The EMBO Journal vol.6 no.11 pp.3465–3470, 1987

# Topology analysis of the SecY protein, an integral membrane protein involved in protein export in *Escherichia coli*

**Yoshinori Akiyama and Koreaki Ito**

Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Communicated by M.Schwartz

**The *secY* (*prlA*) gene product is an essential component of the *Escherichia coli* cytoplasmic membrane, and its function is required for the translocation of exocytoplasmic proteins across the membrane. We have analyzed the orientation of the SecY protein in the membrane by examining the hydropathic character of its amino acid sequence, by testing its susceptibility to proteases added to each side of the membrane, and by characterizing SecY–PhoA (alkaline phosphatase) hybrid proteins constructed by Tn*phoA* transpositions. The orientation of the PhoA portion of the hybrid protein with respect to the membrane was inferred from its enzymatic activity as well as sensitivity to external proteases. The results suggest that SecY contains 10 transmembrane segments, five periplasmically exposed parts, and six cytoplasmic regions including the amino- and carboxy-terminal regions.**

*Key words:* SecY protein/protein export/membrane protein/transmembrane segment/Tn*phoA*

## Introduction

During the course of biogenesis of cell surface or secreted proteins, they must exit from the cytoplasm by translocating across the endoplasmic reticulum membrane (in eukaryotic cells) or the cytoplasmic membrane (in prokaryotic cells). Biochemical or genetic evidence suggests that the translocation process is facilitated by some proteinaceous factors (for review see Walter *et al.*, 1984; Ito, 1986). Although some of these factors, such as the signal recognition particle and its receptor protein (docking protein), have been characterized extensively, many questions remain unanswered. In particular, little is known about the machinery and the mechanism responsible for the translocation of polypeptides from the cytoplasmic to the exocytoplasmic sides of the membrane. The earlier (Blobel and Dobberstein, 1975) and recent (Singer *et al.*, 1987) proposals for the existence of the tunnel-forming proteins in the membrane have not been confirmed experimentally.

We have been characterizing an *Escherichia coli* gene *secY* (or *prlA*) whose mutations can prevent the envelope proteins from crossing the cytoplasmic membrane (defective phenotype; Ito *et al.*, 1983; Shiba *et al.*, 1984) or allow export of a mutant envelope protein whose signal sequence is otherwise nonfunctional (*prlA* suppressor phenotype; Emr *et al.*, 1981). Our studies showed that this gene, located in the promoter-distal part of the *spc* ribosomal protein operon, encodes an integral protein of the cytoplasmic membrane (Akiyama and Ito, 1985).

The SecY protein has some unusual properties, such as aggregation in SDS solution upon heating, anomalous migrations in SDS–PAGE, and strong interaction with some non-ionic detergents. These properties closely resemble those of integral membrane proteins such as the lactose carrier (LacY) protein. Thus, these proteins might share some aspects in their structure or mode of association with the membrane. In fact, both the LacY (Buchel *et al.*, 1980) and the SecY (Cerretti *et al.*, 1983) proteins contain alternating hydrophobic and hydrophilic segments, a feature characteristic of the membrane proteins with multiple membrane-spanning sequences.

In the present work, we have analyzed the orientation of the SecY protein in the membrane with conventional protease digestion experiments, as well as by the genetic approach using Tn*phoA* transposition, which was recently developed by Manoil and Beckwith (1985, 1986).

## Results

### *Hydropathic profile of the SecY sequence*

The amino acid sequence of the SecY protein, which was deduced from the DNA sequence (Cerretti *et al.*, 1983) and subsequently confirmed for the amino-terminal 12 residues (except for the lack of the first methionine; Akiyama and Ito, 1986), was analyzed by the procedure of Kyte and Doolittle (1982) for distribution of hydrophobic amino acids. This protein contains 10 regions that are 20–30 residues long and rich in hydrophobic amino acids (Figure 1). For the purpose of clarity we number these hydrophobic segments 1–10 in the order of their locations from amino to carboxy termini, whereas hydrophilic parts are similarly referred to as hydrophilic segments 1–11. The length of each hydrophobic segment is sufficient for spanning the membrane in an $\alpha$-helical structure, suggesting that SecY traverses the membrane 10 times.

To evaluate more quantitatively the probability at which these hydrophobic segments are in the transmembrane configuration,

**Table I.** Predicted transmembrane segments of the SecY protein

| Hydrophobic segment[a] | Probability of integral association with membrane[b] | Probable membrane-spanning residues[c] |
|---|---|---|
| 6 | 0.999999 | 215–238 |
| 1 | 0.9999 | 22–43 |
| 5 | 0.998 | 184–205 |
| 10 | 0.997 | 396–419 |
| 8 | 0.997 | 314–336 |
| 7 | 0.992 | 271–292 |
| 2 | 0.97 | 78–98 |
| 9 | 0.97 | 372–391 |
| 4 | 0.90 | 158–175 |
| 3 | 0.14 | 122–138[d] |

[a]Numbers defined as in Figure 1.
[b]Calculated by the procedure of Klein *et al.* (1985) using the program IDEAS distributed by the National Institutes of Health, USA.
[c]Half-way between the inner and outer boundary (Klein *et al.*, 1985) is used as a residue of boundary. The residue number starts at the initiator methionine (although it is removed).
[d]This assignment is arbitrary since this segment was classified as peripheral by the computer.

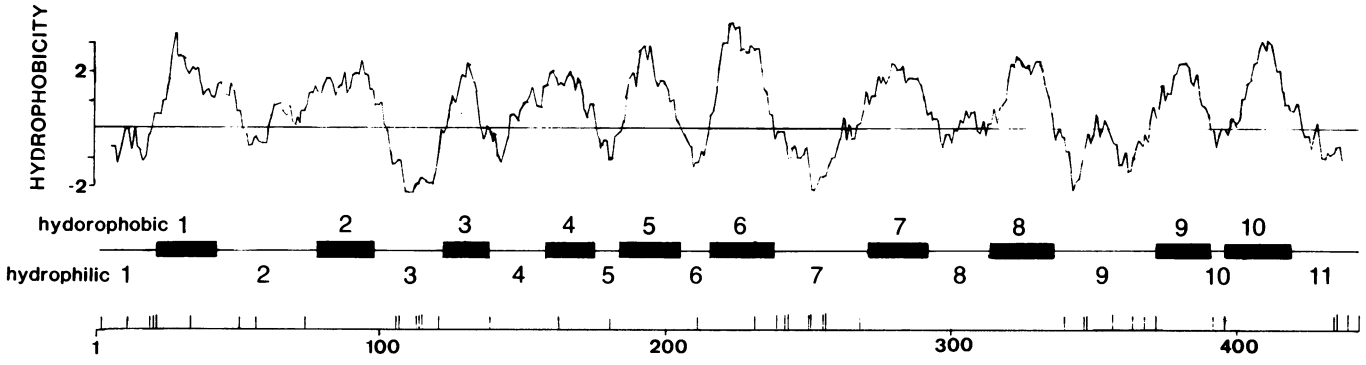


**Fig. 1.** Hydropathy profile of the SecY sequence. The amino acid sequence of SecY (Cerretti *et al.*, 1983) was analyzed by the method of Kyte and Doolittle (1982), with a moving window of 11 residues. The hydrophobic segments are boxed and numbered in the order of $NH_2$ to COOH termini. The hydrophilic parts are similarly numbered. The vertical lines on the bottom line indicate the locations of lysine and arginine residues. The scale at the bottom indicates the residue number including the initiator methionine.

we applied the algorithm developed by Klein *et al.* (1985). This allocation procedure predicted the existence of nine transmembrane segments in the SecY sequence (Table I). The hydrophobic segment 3 gained a lower probability by this method.

*Protease sensitivity of the SecY protein in the membrane*

When the SecY-overproducing strain carrying plasmid pNO1573 was induced and pulse-labeled with [$^{35}$S]methionine, the SecY protein was a major radioactive protein of the cell, and was the most intensively labelled component of the cytoplasmic membrane (Akiyama and Ito, 1985). The cytoplasmic membrane vesicles, which should have been inverted by sonication (Seckler and Wright, 1984), were incubated with trypsin. The SecY protein was digested and a fragment was generated, which migrated in SDS−PAGE at the position corresponding to a mol. wt of ~ 20 000 (Figure 2, lanes 3−5). This faster migrating fragment should have been protected by the membrane because its trypsin resistance was abolished when the membranes were solubilized by detergent Triton X-100 (Figure 2, lanes 7−10). We assume that this fragment was derived from the SecY protein because a control strain with the vector plasmid never yielded such a fragment (data not shown), and the other proteins overproduced from the cloned fragment were all <20 000 daltons.

In contrast, the SecY protein in the intact spheroplasts resisted the trypsin action up to the concentration examined, whereas it was degraded in the presence of the detergent; other proteases, such as proteinase K, pronase and subtilisin, gave similar results (data not shown). These results indicate that SecY has domain(s) exposed to the cytoplasm. The results also suggest that SecY contains at least one membrane-embedded region that can resist trypsin in the absence of detergent. However, it is not clear from these analyses whether SecY contains any regions exposed to the periplasmic side of the membrane.

*Analysis of the SecY protein topology by TnphoA transposition*

To overcome the limitations inherent in the protease digestion experiments (see Discussion), we used the alkaline phosphatase (the *phoA* gene product), which can fold into the enzymatically active structure only when exported to the periplasmic space (Boyd *et al.*, 1987a) as a probe of protein localization (Manoil and Beckwith, 1985). Tn*phoA* is a derivative of transposon Tn*5*, and carries the *phoA* gene missing its own expression and export signals. It can direct the synthesis of an enzymatically active (periplasmically exposed) PhoA fusion protein when it is inserted in frame within the coding sequence of another periplasmic protein, or within a membrane protein's segments that normally face the periplasm. A fusion to a cytoplasmic protein or the cytoplasmic domains of a membrane protein will produce an internalized PhoA moiety that is almost devoid of enzyme activity (Manoil and Beckwith, 1985, 1986).

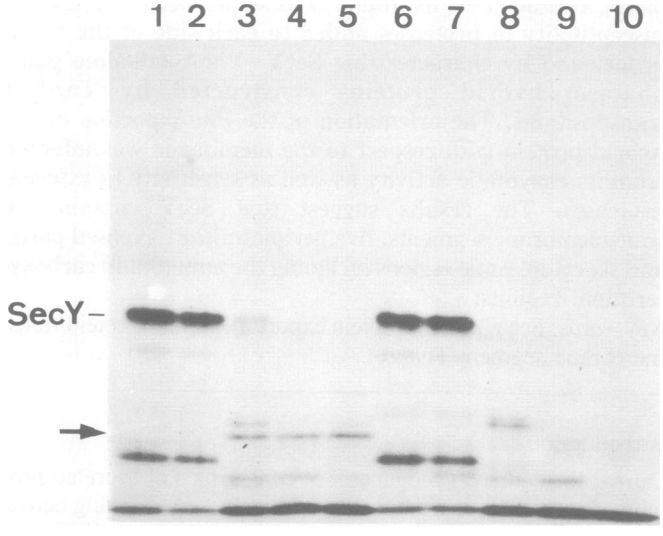


**Fig. 2.** Trypsin sensitivity of the SecY protein in the inverted membrane vesicles. Cytoplasmic membrane vesicles (inverted by sonication) were prepared from [$^{35}$S]methionine-labelled cells of KI269/pNO1573, which had been induced by cyclic AMP and isopropyl-β-D-thiogalactoside. Trypsin digestion was done in the absence (**lanes 1−5**) or presence (**lanes 6−10**) of 1% Triton X-100, at concentrations of 0 (**lanes 1** and **6**), 0.15 (**lanes 2** and **7**), 1.5 (**lanes 3** and **8**), 15 (**lanes 4** and **9**) or 150 (**lanes 5** and **10**) μg/ml. The arrow indicates the position of the putative membrane-protected fragment of SecY.

We selected transpositions of Tn*phoA* onto a multi-copy plasmid pKY4, on which the *secY* gene flanked by artificial *Eco*RI sites had been cloned under the lactose (*lac*) promoter control (Figure 3). The PhoA enzyme activity was monitored by the blue color of a chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate added to the agar medium. The position of the insertion was determined by *Eco*RI digestion pattern of the plasmid.

We obtained a number of insertions which mapped within *secY* and exhibited high enzyme activity of PhoA. These insertions (shown in Figure 3 by downward arrows with filled boxes) fell within or near the five alternate hydrophilic segments numbered 2, 4, 6, 8 and 10. The exact fusion points of the representative plasmids were determined by DNA sequencing (Table II). The sequencing results agree with those of restriction analysis within the error of at most 30 bases or 10 amino acid residues (much

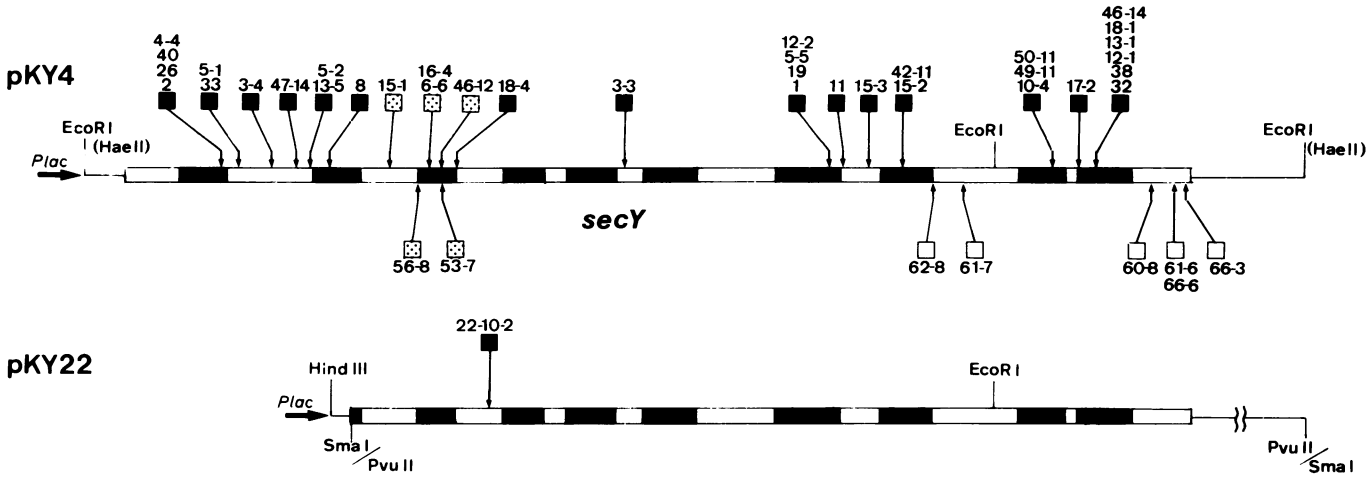


**Fig. 3.** Locations of the Tn*phoA* transpositions on the *secY* gene carried on the plasmid. The *secY* gene was boxed and the portions corresponding to the hydrophobic segments (Table I) are filled. Arrows with filled squares indicate transpositions which produced SecY−PhoA hybrid proteins of high enzyme activities. Arrows with open squares indicate those of low activities. Arrows with dotted squares indicate those of intermediate activities.

less error in many cases). The maintenance of the reading frame in the fusion joint was also confirmed for those sequenced. Indeed, these plasmids directed the synthesis of SecY−PhoA hybrid proteins which were precipitated by antiserum against the PhoA protein (Figure 4A; Table II). The apparent mol. wts of the hybrid proteins in SDS−PAGE were somewhat lower than those expected from the DNA analysis (Table II), but their relative sizes generally correlated with the fusion joints determined, except for some (e.g. 3-3) which gave an apparent degradation product (~46 000 daltons; Figure 4). The deviation in the apparent mol. wts may be explained by the faster migrating property of the SecY protein (Ito, 1984).

The fusions 15-1, 16-4, 46-12 and 6-6 gave pale blue color and intermediate levels of enzyme activity (~30% of the highest class; Table II). They mapped at the hydrophilic segment 3 (15-1) or possibly within the hydrophobic segment 3 (the others) (Figure 3, downwards arrows with dotted boxes).

We tried to obtain fusions which produce a SecY−PhoA hybrid protein with low or negligible enzyme activity by randomly examining the restriction patterns of hundreds of white colonies or of colonies which turned pale blue after storage at 4°C for about a week. Further analysis by immunoprecipitation (Figure 4B) left only eight clones as hybrid protein producers (presumably in-frame fusions). Their fusion positions are indicated in Figure 3 by upward arrows. Fusions 53-7 and 56-8 gave enzyme activities comparable with the intermediate class discussed above and mapped in the same region. Other fusions were only 1−5% as active as the highest class (Table II) and mapped in the hydrophilic segments 9 and 11. Technically, it appeared to be difficult to obtain fusions of low enzyme activity to saturate all the possible sites of insertion.

The occurrence of the enzymatically active fusions at the even numbered hydrophilic segments suggests that these regions are normally facing the periplasm. In contrast, the hydrophilic segments 9 and 11 should be cytoplasmic.

*Protease accessibility of the SecY−PhoA fusion joints*

As an independent test for the localization of the PhoA domains of the SecY−PhoA hybrid proteins, we tested their sensitivities to proteases added to the spheroplasts. The hybrid proteins from fusions 3-4, 18-4, 11 and 12-1 were sensitive to either trypsin (3-4 and 12-1) or proteinase K (all of the above) (Figure 5). Cytoplasmic proteins such as the *groE* gene product and the large

**Table II.** Representative PhoA−SecY hybrid proteins

| SecY−PhoA fusion on pKY4 | Alkaline phosphatase activity | Junction point[a] | Size of protein (kd) | |
|---|---|---|---|---|
| | | | Predicted[b] | SDS−PAGE |
| 3-4 | 260 | 61 | 55.0 | 53 |
| 18-4 | 217 | 138 | 63.2 | 61 |
| 3-3 | 226 | 208 | 70.7 | 68 |
| 11 | 246 | 299 | 80.6 | 73 |
| 12-1 | 174 | 402 | 92.5 | 81 |
| 15-1 | 82.7 | | (60.4) | 58 |
| 61-7 | 10.8 | | (86.4) | 75 |
| 66-6 | 2.6 | | (95.9) | 83 |

[a]Determined by DNA sequencing and represented by residue number of SecY (including the first methionine).
[b]The sum of the SecY part, determined by sequencing or by restriction analysis (in parentheses), and the PhoA moiety encoded by Tn*phoA* (assumed to be 48 500 daltons; Manoil and Beckwith, 1985).

subunits of RNA polymerase were not degraded under the conditions of these experiments (data not shown). Since the SecY protein itself in the membrane resists the external proteases, the hybrid proteins may have been cleaved at or near the SecY−PhoA fusion joints. Consistent with this notion were the results that among those tested only fusions 3-4 and 12-1 were cleaved by trypsin and they contained a lysine or arginine residue in the SecY sequence immediately preceding PhoA. After cleavage by an external protease, a lower mol. wt fragment was observed in the immunoprecipitates for at least fusions 3-4, 18-4 and 11 (Figure 5). Since the normal alkaline phosphatase is resistant to proteases (Roberts and Chlebowski, 1984; our unpublished data), we believe that these fragments represented the PhoA moiety that was exported and enzymatically active.

Another enzymatically active class of hybrid protein, 3-3, was cleaved by some cellular protease, producing a fragment similar in size to those discussed above (Figure 4). The anti-PhoA reacting fragment was recovered from the periplasmic fraction (data not shown for the cell fractionation). Similar periplasmically located degradation product was observed previously with some of the Tsr−PhoA hybrid proteins (Manoil and Beckwith, 1986).

In contrast, the enzymatically inactive hybrid proteins (61-7 and 66-6) in the intact spheroplasts were not cleaved by the

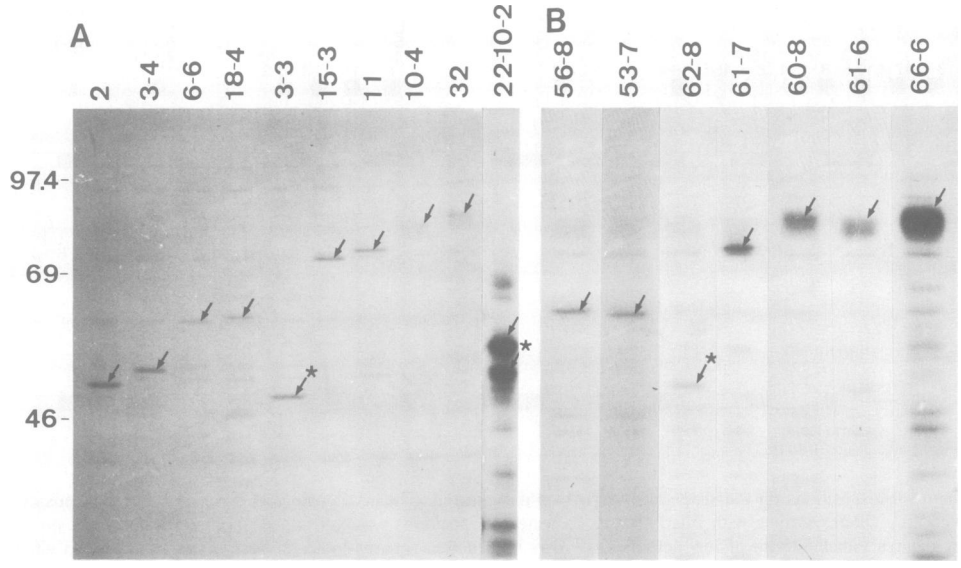


**Fig. 4.** SecY−PhoA hybrid proteins. Cells carrying a pKY4 derivative with Tn*phoA* transposition as indicated (see Figure 3) were induced by cyclic AMP and pulse-labelled with [$^{35}$S]methionine for 3 min. Immunoprecipitates with anti-PhoA serum were electophoresed. **A**, fusions with high enzyme activity; **B**, fusions with intermediate or low enzyme activity. The arrows indicate SecY−PhoA hybrid proteins, while * indicates a probable degradation product.

protease (Figure 5, lanes 10 and 13), whereas they were fully sensitive in the presence of Triton X-100 (lanes 11 and 14). These biochemical data support the notion that the PhoA enzyme activity is a reliable indicator for the export of the PhoA part in the hybrid protein.

*The hydrophobic segment 3 can exhibit an export signal activity*

The SecY−PhoA fusions in the region covering the hydrophilic segment 3 and hydrophobic segment 3 exhibit intermediate enzyme activities. According to the prediction in Table I, the hydrophobic segment 3 has a lower probability of being in the transmembrane structure than other hydrophobic stretches. However, it may be easier to construct a model of SecY topology with this hydrophobic segment also traversing the membrane (see Figure 6 and Discussion).

Some of the transmembrane segments of a membrane protein can act as uncleavable signal peptide (Blobel, 1980; Friedlander and Blobel, 1985; Manoil and Beckwith, 1986). We examined whether the hydrophobic segment 3 alone can exhibit an activity of translocating the PhoA moiety because such an activity may be taken as supporting evidence for a transmembrane orientation of this segment in the normal SecY protein. Tn*phoA* was allowed to transpose onto plasmid pKY22, in which the first and second hydrophobic segments have been deleted from the SecY sequence (Figure 3). We obtained a fusion, 22-10-2, which mapped in the region following the hydrophobic segment 3 (Figure 3). The PhoA enzyme activity in the fusion 22-10-2 was about nine times higher than that in the fusion 3-4, the highest class previously obtained, under the conditions measured. This fusion also produced a large amount of a hybrid protein (Figure 4A). The extraordinarily high enzyme activity in 22-10-2 may be due to the high copy number of the plasmid (see Materials and methods). In any event, the result indicates that the hydrophobic segment 3 without other hydrophobic sequences of SecY can export the PhoA moiety.

## Discussion

The SecY protein in the inverted membrane vesicles is subject to digestion by trypsin, indicating that it contains some cytoplasmic domains. Although the sites of trypsin cleavage have not

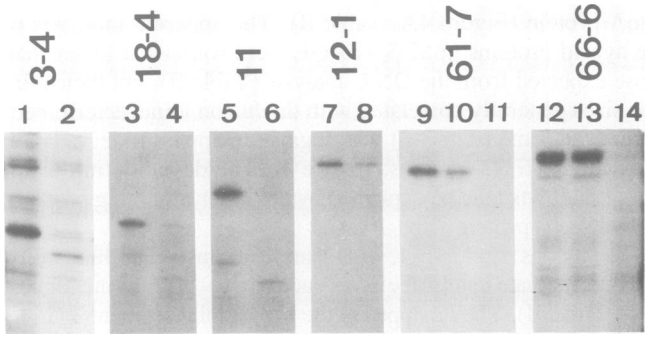


**Fig. 5.** Protease accessibility of the hybrid proteins. Cells of AD90 carrying a pKY4 derivative plasmid with Tn*phoA* insertion as indicated were induced, pulse-labelled with [$^{35}$S]methionine for 2 min, and converted into spheroplasts. They were incubatd with 50 μg/ml of trypsin (**lanes 2** and **8**) or 100 μg/ml of proteinase K (**lanes 4, 6, 10, 11, 13** and **14**). Samples for lanes 11 and 14 received 1% Triton X-100 before the incubation. After the incubation, samples were treated with protease inhibitors and TCA and subjected to precipitation with anti-PhoA serum as described in Materials and methods.

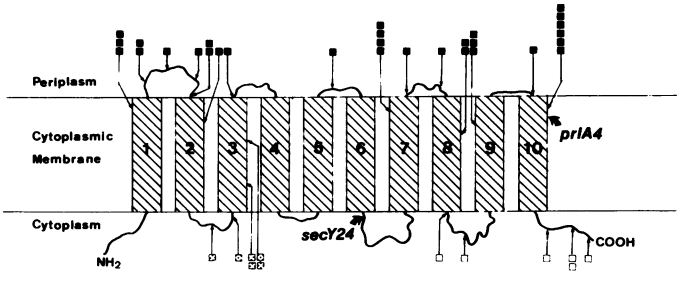


**Fig. 6.** A model for the orientation of the SecY protein in the membrane. Transmembrane segments are represented by hatched boxes. Filled squares with arrows indicate sites at which a highly active SecY−PhoA hybrid protein was generated, whereas dotted and open squares indicate those of intermediate and low enzyme activity respectively. Also shown are the locations of the amino acid alterations by the *secY24* (Shiba *et al.*, 1984) and the *prlA4* (Stader *et al.*, 1986) mutations.

been identified, this protein has a sum of 41 arginine and lysine residues, 31 of which are located in the hydrophilic segments of odd numbers (Figure 1). The resistance of this protein to proteases added to the periplasmic side suggests that SecY does not contain domains extensively exposed to the periplasm. Alternatively, such domains, if they exist, may be intrinsically resistant to proteases.

The PhoA moiety used in the Tn*phoA* analysis can faithfully reflect the local disposition of the target protein attached to the amino-terminal side (Hoffman and Wright, 1985; Manoil and Beckwith, 1985, 1986). It was assumed, and partly proved (Boyd *et al.*, 1987a), that the PhoA enzyme activity is a reliable parameter of its export to the periplasmic side. The Tn*phoA* approach has further been applied to a membrane protein of complex hydropathic characters such as the *malF* gene product (Boyd *et al.*, 1987b). In our similar analysis reported here, the SecY−PhoA fusions with high phosphatase activity have been obtained at five distinct regions along the SecY sequence, although the frequencies of the occurrence of such fusions varied from one region to another. These regions coincide with the alternate (even numbered) hydrophilic segments which should face the periplasm. The periplasmic exposure of these fusion joints was further supported by their accessibility to external proteases.

Based on the discrimination test (Table I) and the observation that even the hydrophobic segment 3 can exhibit an independent translocation activity, we propose that all the hydrophobic segments of SecY span the membrane. Taken together with the periplasmic assignment of the five hydrophilic regions, it follows that SecY traverses the membrane 10 times with both its termini facing the cytoplasm (Figure 6). Consistent with this model, the hydrophilic segments 9 and 11 have been assigned as cytoplasmic by our Tn*phoA* analysis. Also consistent is the enrichment of the potential cleavage sites of trypsin in the hydrophilic segments of odd numbers (Figure 1). The amino acid alteration (Gly240 to Asp) by the temperature-sensitive *secY24* mutation (Shiba *et al.*, 1984) is located in a cytoplasmically disposed region in this model (Figure 6). This is consistent with an idea that the functionally important domain of SecY is located in the cytoplasmic side of the membrane.

The fusions in the region near the hydrophilic segment 3 gave intermediate enzyme activities, despite the fact that this segment is supposed to be cytoplasmic in the proposed model. According to a theory of membrane protein insertion, the odd-numbered hydrophobic segments in our model serve as the export signal, whereas those of even numbers act as 'stop−transfer' sequences (Blobel, 1980). Although the hydrophobic segment 2 appears sufficiently hydrophobic, it is possible that its complete halt in the membrane depends on its interaction with other transmembrane segments located in the C-terminal side, or on the interaction of its C-terminal side with some other component. Alternatively, the hydrophobic segment 2 in the hybrid protein might function, at certain frequency, as an export signal rather than a stop transfer sequence. Expression of such 'cryptic' signal activity would export the PhoA moiety. The membrane anchor sequence of the immunoglobulin M heavy chain, which normally acts to halt transfer, can mediate protein translocation across the endoplasmic reticulum membrane in certain constructions *in vitro* (Mize *et al.*, 1986). In another artificial system of hybrid proteins with two signal sequences, the proteins could assume one of two alternative orientations in the membrane depending upon which signal sequence dominates in initiating translocation (Coleman *et al.*, 1985).

The above discussion reminds us of the need for caution in interpreting the results obtained in the system of Tn*phoA* transposition as already pointed out by Manoil and Beckwith (1986). Although our biochemical data to complement such analysis are still fragmental, we feel that the model presented in Figure 6 is largely correct on the grounds that it is consistent with many observations already discussed. von Heijne (1986) recently proposed a similar structure for the SecY protein based solely on the consideration of its amino acid sequence with special emphasis on the distribution of positively-charged residues.

The SecY protein is unique among the known factors of protein translocation in that it is deeply embedded in the membrane by multiple hydrophobic sequences. The *secA* (Oliver and Beckwith, 1982) and *secB* (Kumamoto and Beckwith, 1985) gene products are soluble proteins; a part of SecA may, peripherally, be associated with the membrane. The eukaryotic membrane components such as the docking protein (Lauffer *et al.*, 1985) and ribophorins (Crimaudo *et al.*, 1987) traverse the membrane once or, at most, twice respectively. The proposed structure of SecY is rather similar to bacteriorhodopsin or the lactose carrier protein, which catalyze translocation of protons or small molecules possibly by forming specialized channels in the membrane (Henderson and Unwin, 1975). In this respect, it might be envisaged that the SecY protein forms a 'channel' for the passage of polypeptides. Although only some of the putative transmembrane segments contain (one or two) charged residues, the possibility of their forming some amphiphilic structure in the membrane should be considered.

While the *secY24* mutation maps in one of the putative cytoplasmic domains, the *prlA4* suppressor allele changes Ile408 (Stader *et al.*, 1986) located within the most C-terminal transmembrane segment (Figure 6). More systematic isolation of mutations affecting the SecY function and their characterization would help define the role of each domain of the SecY protein. Such analysis would be particularly rewarding when combined with the cell-free assay of the SecY activity in the isolated membrane vesicles (Bacallao *et al.*, 1986).

We have shown that a single transmembrane segment, at least the first or the third one, could suffice as the export signal for translocation of the PhoA moiety *in vivo*. It is conceivable that many, if not all, of the alternate transmembrane sequences have independent signal peptide-like activity, as has been demonstrated for the membrane anchors of opsin in the eukaryotic cell-free sysem (Friedlander and Blobel, 1985). SecY−PhoA fusions and their various derivatives will be useful for analysis of the mode of membrane insertion of each transmembrane sequence, and for our understanding of protein−membrane interactions that occur during biogenesis of complex membrane proteins.

## Materials and methods

*Bacterial strains and plasmids*

The *E. coli* K-12 strains used were KI269 [F'*lacI*$^q$ *PL8 Z*$^+$ *Y*$^+$ *A*$^+$ *pro*$^+$/Δ(*argF-lac*)U169 *cya283*, and other irrelevant markers] (Akiyama and Ito, 1985), CC118 (Δ *lacX74 phoAΔ20 recA1* and other irrelevant markers), CC202 (F42 *lacI3 zzf-2*::Tn*phoA*/CC118) (Manoil and Beckwith, 1985), and AD90 (Δ *phoA lon100 tsx*::Tn*5*, constructed in this study). Plasmid pNO1573 was originally provided by M.Nomura and used for *lac* promoter-directed overproduction of the SecY protein (Akiyama and Ito, 1985). Plasmid pKY4 was constructed as follows. The 1.5 kb *Hae*II fragment (which contains *secY*) of the *spc* operon (Cerretti *et al.*, 1983) was linked with the *Eco*RI linker (Genex) after protection of the internal *Eco*RI site by *Eco*RI methylase (New England Biolabs), and cloned into the *Eco*RI site of pNO1575 (Ito *et al.*, 1983). pKY22 was constructed by ligating the 1.35 kb *Pvu*II fragment (which lacks the 5' region of *secY*) of pNO1573 into the *Sma*I site of pUC9 (Vieira and Messing, 1982); the translational reading frame is maintained between the *lacZ'* and *secY* sequences.

*Isolation and characterization of TnphoA insertions*

The procedures of Manoil and Beckwith (1985) were followed for selecting Tn*phoA* transpositions. CC202 was first transformed by pKY4 to ampicillin (50 μg/ml) resistance, and then plated on LB broth (Davis *et al.*, 1980) agar containing 300 μg/ml of kanamycin in addition to ampicillin to select Tn*phoA* transpositions onto the multi-copy plasmid. Resistant colonies were pooled and a mixture of plasmid DNA was extracted, which was then used to transform CC118, using peptone agar plates (Ito *et al.*, 1983) containing ampicillin, kanamycin and 5-bromo-4-chloro-3-indolyl phosphate (20 μg/ml). Since CC118 is *lacI*$^-$, the *lac* promoter on the plasmid is fully expressed in the peptone medium without glucose. Transpositions onto pKY22 were similarly selected except that LB agar was used throughout. pKY22 is deleterious to the cell in the peptone medium. This is because the expression of the *lac* system is higher in the peptone medium than in the LB medium, and the copy number of this pUC9-based plasmid is several fold higher than the pBR322 based pKY4.

The positions of Tn*phoA* insertions in pKY4 were determined by *Eco*RI digestion patterns of the plasmids, based on the known locations of the *Eco*RI sites in pKY4 (Figure 3) and in Tn*phoA* [one at 770 bases and another at 1100 bases from the left (N-terminal) end; Tn*phoA* is 7.61 kb long] (Manoil and Beckwith, 1985; and personal communication). Transpositions onto pKY22 were mapped by digestion with *Hin*dIII and *Eco*RI (see Figure 3).

For sequencing the regions around the *secY–phoA* fusion joints by the chain termination method (Messing, 1983), the *Eco*RI–*Dra*I (located at 250 bases from the left end of Tn*phoA*) fragments of the plasmid derivatives were subcloned into M13mp18 that had been cut with *Sma*I and *Eco*RI.

Alklaline phosphatase enzyme activity was determined with cells grown in peptone medium with kanamycin and ampicillin, as described by Manoil and Beckwith (1985). In the case of the pKY22 derivatives, LB–glucose medium was used to overcome the growth inhibition.

*Trypsin digestion of the SecY protein in the membrane vesicles and spheroplasts*

Cells of KI269/pNO1573 were grown, induced, labelled and fractionated essentially as described previously (Akiyama and Ito, 1985), except that no protease inhibitors were included. The spheroplast suspension or the cytoplasmic membrane peak fraction of the sucrose step gradient centrifugation were mixed with varying concentrations of trypsin, and the mixtures were incubated at 0°C for 30 min. Reactions were stopped by addition of 3 mM toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride (TLCK). Samples were subjected to SDS–PAGE as described previously (Akiyama and Ito, 1985).

*Detection and protease accessibility test of SecY–PhoA hybrid proteins*

For detecting SecY–PhoA hybrid proteins, cells were grown (with appropriate drugs), induced with 5 mM cyclic AMP, and pulse-labelled with [$^{35}$S]methionine, essentially as described previously (Akiyama and Ito, 1985). Antibody precipitation was done as described (Ito *et al.*, 1981) except that SDS solubilization of the trichloroacetic acid (TCA)-precipitated proteins was done at 37°C. Antisera against the PhoA protein were generously provided by J.Garwin and K.Yoda. The sensitivity of the pulse-labelled hybrid protein to proteases was examined by treating spheroplasts with an appropriate protease (at 0°C for 30 min), followed by TCA precipitation and antibody precipitation in the presence of 1 mM phenylmethylsulfonyl fluoride, 3 mM TLCK and 100 μg/ml of soybean trypsin inhibitor. Labelled proteins were visualized by autoradiography or fluorography after SDS–PAGE (Akiyama and Ito, 1985).

## Acknowledgements

We thank Drs Colin Manoil and Jon Beckwith for providing strains, protocols and a restriction map of the Tn*phoA* system, Drs Jeff Garwin and Koji Yoda for anti-PhoA sera, Drs Kiyotaka Shiba and Akira Ito for help in the computer analysis of SecY sequence, Dr Takashi Yura for encouragement, and Ms Mariko Kobayashi for technical assistance. This work was supported by grants from the Ministry of Education, Science and Culture, Japan. Y.A. was a recipient of JSPS Fellowships for Japanese Junior Scientists.

## References

Akiyama,Y. and Ito,K. (1985) *EMBO J.*, **4**, 3351–3356.
Akiyama,Y. and Ito,K. (1986) *Eur. J. Biochem.*, **159**, 263–266.
Bacallao,R., Crooke,E., Shiba,K., Wickner,W. and Ito,K. (1986) *J. Biol. Chem.*, **261**, 12907–12910.
Blobel,G. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 1496–1500.
Blobel,G. and Dobberstein,B. (1975) *J. Cell Biol.*, **67**, 835–851.
Boyd,D., Guan,C.-D., Willard,S., Wright,W., Strauch,K. and Beckwith,J. (1987a) In Torriani-Gorini,A. *et al.* (eds), *Phosphate metabolism and Cellular Regulation in Microorganisms*. ASM Press, Washington DC, in press.
Boyd,D., Manoil,C. and Beckwith,J. (1987b) *Proc. Natl. Acad. Sci. USA*, **84**, in press.
Buchel,D.E., Gronenborn,B. and Muller-Hill,B. (1980) *Nature*, **283**, 541–545.
Cerretti,D.P., Dean,D., Davis,G.R., Bedwell,D.M. and Nomura,M. (1983) *Nucleic Acids Res.*, **11**, 2599–2619.
Coleman,J., Inukai,M. and Inouye,M. (1985) *Cell*, **43**, 351–360.
Crimaudo,C., Hortsch,M., Gausepohl,H. and Meyer,D.I. (1987) *EMBO J.*, **6**, 75–82.
Davis,R.W., Botstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, New York.
Emr,S.D., Hanley-Way,S. and Silhavy,T.J. (1981) *Cell*, **23**, 79–88.
Friedlander,M. and Blobel,G. (1985) *Nature*, **318**, 338–342.
Henderson,R. and Unwin,P.N.T. (1975) *Nature*, **257**, 28–32.
Hoffman,C.S. and Wright,A. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 5107–5111.
Ito,K. (1984) *Mol. Gen. Genet.*, **197**, 204–208.
Ito,K. (1986) *Adv. Biophys.*, **21**, 267–280.
Ito,K., Bassford,P. and Beckwith,J. (1981) *Cell*, **24**, 707–717.
Ito,K., Wittekind,M., Nomura,M., Shiba,K., Yura,T., Miura,A. and Nashimoto, H. (1983) *Cell*, **32**, 789–797.
Klein,P., Kanehisa,M. and DeLisi.C. (1985) *Biochim. Biophys. Acta*, **815**, 468–476.
Kumamoto,C.A. and Beckwith,J. (1985) *J. Bacteriol.*, **163**, 267–274.
Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Lauffer,L., Garcia,P.D., Harkins,R.N., Coussens,L., Ullrich,A. and Walter,P. (1985) *Nature*, **318**, 334–338.
Manoil,C. and Beckwith,J. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 8129–8133.
Manoil,C. and Beckwith,J. (1986) *Science*, **233**, 1403–1408.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20–78.
Mize,N.K., Andrews,D.W. and Lingappa,V.R. (1986) *Cell*, **47**, 711–719.
Oliver,D.B. and Beckwith,J. (1982) *Cell*, **30**, 311–319.
Roberts,C.H. and Chlebowski,J.F. (1984) *J. Biol. Chem.*, **259**, 729–733.
Seckler,R. and Wright,J.K. (1984) *Eur. J. Biochem.*, **142**, 269–279.
Shiba,K., Ito,K., Yura,T. and Cerretti,D.P. (1984) *EMBO J.*, **3**, 631–635.
Singer,S.J., Maher,P.A. and Yaffe,M.P. (1987) *Proc. Natl. Acad. Sci. USA.*, **84**, 1015–1019.
Stader,J., Benson,S.A. and Silhavy,T.J. (1986) *J. Biol. Chem.*, **261**, 15075–15080.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.
von Heijne,G. (1986) *EMBO J.*, **5**, 3021–3027.
Walter,P., Gilmore,R. and Blobel,G. (1984) *Cell*, **38**, 5–8.

*Received on 21 April 1987; revised on 10 August, 1987*