short amphipathic sequence at the beginning of the C-terminal domain be also absent. These findings document the importance of the positive character of the N-terminal domain in chemoreceptor membrane insertion and imply that partially redundant sequence information controls the orientation of the second transmembrane segment.

The *Escherichia coli* serine chemoreceptor (Tsr) is a cytoplasmic membrane protein of simple, well-established topology (Fig. 1). The polypeptide has two membrane-spanning sequences (TM1 and TM2), with a small N-terminal cytoplasmic domain, a large periplasmic domain, and a large C-terminal cytoplasmic domain. The short N-terminal sequence is positively charged, with three of six residues basic (Fig. 1).

The membrane topology of Tsr has been analyzed by using alkaline phosphatase (AP) gene fusions (5, 12). AP requires export from the cytoplasm to be enzymatically active (10). Thus, cells expressing hybrid proteins with AP fused to the Tsr periplasmic domain exhibit much greater activity than those with AP fused to the C-terminal cytoplasmic domain. Mutants with altered Tsr membrane topology have been isolated by using such Tsr-AP (Tsr-PhoA) gene fusions. These studies have identified a novel amphipathic sequence element (AS) adjacent to TM2 which helps prevent export of the C-terminal domain to the periplasm (Fig. 1) (14). Other experiments involving gene fusions have provided a detailed analysis of the protein export function of TM1 (8).

Studies of cleavable signal sequences and of integral membrane proteins have shown that small polar domains rich in positive amino acids may play an essential role in membrane insertion (2, 3). For example, altering the distribution of basic amino acids in the polar regions of the *E. coli* leader peptidase causes the protein's membrane topology to invert (15). In the experiments described here, we sought to determine whether the appropriate membrane insertion of Tsr was dependent upon the positive character of its N-terminal cytoplasmic domain.

We first generated three triple mutations altering the N-terminal positive residues using site-directed mutagenesis. The three residues were simultaneously mutated to asparagine, alanine, and aspartate. The mutations were originally introduced into a phage M13 derivative carrying 5' sequences of *tsr* (M13mp18-*tsr*) (8) by using the procedure described by Kunkel

et al. (7). The appropriate mutants were identified by DNA sequence analysis and transferred into a pBR322 derivative (pEP309) carrying a *tsr-phoA* fusion with a periplasmic fusion junction (residue 89) (8). The presence of the mutations in the final plasmid constructs was confirmed by DNA sequence analysis in all cases. The mutant plasmids were transformed into strains that maintain pBR322 derivatives at a reduced copy number (PcnB⁻) (9), and the AP activities and rates of hybrid protein synthesis of these cells were measured. All three triple mutations dramatically reduced AP activity, without significantly reducing hybrid protein synthesis (Fig. 2). These results imply that all three triple mutations reduced TM1-mediated export. Export activity of the mutants decreased in the order wild type > Asn > Ala > Asp. Thus, the positive character of the Tsr N-terminal segment is essential for TM1-mediated export, and reversing the charge of the segment is more detrimental to export than is neutralizing it. The

FIG. 1. Membrane topology of the serine chemoreceptor. The N-terminal cytoplasmic domain is thought to consist of residues 1 to 6 (whose sequence is shown), the periplasmic domain consists of residues 31 to 191, and the C-terminal cytoplasmic domain consists of residues 215 to 551 (1). The transmembrane sequences (TM1 and TM2) and the AS are indicated. Experimental support for this structure has been provided by a number of studies (4, 6). Bacterial chemoreceptors are thought to function as dimers (13).

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FIG. 2. Effects of N-terminal substitutions on a periplasmic-domain Tsr-PhoA hybrid protein. The diagram represents a periplasmic-domain Tsr-PhoA hybrid protein with a junction at Tsr-89. The positions of the three positive residues in the N-terminal segment are indicated. AP activities of the mutant hybrids are expressed relative to the wild-type (wt) TM1 hybrid (270 U) and are the means of six measurements each (11). Standard deviations were less than 20% of the mean values in all cases. The rates of hybrid protein synthesis were determined by using precipitation by AP antibody after exposure of cells to [35 S]methionine for 2 min (11). Rates of synthesis are expressed relative to the wild-type hybrid and are the mean values of at least two measurements. Standard deviations were less than 15% of the mean values in all cases. AP activities and rates of protein synthesis were determined for cells with plasmids at a low copy number in PcnB⁻ strains CC701 (AP assays) and CC703 (hybrid protein synthesis) (8, 9). Tsr-PhoA hybrid protein recoveries in the soluble and membrane fractions were quantitated by Phosphorimager analysis, and the proportion of the total present in the membrane fraction is shown. β -Galactosidase produced constitutively by the same cells was recovered (86 to 92%) in the soluble fraction, implying efficient separation of the cytoplasm from the membrane.

membrane association of the hybrid proteins was assessed after separation of plasmid-bearing cells into soluble and membrane fractions as described earlier (12) except that cells were exposed to [35 S]methionine for 2 min followed by excess nonradioactive methionine for 1 min, the osmotic shock step was omitted, and hybrid protein was detected directly after electrophoresis without immunoprecipitation. All four hybrids were fractionated to the membrane (Fig. 2), indicating that loss of the N-terminal positive residues does not eliminate TM1 interaction with the bilayer. This result is compatible with membrane insertion of the mutant TM1 sequences in an orientation opposite to the normal orientation (see below), although it does not rule out other types of membrane association.

Next, we wished to analyze the effects of the N-terminal substitutions on Tsr insertion when both TM1 and TM2 were present. To do this, the three triple mutations were transferred to C-terminal-domain *tsr-phoA* gene fusions (Fig. 3). The fusions encoded a wild-type AS or the AS was deleted (Fig. 1). The mutant C-terminal domain *tsr-phoA* fusion plasmids were constructed by conversion of the mutant periplasmic-domain *tsr-phoA* fusion plasmids (Fig. 2) into C-terminal-domain *tsr-phoA* fusions. This was done by the replacement of a restriction fragment (*Mlu*I-*Bst*EII) carrying the fusion junction sequences of each of the periplasmic-domain fusions with corresponding fragments from C-terminal-domain *tsr-phoA* fusion plasmids (pLS210 and pLS211) (14). All three substitutions led to elevated AP activity (wild type < Asn < Ala < Asp), implying increased export of the C-terminal domain to the periplasm. However, even the mutations with the greatest effect (the aspartate substitutions) showed only partial AP export (about 24% of the maximum). The export of the AP was uniformly higher when the AS was also absent from the N-terminal mutant hybrids, and the hybrid carrying the aspartate substitutions and lacking the AS exported nearly all of the C-terminal AP. These findings are compatible with those of earlier studies of a TM1 deletion mutant (14).

These results can be simply accommodated by a model proposing that, in the absence of a positive N-terminal region, TM1 inserts into the membrane in an orientation opposite to its normal orientation (Fig. 4). This misinsertion reduces export (and therefore activity) of the AP moiety of periplasmic-domain Tsr-PhoA hybrids (Fig. 4A). In C-termi-

nal-domain Tsr-PhoA hybrids, the misinsertion of TM1 causes TM2 sometimes to insert into the membrane in an orientation opposite to its normal orientation, leading to a topologically mixed population of molecules (and partial AP activity) (Fig. 4B). When both the N-terminal sequence is negatively charged and the AS is absent, there is essentially complete misinsertion of TM2 and maximal AP activity (Fig. 4C).

These findings thus demonstrate the importance of the positive character of the N-terminal domain in the establishment of Tsr membrane topology. In addition, the results indicate that both the normal insertion of TM1 and the functioning of the AS contribute independently to the appropriate insertion of TM2. This partial redundancy in the sequence information promoting TM2 insertion in the correct orientation may help ensure that Tsr membrane assembly is normally a high-fidelity process (14).

FIG. 3. Effects of N-terminal substitutions on C-terminal-domain Tsr-PhoA hybrid proteins. The diagram represents the cytoplasmic-domain Tsr-PhoA hybrid protein, with a junction at Tsr-240. AP activities were normalized to the activity of a wild-type (wt) periplasmic-domain Tsr-PhoA hybrid (270 U) (Fig. 2) and represent the mean values of six measurements. Standard deviations were less than 20% of the mean values in all cases. The rates of hybrid protein synthesis were normalized to the rate of synthesis of the wild-type periplasmic-domain hybrid (Fig. 2). Standard deviations were less than 25% of the mean values with the exceptions of the Asp (+AS) and Ala (-AS) substitutions, which had standard deviations of 37 and 28%, respectively, of their mean values. AP activities and rates of protein synthesis were determined for cells with plasmids at a low copy number in PcnB⁻ strains CC701 and CC703 (8, 9).

FIG. 4. Models for mutant Tsr-PhoA membrane insertion. (A) A negatively charged N terminus of a periplasmic-domain Tsr-PhoA hybrid causes TM1 to insert in an orientation opposite to its normal orientation, leading to cytoplasmically disposed AP. cyt, cytoplasm. (B) A negatively charged N terminus of a C-terminal cytoplasmic-domain Tsr-PhoA hybrid leads to a mixed population with molecules inserted in the two topologies shown. (C) Elimination of both the N-terminal positive charge and the AS causes nearly complete misorientation of TM2 with accompanying C-terminal-domain export.

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REFERENCES

- Boyd, A., K. Kendall, and M. I. Simon. 1983. Structure of the serine chemoreceptor in *Escherichia coli*. *Nature (London)* **301**: 623–626.
- Boyd, D., and J. Beckwith. 1990. The role of charged amino acids in the localization of secreted and membrane proteins. *Cell* **62**: 1031–1033.
- Dalbey, R. 1990. Positively charged residues are important determinants of membrane protein topology. *Trends Biochem. Sci.* **15**: 253–257.
- Falke, J. J., and D. E. Koshland. 1987. Global flexibility in a sensory receptor: a site-directed cross-linking approach. *Science* **237**: 1596–1600.
- Gebert, J., B. Overhoff, M. Manson, and W. Boos. 1988. The Tsr chemosensory transducer of *Escherichia coli* assembles into the cytoplasmic membrane via a SecA-dependent process. *J. Biol. Chem.* **263**: 16652–16660.
- Hazelbauer, G. 1992. Bacterial chemoreceptors. *Curr. Opin. Struct. Biol.* **2**: 505–510.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**: 367–382.
- Lee, E., and C. Manoil. Mutations eliminating the protein export function of a membrane-spanning sequence. *J. Biol. Chem.*, in press.
- Lopilato, J., S. Bortner, and J. Beckwith. 1986. Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen. Genet.* **205**: 285–290.
- Manoil, C. 1990. Analysis of protein localization by use of gene fusions with complementary properties. *J. Bacteriol.* **172**: 1035–1042.
- Manoil, C. 1991. Analysis of membrane protein topology using alkaline phosphatase and β -galactosidase gene fusions. *Methods Cell Biol.* **34**: 61–75.
- Manoil, C., and J. Beckwith. 1986. A genetic approach to analyzing membrane protein topology. *Science* **233**: 1403–1408.
- Milligan, D., and D. E. Koshland. 1988. Site-directed cross-linking: establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. *J. Biol. Chem.* **263**: 6268–6275.
- Seligman, L., and C. Manoil. 1994. An amphipathic sequence determinant of membrane protein topology. *J. Biol. Chem.* **269**: 19888–19896.
- von Heijne, G. 1989. Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature (London)* **341**: 456–458.