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In vivo membrane assembly of split variants of the *E.coli* outer membrane protein OmpA

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The two-domain, 325 residue outer membrane protein OmpA of *Escherichia coli* is a well-established model for the study of membrane assembly. The N-terminal domain, consisting of ~170 amino acid residues, is embedded in the membrane, presumably in the form of a β-barrel consisting of eight antiparallel transmembrane β-strands. A set of 16 gene variants carrying deletions in the membrane-embedded domain of OmpA was constructed. When pairs of these mutant genes were co-expressed in *E.coli*, it was found that a functional OmpA protein could be assembled efficiently from two complementary protein fragments. Assembly was found when the polypeptide chain was split at the second or third periplasmic turn. All four protein termini were located in the periplasmic space. Interestingly, duplication of transmembrane strands five and six led to a variant with an unusual topology: the N-terminus of one fragment and the C-terminus of the other fragment were exposed at the cell surface. This is the first demonstration of correct membrane assembly of split β-structured membrane proteins. These findings are important for a better understanding of their folding/assembly pathway and may have implications for the development of artificial outer membrane proteins and for the cell surface display of heterologous peptides or proteins.

Keywords: membrane assembly/membrane proteins/ protein design/protein folding/surface display

Introduction

The process of membrane assembly of outer membrane proteins *in vivo* is only poorly understood (for a recent review, see Henning and Koebnik, 1994). The *Escherichia coli* outer membrane protein OmpA is well suited for the study of this problem (Freudl *et al.*, 1986; Klose *et al.*, 1988, 1993; Ried *et al.*, 1994; Koebnik and Krämer, 1995). Although its three-dimensional structure has not yet been solved, a detailed model has been proposed by means of phage mapping, linker insertion mutagenesis in combination with protease digestion experiments, Raman spectroscopy and computer-based predictions (Morona *et al.*, 1984; Vogel and Jähnig, 1986; Ried *et al.*, 1994). This model predicts a two-domain organization of OmpA with 170 N-terminal amino acids residing in the outer membrane and the remaining 155 C-terminal residues extending into the periplasmic space (Figure 1). The membrane-embedded domain was predicted to consist of eight antiparallel β-strands which are connected by four external loops and three periplasmic turns. It is thought that the eight β-strands form a β-barrel. Recently, a truncated variant consisting of only the 171 N-terminal amino acid residues has been constructed and shown to be assembled efficiently into the outer membrane *in vivo* (Ried *et al.*, 1994). For the study of membrane assembly, this variant has the advantages over the porins of being a monomeric protein and of having a much smaller membrane moiety.

In analogy to the three-dimensional structures of porins (Weiss *et al.*, 1991; Cowan *et al.*, 1992), integral outer membrane proteins are often modeled as β-sheeted proteins, e.g. the ferrichrome receptor FhuA (Koebnik and Braun, 1993), the nucleoside-specific channel protein Tsx (Fsihi *et al.*, 1993) or the outer membrane phospholipase A (Brok *et al.*, 1994), all from *E.coli*. These models have in common that membrane-spanning β-strands are meander-like connected by short periplasmic turns and relatively long surface-exposed loops. The protein termini are usually located in the periplasmic space, as found with the porins. Both turns and loops are often permissive for the insertion of small peptides (Charbit *et al.*, 1991; Koebnik and Braun, 1993; Ried *et al.*, 1994). Taken together, these models suggest some common structural and/or folding principles. Therefore, examination of the model protein OmpA should have general implications for this class of integral outer membrane proteins.

Recently, all three periplasmic turns of OmpA have been converted into new protein termini by construction of circularly permuted variants. These OmpA variants were assembled in the outer membrane *in vivo* (Koebnik and Krämer, 1995). In this work, I examined if co-expression of segments of the OmpA β-barrel domain can lead to a functional protein that is correctly assembled in the outer membrane.

Results

Construction of split OmpA variants

I decided to study the folding of the membrane-embedded β-barrel domain of OmpA by splitting its polypeptide chain. In a previous study (Koebnik and Krämer, 1995), four pairs of transmembrane β-strands were envisaged as minimal folding units in the form of β-hairpins and, therefore, were used as segments during construction of permuted OmpA variants. Consequently, DNA regions corresponding to the three periplasmic turns separating the four β-hairpins were used as sites for interrupting the polypeptide chain (Figure 1). The location of these turns in the amino acid sequence has been verified experimentally by the insertion of small peptides before residues Asn46, Thr88 and Thr132, and their proteolytic digestion in spheroplasts (Ried *et al.*, 1994). Here, the same regions

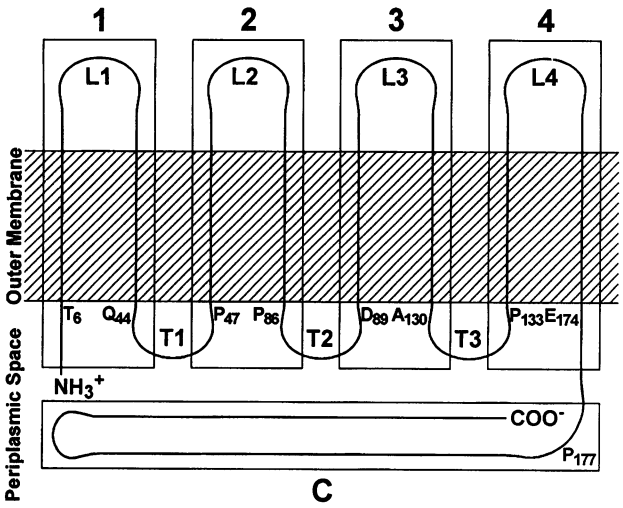

Fig. 1. Two-dimensional model of the arrangement of wild-type OmpA protein in the outer membrane. The first and last amino acid of each β-hairpin are indicated according to the one-letter code, and numbers indicate their position in the mature protein. In view of the unsolved three-dimensional structure, these residues need not be located exactly at the periplasmic ends of the membrane-spanning β-strands. The extramembranous turns and loops have been labeled T1–T3 and L1–L4, respectively. β-Hairpins are boxed and numbered from 1 to 4. The C-terminal periplasmic domain, starting with Pro177, is boxed and marked 'C'.

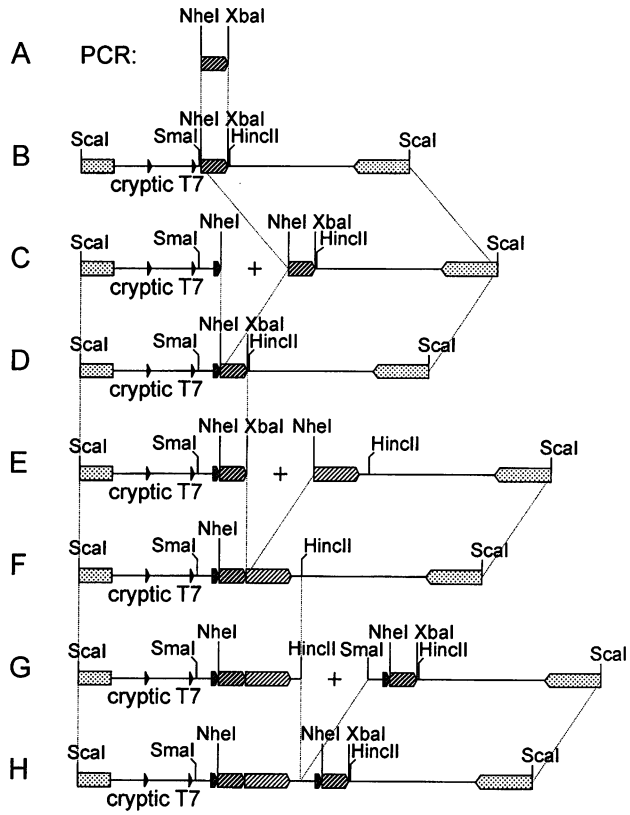

Fig. 2. Construction scheme of plasmids expressing split OmpA variants, as exemplified by the construction of a plasmid co-expressing OmpA(12) and OmpA(34C). (**A**) and (**B**) PCR-amplified DNA fragments encoding two β-hairpins were cloned into plasmid pK(1234), resulting in plasmids pK(12) and pK(34). (**C**) and (**D**) The translation initiation region and the DNA region encoding the signal peptide for secretion were added from plasmid pKONN, leading to plasmids pKOS(12) and pKOS(34). (**E**) and (**F**) The region encoding the periplasmic domain of OmpA was added from pKONN to pKOS(34), resulting in plasmid pKOL(34). (**G**) and (**H**) Finally, the two truncated *ompA* genes (D and F) were cloned in tandem, leading to plasmid pKOLS(34)+(12). The β-lactamase gene containing a unique *Sca*I site (dotted boxes), β-barrel segment-encoding regions (hatched boxes), the signal peptide-encoding region (bold-hatched boxes), the periplasmic domain-encoding region of *ompA* (fine-hatched boxes) and both the T7-specific and the cryptic promoters are shown. The exact location of the cryptic promoter is unknown. For further explanations, see Materials and methods.

corresponding to periplasmic turns were used for the construction of split OmpA variants.

Split variants of the β-barrel domain of OmpA were constructed using PCR (Saiki *et al.*, 1985). First, mutant genes encoding truncated OmpA variants were constructed. For this purpose, individual DNA regions, each encoding a different segment of the OmpA β-barrel, were PCR amplified and subsequently cloned into an expression vector (Materials and methods and Figure 2). The encoded OmpA fragments were designated by their individual β-hairpins (Figure 1) in brackets, i.e. OmpA(12) symbolizing a C-terminally truncated variant lacking the last two β-hairpins. In some cases (see below), the periplasmic domain of OmpA was attached to the C-terminus of the β-barrel segment (Materials and methods and Figure 2). This fusion was symbolized by a 'C', e.g. OmpA(34C) when the periplasmic domain was fused to the fourth β-hairpin. In all cases, the N-terminal signal peptide for secretion was placed in front of the individual β-barrel segment. The amino acid sequences of the resulting OmpA variants are summarized in Table I.

To achieve co-expression of truncated *ompA* genes in the same cell, they were cloned in tandem (Figure 2F and G). The expression of these split OmpA variants was driven by a (relatively weak) cryptic promoter located in the vector (data not shown). Using the bacteriophage T7 RNA polymerase/promoter system (Tabor and Richardson, 1985), exclusive expression was induced from these plasmids. Membrane-assembled molecules are indicated by the two OmpA fragments. For instance, OmpA(12+34C) indicates membrane-assembled molecules resulting from co-expression of variants OmpA(12) and OmpA(34C). This corresponds to wild-type OmpA that was dissected at its second periplasmic turn.

Location and membrane topology of split OmpA variants

The correct assembly of split OmpA variants in the outer membrane of *E.coli* was investigated using three well-established methods: (i) a phage assay; (ii) the detection of a heat-modifiable form, which is typical for an OmpA assembled in the outer membrane; and (iii) the protection of this form against protease in spheroplasts. These assays allow a quantitative estimate of membrane assembly.

For the phage assay, an *ompA* mutant (not expressing the chromosomal *ompA* gene) transformed with the appropriate plasmids was assayed for its sensitivity to the OmpA-specific bacteriophage K3h1. This phage requires only a low concentration of its receptor protein OmpA for plaque formation.

OmpA, like several other outer membrane proteins, exhibits the phenomenon of heat modifiability: the apparent molecular mass from SDS–PAGE depends on the temperature at which the protein has been incubated in

Table I. Molecular masses and amino acid sequences of truncated OmpA variants

OmpA variant	M_r (kDa)	Amino acid sequence of the mature polypeptide				
		N-terminus	Linker	β-Barrel segment	Linker	C-terminus
OmpA β-barrel	18.7	Ala1-Lys3	Asp4-Asn5	Thr6-Gly171		
C-terminally truncated variants:						
OmpA(123)	14.4	Ala1-Lys3	Ala-Ser	Thr6-Ala130	Ser-Arg	
OmpA(12)	9.6	Ala1-Lys3	Ala-Ser	Thr6-Pro86	Ser-Arg	
OmpA(1)	5.0	Ala1-Lys3	Ala-Ser	Thr6-Gln44	Ser-Arg	
N-terminally truncated variants:						
OmpA(234)	14.8	Ala1-Lys3	Ala-Ser	Pro47-Glu174	Ser-Arg	
OmpA(34)	10.1	Ala1-Lys3	Ala-Ser	Asp89-Glu174	Ser-Arg	
OmpA(4)	5.4	Ala1-Lys3	Ala-Ser	Pro133-Glu174	Ser-Arg	
N-terminally and C-terminally truncated variants:						
OmpA(2)	5.1	Ala1-Lys3	Ala-Ser	Pro47-Pro86	Ser-Arg	
OmpA(3)	5.3	Ala1-Lys3	Ala-Ser	Asp89-Ala130	Ser-Arg	
OmpA(23)	9.9	Ala1-Lys3	Ala-Ser	Pro47-Ala130	Ser-Arg	
C-terminally enlarged variants (see text):						
Wild-type	35.1	Ala1-Lys3	Asp4-Asn5	Thr6-Glu174	Ala175-Ala176	Pro177-Ala325
OmpA(1C)	20.8	Ala1-Lys3	Ala-Ser	Thr6-Gln44	Ser-Ser	Pro177-Ala325
OmpA(2C)	21.0	Ala1-Lys3	Ala-Ser	Pro47-Pro86	Ser-Ser	Pro177-Ala325
OmpA(3C)	21.2	Ala1-Lys3	Ala-Ser	Asp89-Ala130	Ser-Ser	Pro177-Ala325
OmpA(4C)	21.2	Ala1-Lys3	Ala-Ser	Pro133-Glu174	Ser-Ser	Pro177-Ala325
OmpA(12C)	25.5	Ala1-Lys3	Ala-Ser	Thr6-Pro86	Ser-Ser	Pro177-Ala325
OmpA(34C)	26.0	Ala1-Lys3	Ala-Ser	Asp89-Glu174	Ser-Ser	Pro177-Ala325
OmpA(234C)	30.6	Ala1-Lys3	Ala-Ser	Pro47-Glu174	Ser-Ser	Pro177-Ala325

For each variant, the complete sequence of the mature protein is summarized from left to right. For nomenclature of OmpA variants see text. The linker sequences result from ligation of two *Nhe*I sites (Ala-Ser), two *Xba*I sites (Ser-Arg) or an *Xba*I site with an *Nhe*I site (Ser-Ser).

the presence of SDS (Beher *et al.*, 1980). Membrane-assembled wild-type OmpA incubated at 50°C migrates as a 32 kDa species but, when boiled for 5 min, it migrates as a 36 kDa species, as would be expected from the calculated molecular mass. It is assumed that only prolonged heating disrupts the secondary/tertiary structures of the protein completely. Only the membrane-assembled form of wild-type OmpA exhibits this heat modifiability and is protected from digestion by proteases. In contrast, a non-heat-modifiable form (which migrates electrophoretically as a 36 kDa species regardless of the solubilization temperature) represents a periplasmic folding/assembly intermediate (imp-OmpA, for *im*mature *p*rocessed), which is not yet assembled in the outer membrane (Freudl *et al.*, 1986). This wild-type imp-OmpA is converted rapidly to the heat-modifiable form; at least 95% is assembled after a chase of 15 min at 30°C.

Dissection of OmpA at its first or third periplasmic turn. An *ompA* mutant host strain carrying plasmids coding for OmpA variants was assayed for phage sensitivity. Neither co-expression of variants OmpA(1) and OmpA(234) nor of variants OmpA(123) and OmpA(4) mediated any phage sensitivity (Table II). It is known that the smaller the peptide the less efficiently it is secreted across the cytoplasmic membrane, if at all (Freudl *et al.*, 1989). The variants OmpA(1) and OmpA(4) are rather short (46 and 49 amino acid residues, respectively). By pulse–chase experiments no processed species could be detected for such small OmpA variants consisting of only the second [OmpA(2), 47 amino acids], third [OmpA(3), 49 amino acids] or fourth β-hairpin, although the precursor proteins disappeared rapidly (data not shown). It appears that both the precursor and the processed species (if there is such a species) are very unstable. However, these experiments did not exclude the possibility that some mature protein was formed. The first β-hairpin, lacking methionine residues, was not examined, but from earlier experiments it appears that it also is not secreted across the cytoplasmic membrane (Freudl *et al.*, 1989).

In order to facilitate the translocation of the first or

Table II. Membrane assembly of split OmpA variants as judged by their heat modifiability and their phage receptor function

Co-expressed OmpA fragments		Heat-modifiable fraction (%)	Sensitivity to phage K3h1
1	234	0	R
1C	234	<1	R
123	4	0	R
123	4C	6	I
12	34	>50	10^{-1}
12	34C	30	10^{-4}
12C	34	1–2	R
12C	34C	1–2	R
123	34	15	I
12	234	0	R
123	234	0	R
Any single OmpA fragment alone		0	R
Wild-type OmpA		>95	1

The amount of heat-modifiable molecules was assayed after a chase of 15 min (Figure 3). Abbreviations: R, complete phage resistance; I, growth inhibition when phage was added at high concentration. Numbers indicate efficiency of plating. All experiments were performed at least three times.

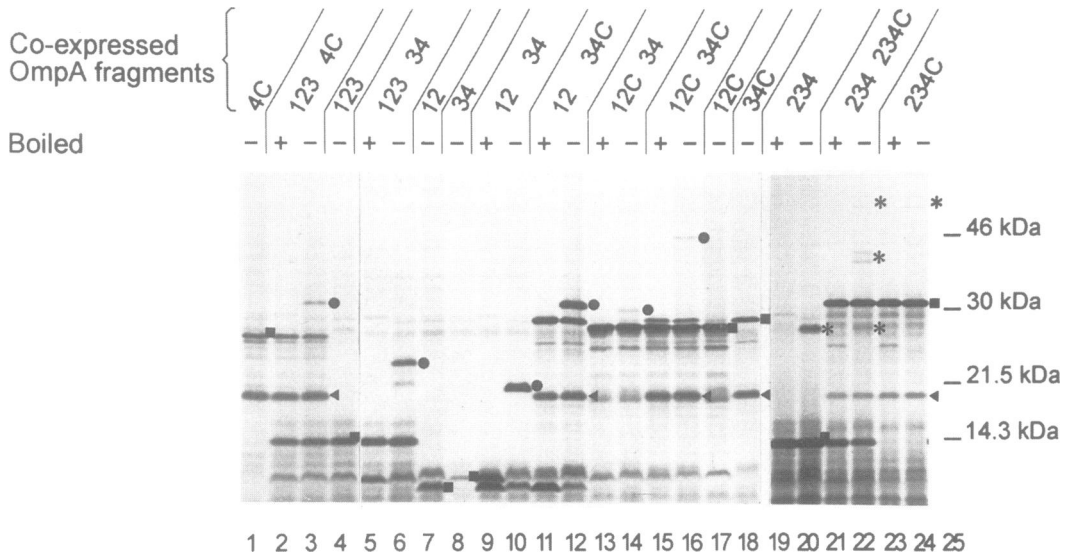

Fig. 3. Autoradiographs of SDS–polyacrylamide gel electrophoretograms of cells expressing different combinations of truncated OmpA variants. Proteins were radioactively labeled using the T7 RNA polymerase/promoter system, and chased for 15 min. Heat-modifiable forms are marked by a dot, non-heat-modifiable forms by a square. Presumptive dimeric forms of N-terminally truncated OmpA variants (see text) are marked by an asterisk. Triangles mark a band thought to originate from internal translation initiation at Met161.

fourth β-hairpin, these two segments were enlarged at the C-terminus by the addition of the periplasmic domain of OmpA, leading to variants OmpA(1C) and OmpA(4C). This protein domain was chosen since it can be stably produced *in vivo* (our unpublished results), indicating its independent folding as a single protein domain. Thus, the periplasmic domain is unlikely to interfere with folding of the attached OmpA β-barrel segment. Co-expression of variants OmpA(123) and OmpA(4C) caused some growth inhibition when phage K3h1 was added at high concentration, indicating that a small amount of the protein was assembled in the outer membrane. In contrast, no phage sensitivity was observed with co-expression of variants OmpA(1C) and OmpA(234) (Table II). Pulse–chase experiments showed that clearly detectable amounts of the co-expressed OmpA(123) and OmpA(4C) variants became heat modifiable (Figure 3, lanes 2 and 3, and Table II). Using protease protection experiments, I examined whether the heat-modifiable form represents membrane-assembled molecules. After conversion of cells to spheroplasts and digestion with subtilisin, SDS–PAGE demonstrated that the heat-modifiable form was protected from proteolytic digestion, while the non-heat-modifiable form was completely degraded (data not shown). This behavior is identical to that of the wild-type OmpA. These observations (phage receptor function, heat modifiability and protease protection) indicated that the membrane-assembled OmpA species that was dissected at the third periplasmic turn [OmpA(123+4C)] assumes the same membrane topology as the wild-type OmpA protein. After radioactive labeling of the co-expressed OmpA(1C) and OmpA(234) variants, a very faint band of a heat-modifiable species of the corresponding apparent molecular mass could be detected in an SDS–PAGE autoradiogram (Table II). Therefore, it appears that a small fraction of these variants was assembled in the outer membrane [OmpA(1C+234)]; however, its amount was apparently too low to mediate phage sensitivity when expressed from the cryptic promoter. Thus, while dissection of OmpA at the third periplasmic turn allows the membrane-assembled species to assume wild-type membrane topology, dissection at the first turn allows only inefficient membrane assembly.

Dissection of OmpA at its second periplasmic turn. A strain co-expressing variants OmpA(12) and OmpA(34) was almost fully sensitive to phage K3h1 (Table II). Control strains expressing only the N-terminal [OmpA(12)] or C-terminal [OmpA(34)] half of the β-barrel domain were resistant. Addition of the periplasmic domain to OmpA(12) and OmpA(34) at their C-termini led to a reduction of phage sensitivity when the variants OmpA(12C) and OmpA(34C) were co-expressed with OmpA(12) and OmpA(34) (Table II). Radioactive labeling experiments showed that substantial amounts of the co-expressed OmpA(12) and OmpA(34) variants and of the co-expressed OmpA(12) and OmpA(34C) variants became heat modifiable (Figure 3, lanes 9–12, and Table II). Co-expression of variants OmpA(12C) and OmpA(34) or of variants OmpA(12C) and OmpA(34C) resulted only in a faint heat-modifiable band (Figure 3, lanes 13–16, and Table II). Protease protection experiments using spheroplasts confirmed the membrane assembly of the heat-modifiable form (Figure 4, lanes 9–12). Hence, all variants that were split at the second periplasmic turn could at least partially assemble into the outer membrane. The different amounts of membrane-assembled molecules could easily account for the reduced phage sensitivity. As an example, Figure 5A shows a model of the membrane topology of split variant OmpA(12+34).

Since co-expressed variants OmpA(12) and OmpA(34) assembled into the outer membrane with high efficiency, it was interesting to investigate the effect of a duplicated β-hairpin on membrane assembly. To this end, co-expression of variants OmpA(123) and OmpA(34), OmpA(12) and OmpA(234) and OmpA(123) and OmpA(234) were examined. Only co-expression of variants OmpA(123) and OmpA(34) caused some growth inhibition with phage

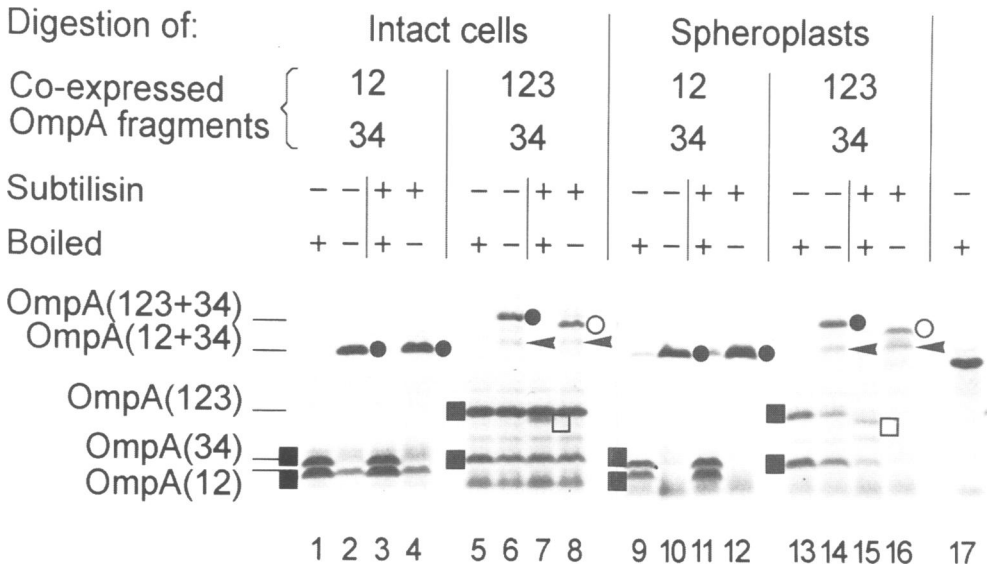

Fig. 4. Autoradiographs of SDS–polyacrylamide gel electrophoretograms demonstrating protease protection of split OmpA variants. [^{35}S]Methionine-labeled and chased variants OmpA(12+34) and OmpA(123+34) were subjected to protease digestion, either in intact cells or after conversion of cells to spheroplasts. For comparison, a C-terminally truncated OmpA derivative consisting of only its β-barrel domain and ending with Gly171 (Ried *et al.*, 1994) is shown in lane 17. The membrane-embedded and, therefore, protected part of variant OmpA(123+34) is indicated by open symbols [open circle for assembled molecules, open square for truncated OmpA(123) molecules after heat denaturation]; this part is also heat modifiable. The arrowheads indicate molecules thought to result from endogenous proteolysis of the OmpA(123+34) variant. For other symbols see Figure 3.

K3h1 and gave rise to a heat-modifiable (Figure 3, lanes 5 and 6, and Table II) and protease-protected form (Figure 4, lanes 13–16). However, the OmpA(123) fragment resulting from heat denaturation of the subtilisin-digested OmpA(123+34) molecules was smaller than expected. Interestingly, this fragment was cleaved to the same size after proteolytic digestion of spheroplasts or intact cells (Figure 4, compare lanes 7 and 15). The thermal stabilities of membrane-assembled OmpA(123+34) and OmpA(12+34) molecules were compared. The half-times necessary for conversion of the heat-modifiable form into the non-heat-modifiable form by incubation at 72°C were ~40 min for variant OmpA(123+34) and 10 min for variant OmpA(12+34) (Figure 6). Thus, only duplication of the third β-hairpin led to assembled OmpA protein, while duplication of the second or second and third was not tolerated.

Characterization of N-terminally truncated OmpA variants. During various co-expression experiments involving variant OmpA(234), heat-modifiable species were observed in an SDS–PAGE. This was also the case when OmpA(234) was expressed alone (Figure 3, lanes 19 and 20). Although the molecular mass of heat-modifiable molecules cannot be determined exactly because of their aberrant migration in SDS–PAGE, it appeared that these species could represent dimeric forms of OmpA(234). Comparison of its heat modifiability with that of the OmpA(12+34) molecules (see above) revealed that the former was much less stable than the latter (data not shown). Protease digestion demonstrated that the heat-modifiable OmpA(234) molecules are not stably integrated in the outer membrane (data not shown).

In order to investigate whether these forms represent dimers, variant OmpA(234C) was constructed. Expression of OmpA(234C) gave rise to two heat-modifiable bands in an SDS–PAGE of a size corresponding to dimers

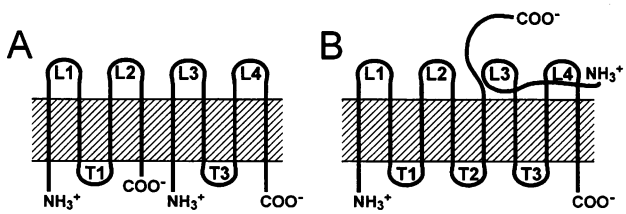

Fig. 5. Models of the membrane topology of split OmpA variants. Variants OmpA(12+34) (**A**) and OmpA(123+34) (**B**) are shown. The extramembranous turns and loops are designated as in Figure 1.

(Figure 3, lanes 23 and 24; the faster migrating species might originate from endogenous proteolytic digestion). Co-expression of truncated proteins with and without the periplasmic domain [OmpA(234) and OmpA(234C)] resulted in the appearance of the presumably dimeric forms observed during expression of both truncated variants alone. In addition, a double band of intermediate size was observed in an SDS–PAGE (Figure 3, lanes 21 and 22).

Provided that these protein species represent dimers, it would be interesting to examine other combinations of truncated OmpA variants, in order to define a minimal dimerization motif. Therefore, the following OmpA variants were co-expressed: OmpA(234) and OmpA(23), OmpA(234) and OmpA(34) and OmpA(23) and OmpA(34). In no case was an additional heat-modifiable form observed (data not shown).

Discussion

Various split variants of the membrane-embedded β-barrel domain of OmpA were constructed and their membrane assembly was assayed by determining the efficiency of plating of the OmpA-specific phage K3h1 and by the detection of a heat-modifiable and protease-protected form. Four variants, causing sensitivity to bacteriophage K3h1,

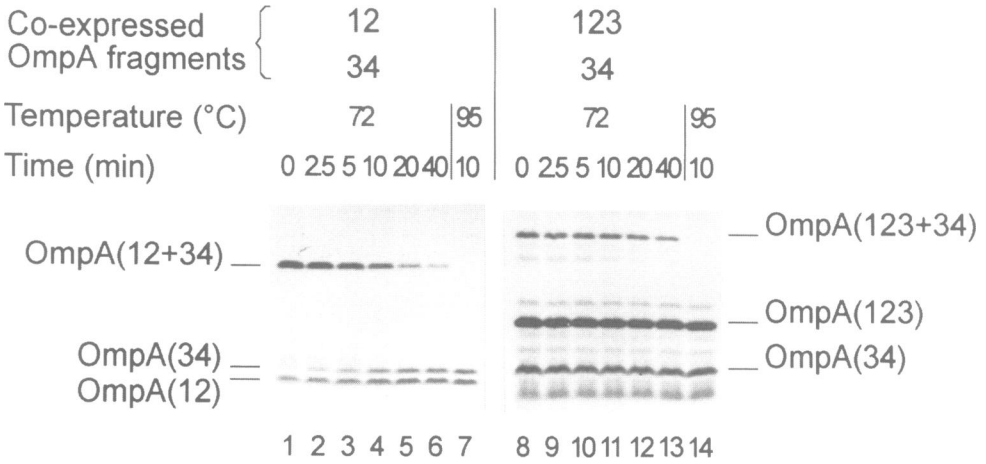

Fig. 6. Autoradiographs of SDS–polyacrylamide gel electrophoretograms comparing the thermal stability of split OmpA variants. Variants OmpA(12+34) and OmpA(123+34) were radioactively labeled and chased for 15 min. Proteins were solubilized in SDS–PAGE sample buffer containing 2% SDS (w/v), and were incubated at 72°C for the time indicated prior to electrophoresis.

were rather efficiently assembled into the outer membrane *in vivo*, although with reduced efficiencies in comparison with the wild type. Variant OmpA(12+34) was most efficiently assembled, followed by variants OmpA(12+34C), OmpA(123+34) and OmpA(123+4C), in this order. These data suggest that dissection at the second periplasmic turn leads to the most efficiently assembling variants. However, this is not necessarily true, since dissection at the other two periplasmic turns required some tricks in order to achieve an efficient secretion across the cytoplasmic membrane. For this purpose, the periplasmic domain of OmpA was fused at the C-terminus of the corresponding β-hairpins. Analogous experiments with variants that were split at the second periplasmic turn clearly demonstrated that the periplasmic domain can interfere with membrane assembly of the attached OmpA β-barrel segment. Therefore, the data are not comparable with respect to the permissiveness of the periplasmic turns for dissection of the polypeptide chain. Another way of secretion of single β-hairpins or *in vitro* experiments might result in a different order of the efficiency of membrane assembly.

The *in vivo* and *in vitro* assembly of functional proteins from two or more fragments have been reported repeatedly (for reviews, see Wetlaufer, 1981; Taniuchi *et al.*, 1986). This applies not only for soluble proteins like maltose binding protein (Betton and Hofnung, 1994), barnase (Kippen and Fersht, 1995) or chymotrypsin inhibitor-2 (Ruizsanz *et al.*, 1995), but also for integral membrane proteins of the cytoplasmic membrane, e.g. bacteriorhodopsin (Liao *et al.*, 1984) or lactose permease (Zen *et al.*, 1994). These membrane proteins traverse the lipid bilayer with α-helices which may adopt this type of secondary structure independently of each other. Their membrane assembly can be considered as a two-stage process. In the first stage, hydrophobic α-helices are established across the lipid bilayer. In the second stage, they interact specifically to form the final transmembrane structure (Popot *et al.*, 1987). The situation is, of course, completely different with a β-barrel protein because the formation of a β-sheeted structure requires much more cooperativity than the formation of an α-helical membrane protein. Saturation of hydrogen bond donors and acceptors requires the formation of the complete β-sheet; usually, individual β-strands cannot exist in solution. Therefore, membrane assembly of split OmpA variants was unexpected. This first demonstration of membrane assembly of an outer membrane protein from two complementing protein fragments may be of considerable significance for the development of artificial outer membrane proteins. It may simplify such a task because now it appears possible to start with only four transmembrane β-strands that could lead to a homodimeric 8-stranded protein.

The atomic structure and the folding pathway of OmpA are not known. Since the membrane-assembled split OmpA variants are heat modifiable and serve as receptors for OmpA-specific bacteriophages, it is very probable that the structure of the split OmpA variants is similar to that of the wild-type. Yet, it is not clear if the folding pathway is similar to that of the wild-type protein, but this certainly appears possible. There are a number of possible pathways which could lead to membrane-assembled molecules. For wild-type OmpA, a periplasmic folding intermediate has been characterized *in vivo* (imp-OmpA, Freudl *et al.*, 1986). Although originally assumed to be in association with the cytoplasmic membrane, this intermediate (or another non-heat-modifiable intermediate) could just as well be associated with the outer membrane (Henning and Koebnik, 1994). A corresponding membrane-adsorbed and β-structured folding intermediate has been observed *in vitro* (Surrey and Jähnig, 1992). On the basis of these facts, I suggest two slightly different models for the sorting and assembly of split OmpA variants (Figure 7). First, both OmpA fragments are secreted across the cytoplasmic membrane individually. Then, either both (Figure 7d) or only one (Figure 7b) of the two OmpA fragments adsorb at the periplasmic face of the outer membrane. Klose *et al.* (1988) showed for a number of OmpA deletion derivatives that only those variants that retained residues 154–180 were associated with the outer membrane. Most probably, this will also apply for this series of truncated OmpA variants; hence, only one OmpA fragment, namely that with the last transmembrane β-strand, will adsorb to the outer membrane. The amphipathic character of this last transmembrane strand is highly conserved among various outer membrane proteins (Struyvé *et al.*, 1991) and plays

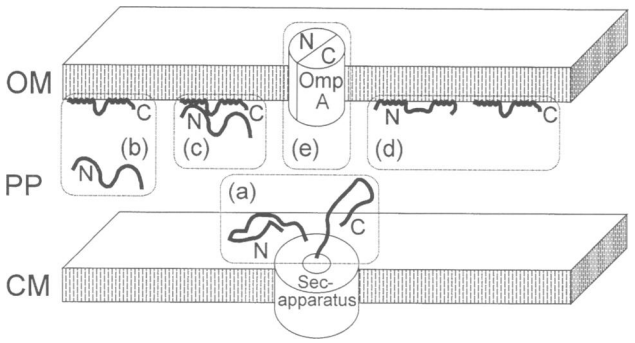

Fig. 7. Model of membrane assembly of split OmpA variants (for details see Discussion). First, both OmpA fragments are secreted across the cytoplasmic membrane by the Sec apparatus (a). Then, either both (d) or only one of the two (b) OmpA fragments adsorb at the periplasmic face of the outer membrane. After association of two complementing fragments (c), both OmpA fragments fold into the membrane, leading to the final protein structure (e). The two OmpA fragments are marked by an 'N' (N-terminal fragment) or a 'C' (C-terminal fragment). CM, cytoplasmic membrane; PP, periplasmic space; OM, outer membrane.

an essential role for outer membrane localization, as has been shown for various proteins, e.g. OmpA (Klose *et al.*, 1988) and the porin PhoE (Bosch *et al.*, 1989). It could be the role of this β-strand to mediate the initial contact with the membrane. In addition to this interaction with the lipid bilayer, both OmpA fragments must find each other in order to fold into the membrane. When two complementing fragments have met they may associate in a conformation equivalent to that of the wild-type folding intermediate. However, attempts to characterize this association of co-expressed variants OmpA(12) and OmpA(34) in more detail by negative complementation with an additional OmpA fragment [variants OmpA(23), OmpA(1C), OmpA(2C), OmpA(3C) or OmpA(4C)] were unsuccessful; neither phage assays nor pulse–chase experiments revealed any effect of these five additional OmpA fragments (data not shown). The alternative possibility, that both complementing protein fragments recognize each other in the periplasmic space prior to membrane interaction, cannot be excluded. However, *in vitro* studies showed that the β-structure of OmpA is adopted only after contact with a membrane (Surrey and Jähnig, 1992). It is therefore conceivable that individual β-strands adopt their conformation while becoming attached at the inner leaflet of the outer membrane. Probably, the OmpA fragments interact in this special conformation. Finally, it is highly unlikely that individual fragments incorporate into the outer membrane and assemble thereafter, since in this case a number of charged residues would have to be exposed to the lipid bilayer. The analysis of the three-dimensional structures of porins (Kreusch and Schulz, 1994) as well as OmpA variants with randomized amino acid residues pointing toward the lipid bilayer (R.Koebnik and L.Krämer, unpublished) indicates that charged residues at such a location are not compatible with membrane assembly.

The OmpA(123+34) variant is of special interest because it has the potential to assume different topologies. For instance, it could form a 10-stranded β-barrel. Heat modifiability most probably reflects the packing of the interior of the β-barrel domain of OmpA. A 10-stranded β-barrel would certainly contain a number of packing defects (cavities). Therefore, since the OmpA(123+34) variant is more stable than the OmpA(12+34) variant, it is highly probable that the membrane-assembled molecules represent 8-stranded β-barrels. In this case, there are three possibilities for folding: (i) β-hairpin '3' of the OmpA(123) molecules resides in the periplasmic space; (ii) β-hairpin '3' of the OmpA(34) molecules resides in the periplasmic space; or (iii) the last transmembrane strand of OmpA(123) and the first transmembrane strand of OmpA(34) are located at the cell surface. All these topologies have the same 8-stranded β-barrel in common. Proteolytic digestion of spheroplasts and of intact cells resulted in the same cleavage of the membrane-assembled OmpA(123) molecules. This behavior is in agreement with only the third topology (Figure 5B). The proteolytic resistance of the membrane-assembled OmpA(34) molecules may be due to tighter folding or shielding by lipopolysaccharide molecules. This topology gives a plausible explanation for the increased thermal stability of this variant (see above): all three periplasmic turns are retained in the OmpA(123+34) variant, whereas one of these turns is destroyed in the OmpA(12+34) variant (Figure 5). A contribution of periplasmic turns to the stability of the OmpA protein or its circularly permuted derivatives has been observed (our unpublished results). Surprisingly, despite the two protruding protein termini at the cell surface, co-expression of variants OmpA(123) and OmpA(34) mediated phage sensitivity. However, it is possible that only molecules resulting from endogenous proteolysis (Figure 4) served as a phage receptor. Normally, the termini of outer membrane proteins are located in the periplasmic space, therefore the OmpA(123+34) variant exhibits a very unusual topology. Further work will be necessary to test if this feature may be used for the exposure of peptides or proteins at the cell surface. The possibility of displaying proteins by fusing only one of its termini (rather than both) would be advantageous over conventional display systems in which the region to be displayed is inserted internally (Charbit *et al.*, 1991; Steidler *et al.*, 1993). With the OmpA(123+34) proteins, no special spatial arrangement of both protein termini in relation to each other would be required and, hence, limit the display approach.

N-terminally truncated OmpA variants lacking the first two transmembrane strands [OmpA(234)] gave rise to a heat-modifiable, presumably homodimeric species which was not protected from proteolytic digestion. Similarly, addition of the periplasmic domain of OmpA [OmpA-(234C)] resulted in presumably dimeric species, although in much reduced amounts. This may be the reason why such species were not detected in Coomassie blue-stained gels in earlier experiments using almost identical constructs (OmpAΔ4–45; Klose *et al.*, 1988). Co-expression of OmpA(234) and OmpA(234C) resulted in several heat-modifiable species. Formation of homo- and heterodimers would be in accordance with their observed electrophoretic migration rates. It is not known whether these presumably dimeric forms are of any physiological significance or if they represent an experimental artifact.

In summary, this study shows that an outer membrane protein can be dissected at its periplasmic turns and still retain its potential to fold into a structure very similar to

that of the wild-type protein. Therefore, periplasmic turns have a 3-fold potential for protein engineering: (i) they are permissive for insertion of small peptides (Ried *et al.*, 1994); (ii) they can be used to cut-and-paste the polypeptide chain for construction of circularly permuted variants (Koebnik and Krämer, 1995); and (iii) they are useful to split the polypeptide chain. Taken together, these studies clearly demonstrate that the periplasmic turns do not possess topogenic information *per se*. However, their structure must be compatible with the productive folding pathway.

Materials and methods

Bacterial strains, plasmids and phages

An *ompA* mutant of strain UH300 (not synthesizing the protein; Klose *et al.*, 1993) was used for most experiments. For labeling experiments, this strain was transformed with plasmid pGP1-2 (Tabor and Richardson, 1985), which allows temperature-induced expression of the T7 RNA polymerase and, hence, of genes under the control of a T7-specific promoter.

Plasmids pK(1234) and pKONN have been described previously (Koebnik and Krämer, 1995). Plasmid pK(1234), double-digested with *Nhe*I and *Xba*I, was used as a vector for the original cloning of non-expressed PCR-amplified *ompA* gene fragments encoding β-barrel segments. Plasmid pKONN is a pBluescript KS+ (Stratagene, La Jolla, CA) derivative, which contains a promoterless wild-type-like *ompA* allele with two *Nhe*I sites (coding for Ala-Ser) flanking the β-barrel domain-encoding region downstream of a T7-specific promoter. This plasmid served as an expression vector for all truncated and split *ompA* variants.

Bacteriophage K3h1 is a T-even-type OmpA-specific coliphage (Morona *et al.*, 1984), which served in an assay for the membrane assembly of various OmpA variants. This assay was performed as described previously (Koebnik and Krämer, 1995).

Assembly of split OmpA-encoding genes

Previously, the *ompA* gene was divided into six modules (Koebnik and Krämer, 1995), namely the translation initiation region plus the signal peptide for secretion up to amino acid Lys3, four transmembrane β-hairpins (Thr6-Gln44, Pro47-Pro86, Asp89-Ala130, Pro133-Glu174) and the complete C-terminal periplasmic domain starting with Pro177 (see also Figure 1). Their rearrangement allowed an easy assembly of split OmpA-encoding genes (Figure 2).

In brief, DNA fragments encoding an OmpA β-barrel segment (i.e. one to three β-hairpins) were amplified by PCR and cloned into plasmid pK(1234), using *Nhe*I and *Xba*I sites provided at the 5′ end of *ompA*-specific PCR primers (Figure 2A and B). Then, the translation initiation region and the signal peptide-encoding region of *ompA* were added as a *Sca*I–*Nhe*I fragment from plasmid pKONN, resulting in plasmids expressing OmpA segments with one, two or three β-hairpins (Figure 2C and D). In some cases, the periplasmic domain-encoding region of *ompA* was added as an *Nhe*I–*Sca*I fragment from plasmid pKONN, resulting in plasmids expressing OmpA β-barrel segments with the periplasmic domain added to their C-termini (Figure 2E and F).

Co-expression vectors encoding different truncated OmpA fragments were obtained as follows (Figure 2G and H): a plasmid encoding one OmpA fragment was digested with *Sca*I and *Hin*cII, which cleaves downstream of each mutant *ompA* gene. A second plasmid encoding another OmpA fragment was digested with *Sca*I and *Sma*I, which cleaves in front of the mutant *ompA* genes. After gel purification of the OmpA variant-encoding DNA fragments, both fragments were ligated and transformed into *E.coli*.

Labeling, pulse–chase and protease digestion experiments

Radioactive labeling of OmpA variants, conversion of cells to spheroplasts and their proteolytic digestion with subtilisin have been performed essentially as described by Koebnik and Braun (1993). The *ompA* mutant of strain UH300 carrying plasmid pGP1-2 served as a host. For pulse–chase experiments, *de novo* synthesized proteins were labeled with [^{35}S]methionine (1200 kBq/ml; NEN Dupont, 43 Tbq/mmol) for 5 min at 30°C, washed once with M9 medium supplemented with 100 mM L-methionine, and resuspended therein. After a chase of 15 min, samples were withdrawn and resuspended in SDS–PAGE sample buffer. For protease protection experiments, cells were converted to spheroplasts using a sucrose–EDTA–lysozyme procedure (Koebnik and Braun, 1993) and subjected to subtilisin digestion (0.2 mg/ml) for 30 min at 30°C. For protease digestion of intact cells, proteins were labeled as described above. After a chase of 15 min, the cells were washed with buffer containing 0.2 M Tris–HCl, pH 8.0, and 150 mM NaCl, and were suspended in 1 ml of buffer supplemented with 10 mM $CaCl_2$. The mixture was incubated at 30°C for 15 min with protease (0.05 mg/ml). In all cases, cells were chased at 30°C and digestions were stopped by addition of phenylmethylsulfonyl fluoride (2 mM). Thermal stability of proteins was estimated by monitoring the conversion of the heat-modifiable form into the non-heat-modifiable form by incubation of the samples at 72°C prior to gel electrophoresis for different time intervals, thus determining the half-time of this conversion.

Proteins were analyzed by Laemmli-type SDS–PAGE (15% acrylamide, w/v) (Laemmli, 1970). Gels were autoradiographed, and protein bands were quantitated using a laser densitometer (UltroScan XL, Pharmacia LKB, Bromma, Sweden). Peaks were integrated using the Pharmacia LKB GelScan XL 2.1 program.

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