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A prokaryotic membrane anchor sequence: Carboxyl terminus of bacteriophage f1 gene *III* protein retains it in the membrane

(filamentous phage/membrane proteins/domain structure/fusion proteins/membrane topogenesis)

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ABSTRACT **Gene *III* protein of bacteriophage f1 is inserted into the host cell membrane where it is assembled into phage particles. A truncated form of gene *III* protein, encoded by a recombinant plasmid and lacking the carboxyl terminus, does not remain in the membrane but instead appears to slip through it. Fusion of a hydrophobic "membrane anchor" from another membrane protein, the gene *VIII* protein, to the truncated gene *III* protein (by manipulation of the recombinant plasmid) restores membrane anchoring. A model for the relationship of gene *III* protein with the *Escherichia coli* membrane is discussed.**

Escherichia coli, like other Gram-negative bacteria, is surrounded by an inner (cytoplasmic) and an outer membrane. The two membranes are separated by the periplasm, an aqueous compartment isolated from the cytoplasm. The inner and outer membranes as well as the periplasm contain different sets of proteins. The mechanism by which proteins are transported to the appropriate cellular destinations has been a topic of great interest recently (1–3). The signal hypothesis (4, 5) explains how proteins synthesized from cytoplasmic precursors might be transferred across lipid bilayers. Indeed, many membrane proteins are synthesized with a hydrophobic NH_2-terminal "signal peptide" which is cleaved upon insertion into the cytoplasmic membrane. Although the signal hypothesis *per se* provides a way to think about how proteins get into and across membranes, it does not help us to understand how an extracytoplasmic bacterial protein gets to its final destination, which may be any of the following: (*a*) anchored in the inner membrane, (*b*) secreted through the inner membrane and into the periplasm, or (*c*) the outer membrane. The factors involved in "deciding" which of these pathways is taken by a given membrane protein are not well understood (3).

One factor distinguishing inner membrane proteins from periplasmic ones may be the presence of a hydrophobic domain which anchors the protein in the membrane. An example of this from a eukaryotic system exists in the secreted and membrane-bound immunoglobulin μ chain molecules. These differ in that the latter contains an extra domain rich in hydrophobic amino acid residues at its COOH terminus (6). Both molecules contain signal peptides at their NH_2 terminus. Several membrane-spanning domains such as the one found at the COOH terminus of membrane-bound immunoglobulin μ chain have been identified and characterized. They share the following structural features: a core sequence of 19–23 uncharged residues, rich in hydrophobic amino acids, flanked by charged residues (7). In this regard, these domains resemble signal sequences.

Bacteriophage f1 gene *III* protein, a minor coat protein, is a 406-amino acid protein bearing an 18-amino acid signal peptide which is cleaved before the protein is assembled into virus particles at the *E. coli* membrane (8, 9). The protein is found in the membrane fraction of infected cells (10) and further studies have shown it to be localized primarily in the inner (cytoplasmic) membrane (ref. 11; R. E. Webster, personal communication).

Gene *III* protein is responsible for a number of effects on the outer membrane of *E. coli*. These include deoxycholate sensitivity, leakage of periplasmic β-lactamase, impaired F pili, and drastic shifts in tolerance to certain colicins. All of these phenotypes are expressed in cells synthesizing either full-length gene *III* protein or its NH_2-terminal fragment. A specific portion of gene *III* has been identified by deletion analysis as the region responsible for these phenotypes (11). This region contains a glycine-rich domain spanning residues 68–87 and composed of four tandem copies of the sequence Glu-Gly-Gly-Gly-Ser.

In this paper we examine the cellular location of full-length gene *III* protein specified by phage f1 and plasmid pJB61 and the truncated form of gene *III* protein encoded by plasmid pJB11.

MATERIALS AND METHODS

Bacteria and Plasmid Strains. All strains used in this paper have been described elsewhere (11) with the exception of plasmid pJB38. Details of its construction are in the legend to Fig. 6.

Cell Fractionation, Immunoprecipitation, and Electrophoresis. Cells were grown as described (11, 12). Fractionation with 0.1 M NaOH was as described (13, 14). Fractionation into periplasmic, cytoplasmic, and membrane fractions was by spheroplast formation and lysis. Cells from 0.2 ml of labeled culture were pelleted by centrifugation and resuspended with cells from 5 ml of unlabeled culture in 0.5 ml of 20% (wt/vol) sucrose/100 mM Tris·HCl, pH 8.0/10 mM Na_3EDTA. Then, 10 μl of lysozyme solution (5 mg/ml, freshly diluted; Sigma) was added and the mixture was incubated on ice for 10 min. The spheroplasts were removed by centrifugation in an Eppendorf microcentrifuge for 2 min, and the supernatant was saved as the periplasmic fraction. The spheroplasts were washed once in the above buffer and resuspended in 50 μl of 100 mM Tris·HCl, pH 8.0/20% sucrose/10 mM $MgCl_2$ and containing 50 μg of DNase (Worthington) per ml. A glass rod was used to resuspend the pellet. Immediately, 200 μl of cold distilled H_2O was added. The spheroplasts were sonicated for 2 sec and then frozen and thawed once for full lysis. The lysed spheroplasts were then centrifuged for 1 min, yielding a cytoplasmic (supernatant) and a membrane (pellet) fraction. All fractions were precipitated with 5% trichloroacetic acid and washed with acetone prior to

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resuspension in 25 μl of 4% $NaDodSO_4$. Aliquots (1 μl) were electrophoresed and autoradiographed to ensure that fractionation was successful; following this, 4-μl fractions were immunoprecipitated as described (11, 14). Electrophoresis was on $NaDodSO_4$/urea/polyacrylamide gels as described (12). Whole-cell Pronase treatment was performed as described (14, 15).

Sequence Determination. The protein sequence was determined radiochemically on a Beckman Sequenator by published procedures (16, 17). The data were compared with the known sequence of pre-gene *III* protein and gene *III* protein as determined by DNA sequence analysis and protein sequence determination (8, 9).

RESULTS

We cloned gene *III* and various NH_2-terminal fragments of it into pBR322. Cells containing such plasmids expressed gene *III* protein at levels similar to those found in phage f1-infected cells (11). Cells bearing the plasmid pJB11 encoded only the NH_2-terminal half (204 amino acids plus signal peptide) of the protein, producing a truncated gene *III* protein of faster electrophoretic mobility than full-length gene *III* protein (Fig. 1).

Because no new bands were obvious in electropherograms of proteins made by cells containing the recombinant plasmids, it was necessary to use a combination of immunoprecipitation with gene *III*-specific antiserum (the kind gift of W. Konigsberg), high-specific-activity [^{35}S]methionine label, short labeling times (3 min), and low temperature (30°C) in order to detect the truncated protein by gel electrophoresis reproducibly. Like many "abnormal" subpeptides in *E. coli*, truncated gene *III* protein was recovered in greater yield when the cells were grown at 30°C.

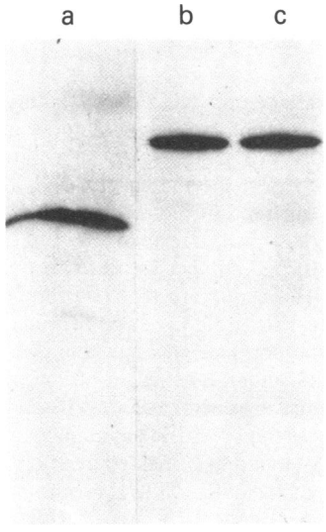

FIG. 1. Plasmid-encoded gene *III* protein and truncated gene *III* protein. Cultures of K38-pJB11 (encoding truncated gene *III* protein), K38-pJB11*am4* [like pJB11 but bearing an early gene *III* amber mutation (11)] infected with phage f1, and K38-pJB61 (encoding a full-length gene *III* protein) were grown in DO medium (as in ref. 12) containing 2 mM amino acids, 0.2% glucose (as in ref. 12), and 100 μg of ampicillin per ml. K38-pJB11*am4* was used as a host for the phage f1 infection so that the same batch of ampicillin-containing medium could be used for growing all of the strains. Because pJB11*am4* bears an early amber mutation in gene *III* (11), it produces no gene *III* protein (see also Fig. 3). Aliquots were labeled with 10 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine and then precipitated with trichloroacetic acid. The pellets were washed with acetone and resuspended in 25 μl of 4% $NaDodSO_4$. An aliquot (4 μl) of such a sample was immunoprecipitated, electrophoresed, and autoradiographed as described (14). Lanes: a, K38-pJB11 (truncated); b, K38-pJB11*am4*/f1-infected; c, K38-pJB61.

That this fragment was processed (i.e., had its signal peptide removed) in the same way as normal gene *III* protein was shown in two ways. First, truncated gene *III* protein was labeled *in vivo* with [^{35}S]cysteine and [^{3}H]lysine. Sequence analysis of the immunoprecipitated labeled truncated gene *III* protein established that cysteine was at position 7 and lysine was at positions 10, 22, and 25 (the unprocessed molecule would have no cysteine until residue 25 and would have lysine at positions 2 and 3) as is found in mature gene *III* protein (Fig. 2) (8, 9). Second, truncated gene *III* protein synthesized in an *in vitro* transcription/translation system (only the precursor is made in this system) had a slower electrophoretic mobility than truncated gene *III* protein made *in vivo* (data not shown).

The cellular location of the truncated protein was examined by two methods—the NaOH technique, which separates integral membrane proteins from other *E. coli* proteins (13, 14), and spheroplast formation and lysis, which separates *E. coli* into cytoplasmic, membrane, and periplasmic fractions.

Electrophoresis of immunoprecipitated gene *III* protein from NaOH-soluble and NaOH-insoluble fractions showed that the truncated protein (encoded by plasmid pJB11) was entirely (>95%) in the NaOH-soluble fraction whereas full-length protein (from either phage f1-infected cells or cells containing plasmid pJB61) was primarily in the NaOH-insoluble (integral membrane protein) fraction (Fig. 3). This suggested that full-length gene *III* protein is an integral membrane protein but truncated gene *III* protein is not. The NaOH technique did not indicate whether truncated gene *III* protein is in the cytoplasmic or the periplasmic compartment. To distinguish between these possibilities, periplasmic, cytoplasmic, and membrane fractions were prepared and immunoprecipitated. The data show that, whereas most of the full-length protein was in the

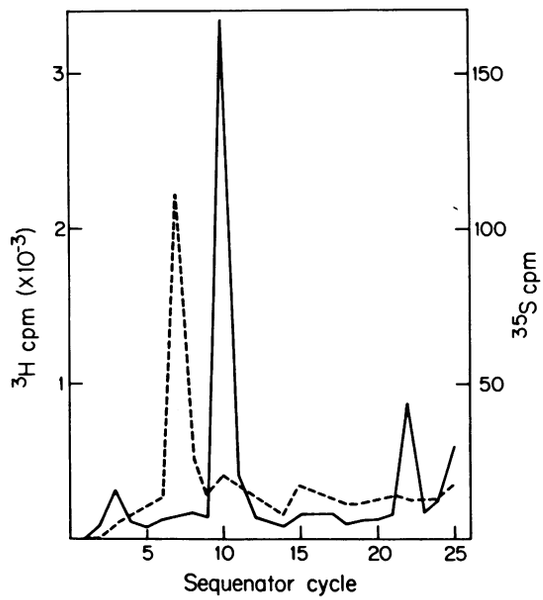

FIG. 2. Sequence analysis of pJB11 truncated gene *III* protein NH_2 terminus. Two K38-pJB11 cultures were grown as described in Fig. 1 (except lacking the amino acid used for labeling). One was labeled with [^{3}H]lysine and the other, with [^{35}S]cysteine. A periplasmic fraction was prepared from each culture and immunoprecipitated. Small aliquots were electrophoresed and found to be electrophoretically homogeneous. The immunoprecipitates were mixed and analyzed in a Beckman Sequenator (16, 17). As expected for mature gene *III* protein (8, 9), the [^{35}S]cysteine peak (broken line) was at position 7 and the [^{3}H]lysine peaks (solid line) were at positions 10, 22, and 25. Unprocessed pre-gene *III* protein would have lysine at positions 2 and 3 and no cysteine until position 25.

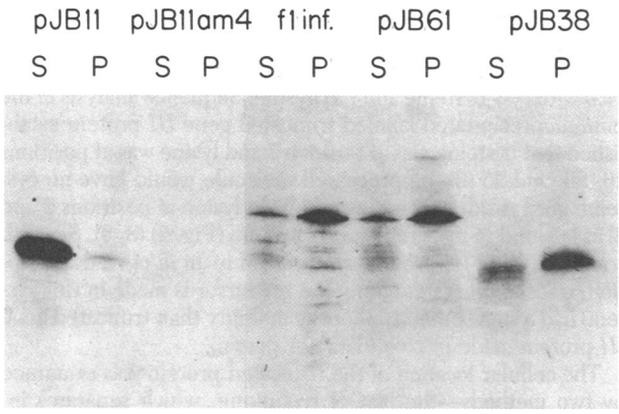

FIG. 3. NaOH fractionation of *E. coli* for localization of gene *III* proteins. Cells grown, infected, and labeled as in Fig. 1 were fractionated with 0.1 M NaOH (14) into supernatant (S) and pellet (P). The host strain for all plasmids was K38 (Su^-). After electrophoresis of small aliquots of each sample to verify successful fractionation (14), aliquots were immunoprecipitated, electrophoresed, and autoradiographed as in Fig. 1.

membrane pellet, essentially all of the truncated protein was in the periplasm (Fig. 4). Thus, the truncated protein apparently passes through the inner membrane and into the periplasm. Some of the truncated protein, like the periplasmic β-lactamase encoded by the plasmids, leaked from the periplasm into the medium (R. E. Webster, personal communication).

The above results suggest that the gene *III* protein is anchored in the membrane by virtue of its COOH-terminal sequence. If this is its only attachment point, then the NH_2 terminus may be thought of as floating free in the periplasm. This idea was tested by treating phage f1-infected cells with Pronase in the presence of EDTA. Others have shown that such treatment selectively removes the portion(s) of membrane proteins exposed to the periplasm (14, 15). Under these conditions, cytoplasmic proteins remained undigested (Fig. 5). Indeed, all the gene *III* protein molecules were protease-sensitive in both phage f1-infected cells and cells producing plasmid-encoded gene *III* protein (Fig. 5). However, a faint band of lower molecular weight appeared subsequent to such digestion. This

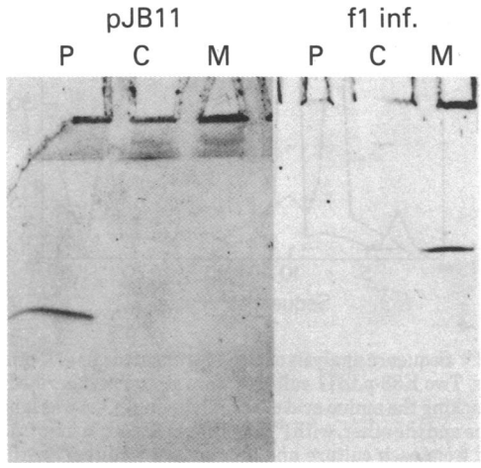

FIG. 4. Spheroplast formation and lysis. Cells grown, infected, and labeled as in Fig. 1 were fractionated into periplasm (P), cytoplasm (C), and membrane (M) by spheroplast formation and lysis. After electrophoresis of small aliquots to confirm successful fractionation of various *E. coli* proteins, aliquots were immunoprecipitated, electrophoresed, and autoradiographed as in Fig. 1.

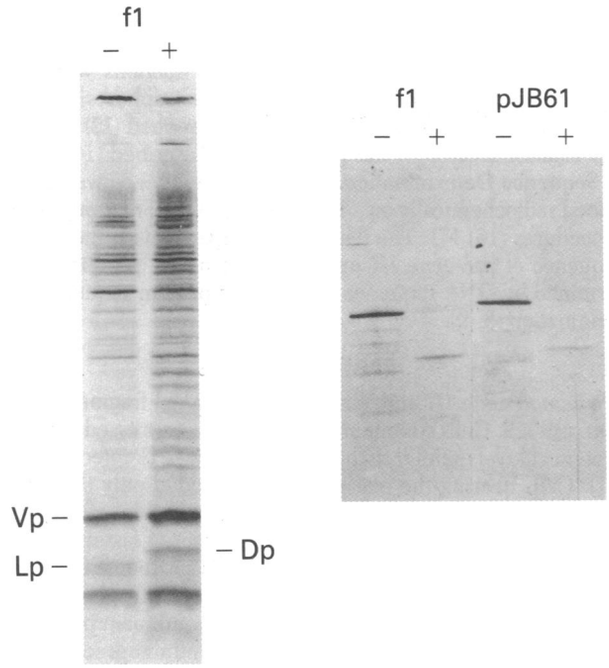

FIG. 5. Cells grown, infected, and labeled as in Fig. 1 were treated with Pronase in Tris/sucrose/EDTA as described (14, 15). Aliquots of the labeled phage f1-infected cell proteins were electrophoresed to confirm successful digestion of lipoprotein (*Left*). Note that lipoprotein (Lp) was converted quantitatively to a digestion product (Dp) of slower mobility by Pronase digestion (14), but cytoplasmic gene *V* protein (Vp) (and most other *E. coli* proteins) are not digested detectably. Aliquots were immunoprecipitated and electrophoresed as described above (*Right*). −, No Pronase added, 0°C, 2 hr; +, 100 μg of Pronase per ml, 0°C, 2 hr. The host strain was K38.

faint band probably represented the NH_2-terminal portion of gene *III* protein. Truncated gene *III* protein remained untouched by the protease under these conditions (data not shown). The protease resistance of the NH_2-terminal portion of gene *III* protein, seen in the electron microscope as a ball-shaped particle, has been described (18, 19).

A plasmid, pJB38, that specifies a hybrid or fusion protein containing the NH_2 terminus of gene *III* protein and the COOH terminus of gene *VIII* protein was constructed (Fig. 6). Gene *VIII* protein is another phage coat protein which is found principally in the inner membrane (21). Plasmid pJB38 caused the same outer membrane effects caused by its parent, pJB11. The hybrid protein specified by pJB38 contained all of the gene *III* protein sequences found in truncated gene *III* protein fused to residues 5–50 (i.e., the entire COOH terminus and membrane-spanning domain) of gene *VIII* protein (Fig. 6). This hybrid protein was found in the NaOH pellet (integral membrane fraction) of *E. coli* (Fig. 3). Thus, the sequence of amino acids responsible for anchoring gene *VIII* protein in the membrane can also anchor truncated gene *III* protein in the membrane.

DISCUSSION

We have shown that plasmid pJB61-encoded gene *III* protein is expressed at a level similar to that produced in phage f1-infected cells [the copy number of the two replicons is similar (22)]. Moreover, the plasmid-encoded protein is inserted into the membrane like the phage-encoded protein, suggesting that only host cell components and not phage-specific gene products are necessary for effecting such insertion. A truncated form of gene *III* protein, encoded by plasmid pJB11, is not only inserted into the membrane but also passes through it, suggesting that

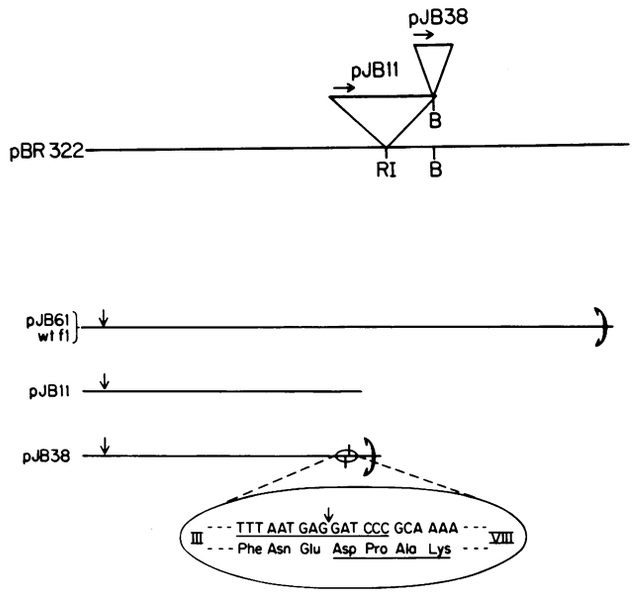

FIG. 6. (*Upper*) Plasmid pJB11 was constructed by ligating the *Hae* III fragment C of phage f1 to *Eco*RI linkers and thence into the *Eco*RI site of pBR322 (pBR322 is shown here cleaved at its *Pvu* II site; see ref. 11 for further construction details). Plasmid pJB38 was constructed by ligating the *Mbo* I fragment D of phage f1, which contains the COOH terminus of gene *VIII*, to a *Bam*HI digest of pJB11 which had been treated with alkaline phosphatase to prevent reformation of pJB11 (20). Thus, pJB38 lacks the small *Bam*HI fragment contained in pJB11 and, as a result, lacks a portion of the tetracycline-resistance gene. RI, *Eco*RI site; B, *Bam*HI site; arrows, direction of transcription of genes *III* and *VIII*. (*Lower*) In-frame fusion between gene *III* and gene *VIII* is formed when the complementary *Bam*HI and *Mbo* I ends are ligated. Because the *Bam*HI site within the pJB11 insert lies near the right end of the insert, only the last seven amino acids of the pJB11 truncated protein are not found in pJB38. Horizontal lines represent the gene products encoded by the plasmid indicated. Straight arrows, signal peptidase cleavage site; curved arrows, position of membrane anchoring sequences; vertical lines in pJB38, transition boundaries in the fusion. The oval shows the DNA and predicted protein sequences of the fusion boundary. In it, the arrow marks the *Bam*HI site, the upper line indicates the extent of gene *III* protein sequence, and the lower line indicates the extent of gene *VIII* protein sequence.

the COOH-terminal portion of gene *III* protein contains a sequence for retaining it in the membrane.

Taken together with the elegant studies of *mal* operon–β-galactosidase fusions (3, 23, 24), our results show that, even though a large portion of a membrane protein is removed by deletion (as we have shown for gene *III* protein) or drastically changed in sequence by formation of a protein fusion [as shown for *lam*B and *mal*E (23, 24)], the initial step in transferring the protein to its extracytoplasmic location—insertion into the membrane—can still take place. Our experiments show that at least the last 200 amino acids of gene *III* protein are dispensable as far as translocation across the membrane is concerned. Plasmid pJB2, which encodes a shorter gene *III* protein fragment (only 98 amino acids plus signal peptide), affects the outer membrane in much the same way as the longer pJB11 fragment (11). We take this as suggestive evidence (but not proof) that this shorter fragment is also able to pass through the membrane. This would indicate that the last 300 amino acids of gene *III* protein are not required for passage through the membrane. In a similar manner, it has been shown that *mal* operon–β-galactosidase fusion proteins lacking extensive COOH-terminal regions of the corresponding *mal* gene product are inserted into the membrane.

Those *mal*E/*lac*Z and *lam*B/*lac*Z fusion proteins that are found in the cytoplasmic membrane can be lethal to the cell. When cells make large quantities of these fusion proteins, they accumulate the precursors of many periplasmic and outer membrane proteins and eventually they die (25). This has been attributed to "jamming" of the limited number of "export sites," presumably by the enormous β-galactosidase moiety of the fusion protein. Blocking export by mutation of the signal sequence (3) or the use of export-deficient cells (26) keeps the cells viable. The membrane perturbation produced by gene *III* protein (11) is entirely different. First, the membrane effects do not depend on the presence of the gene *III* protein membrane anchor sequence; truncated gene *III* protein, which is free in the periplasm, has the same effect. Second, with the gene *III* protein system, we never see membrane protein precursors. Finally, the cells do not die.

Insertion of gene *III* protein into the membrane and subsequent removal of its signal peptide happen independently of membrane anchoring because the truncated protein is translocated and processed in a normal fashion. Thus, membrane insertion and membrane anchoring may be thought of as separate processes.

The COOH terminus of gene *III* protein (residues 379–401) has the highest average hydrophobicity of any domain of gene *III* protein, including the signal peptide (Fig. 7). Its structure, 23 neutral residues flanked by arginine residues, is quite similar to structures identified as membrane anchor sequences in other proteins (7). We propose that this particular domain is required for anchoring gene *III* protein in the membrane. A similar model for the anchoring of gene *III* protein in the membrane via its COOH-terminal sequence has been proposed on the basis of sequence data alone (19). Further experiments will be

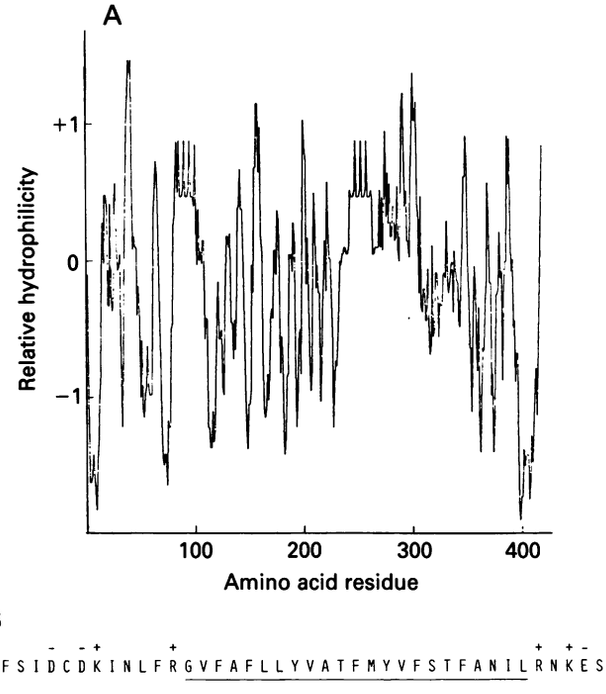

FIG. 7. (*A*) Average hydrophilicity of gene *III* protein. The running average hydrophilicity per 6 residues [based on the data of Levitt (27)] was calculated and plotted as a function of amino acid residue position (including the signal peptide). +1, most hydrophilic; −1, least hydrophilic. Note the COOH terminus (379–401), which is even more hydrophobic than the signal peptide (1–18). Note also the two very hydrophilic glycine-rich domains (68–87; 226–264) composed of repeating sequences rich in glycine (9). (*B*) Amino acid sequence of the putative gene *III* protein COOH-terminal membrane anchor sequence [based on the DNA sequence (9, 19)]. Charged residues are indicated.

required to identify explicitly the particular domain responsible for anchoring. The relationship between truncated and full-length gene *III* proteins is like that between the secreted and membrane-bound immunoglobulin μ and δ chain molecules (6, 28) and may prove to be a general model.

At least two bacterial inner membrane proteins, the carboxypeptidases D of two *Bacillus* species, have COOH-terminal membrane anchor sequences (29). These are similar in general structure to the sequence at the gene *III* protein COOH terminus, except that one does contain a single acidic residue. The NH_2-terminal domain of these proteins contains the carboxypeptidase activity and is functional in the absence of membrane anchoring (30). Because both the truncated and full-length gene *III* proteins cause an outer membrane pleiotropic effect (11), gene *III* protein also may be thought of as having an active NH_2-terminal domain which does not require membrane anchoring for activity. Like gene *III* protein, the *E. coli* carboxypeptidase analogous to the *Bacillus* proteins bears a signal sequence (31).

We call the hydrophobic COOH-terminal domains "membrane anchor sequences" because this describes their effect. These sequences may also act as "stop transfer" or "dissociation" sequences which allow the ribosome to dissociate from the membrane, interrupting vectorial discharge of the protein into the membrane (3, 5, 32, 33). However, because we have no information on the state of the ribosomes when they produce such sequences, we hesitate to apply such terminology at this time.

Like signal sequences, membrane anchor sequences may be discrete, separable domains that are both sufficient and necessary for membrane attachment once the protein is inserted into the membrane. The similarity in structure of signal peptides and membrane anchors suggests such a similar role. Signal peptidases may have arisen evolutionarily as a way to free proteins (or their NH_2-terminal domains) from the outer face of the membrane once it had been crossed.

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