Journal of Bioenergetics and Biomembranes, Vol. 22, No. 3, 1990

MINI-REVIEW

Export and Sorting of the *Escherichia coli* Outer Membrane Protein OmpA

Roland Freudl,[1] Michael Klose,[2] and Ulf Henning[2]

Received November 7, 1989

Abstract

Results of studies, mostly using the outer membrane, 325 residue protein OmpA, are reviewed which concern its translocation across the plasma membrane and incorporation into the outer membrane of *Escherichia coli.* For translocation, neither a unique export signal, acting in a positive fashion within the mature part of the precursor, nor a unique conformation of the precursor is required. Rather, the mature part of a secretory protein has to be export-compatible. Export-incompatibility can be caused by a stretch of 16 (but not 8 or 12) hydrophobic residues, too low a size of the polypeptide (smaller than 75 residue precursors), net positive charge at the N-terminus, or lack of a turn potential at the same site. It is not yet clear whether binding sites for chaperonins (SecB, trigger factor, GroEL) within OmpA are important *in vivo.* The mechanism of sorting of outer membrane proteins is not yet understood. The membrane part of OmpA, encompassing residues 1 to about 170, it thought to traverse the membrane eight times in antiparallel β-sheet conformation. At least the structure of the last β-strand (residues 160–170) is of crucial importance for membrane assembly. It must be amphiphilic or hydrophobic, these properties must extend over at least nine residues, and it must not contain a proline residue at or near its center. Membrane incorporation of OmpA involves a conformational change of the protein and it could be that the last β-strand initiates folding and assembly in the outer membrane.

Key Words: *Escherichia coli*; plasma membrane; outer membrane; OmpA; protein translocation/sorting.

Introduction

The 325 residue OmpA protein (Chen *et al.*, 1980) is one of the abundant proteins of the *E. coli* outer membrane (for a recent review see Nikaido and

[1] Institut für Biotechnologie der Kernforschungsanlage Jülich, D-5170 Jülich, F.R.G.
[2] Max-Planck-Institut für Biologie, D-7400 Tübingen, F.R.G.

0145-479X/90/0600-0441$06.00/0 © 1990 Plenum Publishing Corporation

Vaara, 1987). On the basis of a large body of experimental evidence, a model for the arrangement of the protein in the outer membrane (om) was developed (Braun and Cole, 1982, 1983, 1984; Cole *et al.*, 1983; Freudl and Cole, 1983; Freudl *et al.*, 1986a, Freudl, 1989; Klose *et al.*, 1990; Morona *et al.*, 1984, 1985; Vogel and Jähnig, 1986). OmpA consists of a membrane moiety encompassing residues 1 to about 170; the C-terminal part is periplasmic. The membrane part is thought to cross the om eight times in the form of antiparallel β-strands, forming an amphiphilic β-barrel. The regions around residues 25, 70, 110, and 154 are exposed at the cell's surface. Isolated, denatured (urea) OmpA can be renatured by the addition of lipopolysaccharide (LPS) or the lipid A part of LPS alone; therefore, the protein is most likely associated with LPS in the om (Schweizer *et al.*, 1978). Like most other exported proteins, OmpA is initially synthesized as a precursor (Crowlesmith *et al.*, 1981), possessing a 21-residue signal peptide (Beck and Bremer, 1980; Movva *et al.*, 1980). Translocation of the protein across the plasma membrane involves an export machinery, encoded by the *sec/prl* genes (see the contribution by T. J. Silhavy). Using results mostly obtained with OmpA, we here address the following questions. 1. Is the protein a "blind" passenger of its signal sequence, or does it play an active role in plasma membrane translocation, or are there passive properties which make it an exported protein? 2. How does it recognize the om and what is the mechanism of its membrane assembly?

Translocation Across the Plasma Membrane

The Role of OmpA

A set of 12 overlapping deletions in the *ompA* gene has been constructed and the location of the corresponding OmpA fragments has been determined by immuno electron microscopy (Freudl *et al.*, 1985, 1987; Klose *et al.*, 1988a). Among the mutant proteins, one existed where the signal peptide was fused to the periplasmic part of the protein (Freudl *et al.*, 1987), which certainly differs considerably in its potential to assume a certain conformation from this potential of the membrane moiety. All precursors were processed and all polypeptides were found in the periplasmic space or associated with the om (see below). We concluded that OmpA does not contain, at a unique site, a signal required for export and that, for translocation, the precursor need not assume any unique conformation. In other words, the sequences following the signal peptide appeared not to play any active role in this process but, since the phenomenon of export-incompatibility exists (see below), had to be compatible with it. Essentially

the same conclusion was drawn for the om protein PhoE (Bosch *et al.*, 1986, 1988).

Efficiency of Export

Do the results summarized above provide the answer to our first question? Not entirely. Our data do not tell much about the efficiency of translocation of some OmpA fragments (but see below). As discussed by Ferenci and Silhavy (1987) and Rasmussen and Silhavy (1987), there may be areas in exported proteins which are essential for the most efficient mode of translocation. One way to achieve this could be via the action of one or the other chaperonin, which were originally defined as proteins promoting correct assembly of oligomeric proteins (Hemmingsen *et al.*, 1988). The term has since also been assigned to proteins which stabilize precursors of secretory polypeptides in an export-compatible form (Lecker *et al.*, 1989), i.e., preventing them from folding into a conformation which is no longer export-compatible. (For a detailed discussion of the role of protein folding in translocation, see the contributions by C. Kumamoto and P. J. Bassford.) In *E. coli*, three cytosolic chaperonins have been studied, SecB (Kumamoto and Beckwith, 1983, 1985), trigger factor (Crooke and Wickner, 1987; Crooke *et al.*, 1988), and GroEL (Bochkareva *et al.*, 1988). In the absence of SecB, processing of pro-OmpA was considerably retarded (Collier *et al.*, 1988; Watanabe *et al.*, 1988). It has not yet been tested if the rate of processing of the precursors of the OmpA fragments (see above) is altered in the absence of SecB.

In all cell-free protein translocation system, isolated pro-OmpA has proven to be incapable of translocation into plasma membrane vesicles. It could be made translocation-competent by denaturing it in 8 M urea followed by dilution into buffer containing 0.8 M urea. It rapidly lost this competence unless any of the three chaperonins were present to which the precursor bound (Lecker *et al.*, 1989). This showed that pro-OmpA could assume the competent state spontaneously and that the chaperonins kept it in that form; in other words, they apparently did not actively induce the formation of a translocation-competent conformation. The authors also showed that the three proteins could bind not only to pro-OmpA but also to OmpA. It is not yet known if binding sites in OmpA are also of importance *in vivo* and it remains somewhat puzzling why trigger factor cannot, at least not fully, substitute for the loss of SecB. It should also be noted that the identity of the target for SecB is controversial. Evidence has been presented that SecB binds to the mature part of the precursor of the periplasmic maltose-binding protein (Collier *et al.*, 1988), while other experimental results led to the conclusion that SecB binds to the signal peptide of this precursor (Watanabe

and Blobel, 1989). Possibly both views are correct. In any event, should a chaperonin bind to the mature part of pro-OmpA and should such binding effect an increase in the efficiency of translocation, the answer to our first question would be: the protein does not play an active role in translocation, i.e., it does not contain export signals, but it is also not a blind passenger of its signal peptide; it has the passive property to provide a binding site for chaperonins. Whether or not the chaperonins are absolutely required for export cannot be discerned from the export-compatibility of the OmpA fragments as described above; there may be more than one binding site for a given chaperonin, or different sites for the different chaperonins may exist. For a decision, one would have to construct mutants unable—presumably conditionally so—to synthesize any chaperonin.

The efficiency of processing/translocation can also be altered, however, by mutational alterations within the mature part of precursors. It has been suggested by Ferenci and Silhavy (1987) and Rasmussen and Silhavy (1987) that, for high efficiency of translocation of precursors, the structure of the area immediately following the cleavage site for the signal peptidase is important. This has proven to be correct. Increasing or introducing net positive charge next to this site was found to severely inhibit translocation (Li *et al.*, 1988; Yamane and Mizushima, 1988; MacIntyre *et al.*, submitted). Another important structural feature of the N-terminus of the mature part of precursors is a requirement for a high probability to form a turn; when this turn potential was lowered by site-directed mutagenesis, processing became increasingly defective (Inouye *et al.*, 1986; Duffaud and Inouye, 1988). Did these mutational alterations destroy a positively acting export signal? Most likely not. For example, and as discussed by Li *et al.* (1988) and Yamane and Mizushima (1988), in the cases of the increased positive charge at the N-terminus the "positive-inside" rule may come into play (von Heijne, 1986, 1988). This rule states that in integral plasma membrane proteins, spanning the membrane repeatedly, regions located at the cytoplasmic side of the membrane are enriched for positively charged residues while areas exposed at the periplasmic face are not. Hence, an increase of positive charge at the N-terminus could render the protein export-incompatible and would have a direct negative effect. Other structural features, causing such incompatibility, have been found for OmpA.

Export-Incompatibility Caused by Too Low a Size of OmpA Fragments

When measuring the rates of processing of the smaller OmpA fragments discussed above, it was found that a correlation existed between this rate and the size of the fragments: the smaller the fragment, the slower the rate was (Freudl and Henning, 1988). Still smaller *ompA* fragments were constructed

(Freudl *et al.*, 1989), encoding precursors consisting of 123, 116, 88, 72 or 68 residues. It turned out that the former three were processed and localized to the periplasm, while the latter two were not processed and remained cytosolic (45 residues following the signal peptide, although different from wild type, were the same in all cases). We proposed the following explanation for the failure of the two smallest fragments to leave the cytosol. The ribosome covers about 40 residues of a growing polypeptide chain (Blobel and Sabatini, 1970; Bernabeu and Lake, 1982); thus, the signal peptide of the smallest fragments is just emerging when translation ends. If there is a requirement for this peptide to interact with a component of the export apparatus before translation is finished, not enough time may be left for the small precursors to establish this interaction and they may then no longer be able to enter the export pathway. A similar size limit has been found for eukaryotic secretory proteins; for references, see Freudl *et al.*, (1989).

Export-Incompatibility Caused by Hydrophobic Sequences

Many integral membrane proteins are anchored in their membrane by a stretch of about 20 lipophilic residues (see von Heijne, 1985). The minimum length of such a sequence for anchor function has been determined to be 16 residues (Davis and Model, 1985). None of the outer membrane proteins with known sequences contain such an anchor, presumably in order to avoid being localized to the plasma membrane. This hypothesis has been tested by inserting a linker, encoding the sequence Leu-Ala-Leu-Val, into the *ompA* gene (MacIntyre *et al.*, 1988). Genes were recovered which possessed two, three, or four such linkers between the codons for residues 228 and 229, and four linkers between residues 153 and 154. In the first case, 16 but not 12 or 8 lipophilic residues blocked export of the altered proteins. In the second case, the protein became anchored in the plasma membrane with a periplasmic N-terminus and a cytoplasmic C-terminus; hence, the 16 residues apparently acted as a stop transfer sequence (Blobel, 1980). When the signal peptide was absent, the protein was also anchored in this membrane, but in the opposite orientation. Thus, in this case, the internal lipophilic sequence must have acted as both a signal and an anchor. Interestingly, but unexplained, expression of the plasmid-borne genes coding for these proteins with or without the N-terminal signal peptide caused an accumulation of the chromosomally encoded pro-OmpA. It appeared, therefore, that the export apparatus remained occupied by the altered proteins or at least by a fraction of them. Whatever the reason may be for the inability of these polypeptides to diffuse away from the export machinery, it is obvious that a hydrophobic stretch of 16 residues is not permitted in an outer membrane protein and would also not be permitted in a cytosolic protein unless it were rapidly

hidden by folding. Obviously, om proteins did what they had to do, namely, evolved a type of membrane anchoring quite different from that of many plasma membrane proteins.

Sorting to the Outer Membrane

The route from the plasma membrane to the om and incorporation into the om still represents essentially a black box and this, as far as we know, is true for all om proteins. It is known that during this pathway OmpA undergoes a conformational change (Freudl *et al.*, 1986b). An OmpA species was identified which had already lost the signal peptide, appeared still to be associated with the plasma membrane, and differed substantially in conformation from that of OmpA present in the om. The same species, called imp-OmpA (for *im*mature *p*rocessed), was found attached to the inner face of the om when the protein was overproduced; this imp-OmpA could be brought into the mature conformation by association with LPS *in vitro*. This result suggested that the imp-OmpA recognizes and binds to LPS, possibly to its lipid A moiety (see Introduction) in the om, and that the resulting change in conformation forces the protein into the outer membrane. There is no evidence for this; it is even unknown whether or not the conformational change is causally related to the incorporation of the protein into the om. Since the imp-OmpA does not measurably associate with LPS prior to incorporation into the OM, it seems that this incorporation does not require *de novo* synthesis of LPS, as is probably the case for the om porin OmpF (Bolla *et al.*, 1988). In the latter case, however, absence of lipid synthesis blocked trimerization of the polypeptide, which appears to be a prerequisite for membrane insertion (Boulain *et al.*, 1986); OmpA most likely does not exist in the om as a defined oligomer. We are presently investigating the question of whether newly synthesized OmpA can be correctly inserted into the om in the absence of LPS synthesis. Preliminary experiments indicate that this is so; if substantiated, a transporting role for this component would be excluded. Yet, it should be noted that an answer to this question would tell us next to nothing concerning the mechanism of recognition of the om by the protein, let alone the mechanism of membrane assembly. In fact, if LPS were the receptor, we would be confronted with the chicken-and-egg problem of how LPS finds the om, a mechanism which is also unknown (Osborn, 1984). An *in vitro* system, using isolated om, is very unlikely to work because one cannot isolate om with "empty space" for OmpA; mutants missing the protein produce correspondingly more OmpC and OmpF porins (and vice versa; unpublished). Since presently an experimental alley to solve these questions, using the wild type OmpA, is not visible, we have turned to the relevant behaviour of mutants.

The conspicuous sequence Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro exists in within the periplasmic domain of OmpA, immediately following the last transmembrane strand. Since membrane assembly involves polypeptide folding, we considered the possibility that this repeat may serve as a nucleation site for such folding. The sequence was replaced by another one by introducing frameshift mutants; there was no effect on assembly, and the significance of this sequence remains mysterious (Klose *et al.*, 1988a). Next, the cellular location of the OmpA fragments (described above) was examined (Klose *et al.*, 1988a). Immuno electron microscopy provided a clear answer: all those still possessing an area between residues 154 and 180 were seen associated with the om while all missing this region were present in the periplasm. Since no mutant protein of the former class would be able to form the hypothetical β-barrel in the om, it was not unexpected that none of them was detectably assembled in the membrane. In general, however, synthesis of those fragments found at the om was more toxic than that of the others. Particular toxicity (stop of growth and cell lysis upon induction of gene expression) was exhibited by a protein which missed precisely the third and fourth β-strand (residues 43–84). This may indicate that this protein started membrane assembly and soon disrupted the om because of its inability to assume the conformation of the amphiphilic β-barrel. If so, another area of the polypeptide would dictate om assembly. In any event, it remained an open question whether or not the microscopically observed membrane association represented a step in the physiological sorting mechanism.

If the results just presented do no reflect something fortuitous, the area between residues 154 and 180 should harbor some sort of a sorting signal. The region of OmpA between residues 154 and approximately 160 is exposed at the cell's surface, while that between residues 160 and 170 is thought to represent the last β-strand embedded in the om (Morona *et al.*, 1984; Vogel and Jähnig, 1986). The region around residue 154 can be altered substantially without influence on membrane assembly of the polypeptide (Freudl *et al.*, 1986a). Thus, a sorting signal might exist within or be represented by the last β-strand. The structure of this sequence was altered by site-directed mutagenesis (Klose *et al.*, 1988b). A double mutant, *ompA* ON6, causing the substitutions $\text{Leu}^{164} \rightarrow$ Pro and $\text{Val}^{166} \rightarrow$ Asp was found to be incapable of incorporating into the om. A fair number of other substitutions within the membrane moiety of OmpA and, in particular, a double mutant altering the presumed first β-strand (residues 7–17) in a way almost identical to that of OmpA ON6, were not impaired in membrane assembly. The *ompA* ON6 gene was further altered by inserting linkers between the codons for residues 164 and 165, leading to proteins possessing up to 15 additional residues (Klose *et al.*, 1989). Of 13 different genes obtained, five encoded proteins which had regained the ability to assemble in the membrane. The properties of the

mutant polypeptides permitted deduction of some rules for compatibility of this strand, or possibly initiation of, membrane insertion: (a) it must be amphiphilic or hydrophobic, while its primary structure as such is fairly unimportant, (b) amphiphilicity or hydrophobicity must extend over at least nine residues, and (c) it must not contain a proline residue around its center. While these results show that at least one rather small region of OmpA is crucial for its membrane insertion, they obviously cannot tell us if this area represents *the* starter for this insertion. Work is in progress to violate rule (c) for the remaining six β-strands of OmpA. We are also attempting to isolate, presumably conditional, non-*ompA* mutants with an impaired ability to accept OmpA in the om.

In summary, we are still very much in the dark regarding the mechanisms of recognition of and insertion of OmpA (and all other om proteins) into the om.

Acknowledgment

We thank Sheila MacIntyre for critically reading the manuscript.

References

Beck, E., and Bremer, E. (1980). *Nucleic Acids Res.* **8**, 3011–3027.
Bernabeu, C., and Lake, J. A. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 3111–3115.
Blobel, G., and Sabatini, D. D. (1970). *J. Cell. Biol.* **45**, 130–145.
Blobel, G. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 1496–1500.
Bochkareva, E. S., Lissin, M. N., and Girshovich, A. S. (1988). *Nature* (*London*) **336**, 254–257.
Bolla, J.-M., Lazdunski, C., and Pages, J.-M. (1988). *EMBO J.* **7**, 3595–3599.
Bosch, D., Leunissen, J., Verbakel, J., deJong, M., van Erp, J., and Tommassen, J. (1986). *J. Mol. Biol.* **189**, 449–455.
Bosch, D., Voorhout, W., and Tommassen, J. (1988). *J. Biol. Chem.* **263**, 9952–9957.
Boulain, J., Charbit, A., and Hofnung, M. (1986). *Mol. Gen. Genet.* **205**, 339–348.
Braun, G., and Cole, S. T. (1982). *Nucleic Acids Res.* **10**, 2367–2376.
Braun, G., and Cole, S. T. (1983). *Eur. J. Biochem.* **137**, 495–500.
Braun, G., and Cole, S. T. (1984). *Mol. Gen. Genet.* **195**, 321–328.
Chen, R., Schmidmayr, C., Krämer, C., Chen-Schmeisser, U., and Henning, U. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 4592–4596.
Cole, S. T., Chen-Schmeisser, U., Hindennach, I., and Henning, U. (1983). *J. Bacteriol.* **153**, 581–587.
Collier, D. N., Bankaitis, V. A., Weiss, J. B., and Bassford, P. J., Jr. (1988). *Cell.* **53**, 273–283.
Crooke, E., and Wickner, W. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 5216–5220.
Crooke, E., Brundage, L., Rice, M., and Wickner, W. (1988). *EMBO J.* **7**, 1831–1835.
Crowlesmith, I., Gamon, K., and Henning, U. (1981). *Eur. J. Biochem.* **113**, 375–380.
Davis, N. G., and Model, P. (1985). *Cell* **41**, 607–614.
Duffaud, G., and Inouye, M. (1988). *J. Biol. Chem.* **263**, 10224–10228.
Ferenci, T., and Silhavy, T. J. (1987). *J. Bacteriol.* **169**, 5339–5342.
Freudl, R. (1989). *Gene* **82**, 229–236.
Freudl, R., and Cole, S. T. (1983). *Eur. J. Biochem.* **134**, 497–502.

Freudl, R., and Henning, U. (1988). *J. Mol. Biol.* **203**, 517–519.
Freudl, R., Schwarz, H., Klose, M., Movva, N. R., and Henning, U. (1985). *EMBO J.* **4**, 3593–3598.
Freudl, R., MacIntyre, S., Degen, M., and Henning, U. (1986a). *J. Mol. Biol.* **188**, 491–494.
Freudl, R., Schwarz, H., Stierhof, Y.-D., Gamon, K., Hindennach, I., and Henning, U. (1986b). *J. Biol. Chem.* **261**, 11355–11361.
Freudl, R., Schwarz, H., Degen, M., and Henning, U. (1987). *J. Bacteriol.* **169**, 66–71.
Freudl, R., Schwarz, H., Degen, M., and Henning, U. (1989). *J. Mol. Biol.* **205**, 771–775.
Hemmingsen, S. M., Woodford, C., van der Vies, S. M., Tilly, K., Denis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988). *Nature* (*London*) **333**, 330–334.
Inouye, S., Duffaud, G., and Inouye, M. (1986). *J. Biol. Chem.* **261**, 10970–10975.
Klose, M., Schwarz, H., MacIntyre, S., Freudl, R., Eschbach, M.-L., and Henning, U. (1988a). *J. Biol. Chem.* **263**, 13291–13296.
Klose, M., MacIntyre, S., Schwarz, H. and Henning, U. (1988b). *J. Biol. Chem.* **263**, 13297–13302.
Klose, M., Jähnig, F., Hindennach, I., and Henning, U. (1989). *J. Biol. Chem.* **264**, 21842–21847.
Kumamoto, C. A., and Beckwith, J. (1983). *J. Bacteriol.* **154**, 253–260.
Kumamoto, C. A., and Beckwith, J. (1985). *J. Bacteriol.* **163**, 267–274.
Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P. J., Jr., Kumamoto, C. A., and Wickner, W. (1989). *EMBO J.* **8**, 2703–2709.
Li, P., Beckwith, J., and Inouye, H. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 7685–7689.
MacIntyre, S., Freudl, R., Eschbach, M.-L., and Henning, U. (1988). *J. Biol. Chem.* **263**, 19053–19059.
Morona, R., Klose, M., and Henning, U. (1984). *J. Bacteriol.* **159**, 570–578.
Morona, R., Krämer, C., and Henning, U. (1985). *J. Bacteriol.* **164**, 539–543.
Movva, R. N., Nakamura, K., and Inouye, M. (1980). *J. Mol. Biol.* **143**, 317–328.
Nikaido, H., and Vaara, M. (1987). In *Escherichia coli and Salmonella typhimurium* (Neidhard, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds.), Vol. 1, American Society for Microbiology, Washington, D.C., pp. 7–32.
Osborn, M. J. (1984). *Harvey Lect.* **78**, 87–103.
Rasmussen, B. A., and Silhavy, T. J. (1987). *Genes Dev.* **1**, 185–196.
Schweizer, M., Hindennach, I., Garten, W., and Henning, U. (1978). *Eur. J. Biochem.* **82**, 211–217.
Vogel, H., and Jähnig, F. (1986). *J. Mol. Biol.* **190**, 191–199.
von Heijne, G. (1985). *Curr. Top. Membr. Transp.* **24**, 151–179.
von Heijne, G. (1986). *EMBO J.* **5**, 3021–3027.
von Heijne, G. (1988). *Biochim. Biophys. Acta* **947**, 307–333.
Watanabe, M., and Blobel, G. (1989). *Cell.* **58**, 695–705.
Watanabe, T., Hayashi, S., and Wu, H. C. (1988). *J. Bacteriol.* **170**, 4001–4007.
Yamane, K., and Mizushima, S. (1988). *J. Biol. Chem.* **263**, 19690–19696.