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Review

The expression of outer membrane proteins for crystallization

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Abstract

The production of sufficient amounts of chemically and conformationally homogenous protein is a major requirement for successful crystallization and structure determination. With membrane proteins, this constitutes a particular problem because the membrane volume is limited and the organisms are usually very sensitive to changes in membrane properties brought about by massive protein insertion. Moreover, the extraction of membrane proteins from the membrane with detergents is generally a harsh treatment, which gives rise to conformational aberrations. A number of successful procedures for functional expression followed by purification are reviewed here together with nonfunctional expression into inclusion bodies and subsequent (re)folding to produce functional proteins. Most of the data are for prokaryotic outer membrane proteins, but the outer membrane proteins of eukaryotic organelles are also considered as they do show similar features.

Keywords: Functional expression; Eukaryotic organellar outer membrane; Detergent extraction; Detergent micelle; Inclusion body; In vitro (re)folding

1. Introduction

The majority of membrane proteins occur in the cytoplasmic membrane and they are characterized by membrane-spanning α-helices. Numerous prokaryotes possess an outer membrane in addition to their cytoplasmic or inner membrane. Up to now, all established structures of integral bacterial outer membrane proteins contained β-pleated sheets but no transmembrane α-helices. In all cases, these sheets formed circular β-barrels so that all polar atoms of the peptide bonds were buried, as in a transmembrane α-helix. In contrast to the inner membranes, the outer membranes obviously function essentially as shields against mechanical and chemical stress and are permeable for small solutes. This may be the reason for the application of β-barrel proteins in this membrane because most of them contain a reasonably sized pore if they consist of more than about a dozen strands [1,2]. However, there are a number of proteins with smaller β-barrels that form no pore, indicating that the β-barrel structure has been selected for a more general reason. The β-barrel residues are completely connected by main chain hydrogen bonds and thus tend to form a stable scaffold. This is in contrast to the proteins of the inner membrane where the α-helices form bundles that are merely connected via side chain interactions and are therefore intrinsically less stable. This difference in stability is reflected in the number and resolution of the known membrane protein structures that are still dominated by outer membrane proteins.

As a basic rule for any crystallization attempt, the protein of interest should be chemically and conformationally homogeneous. The larger the amount of the produced protein, the higher the chances of fulfilling these conditions. First, a large quantity usually means a favorable protein/impurity ratio, and furthermore, it allows the retention of only the purest fractions during purification. Accordingly, the initial isolation procedures starting from the naturally expressed protein were soon abandoned in favor of methods that increased the yield by homologous or nearly homologous overexpression into the outer membrane. As an alter-

Abbreviations: ADA, *N*-(2-acetamido)-2-iminodiacetic acid; C_6DAO, hexyl-dimethyl-aminoxide; C_6G, hexyl-glucoside; C_8POE, octyl-poly-oxyethylenoxide; C_8E_4, octyl-tetra-oxyethylenoxide; C_{10}DAO, decyl-dimethyl-aminoxide; $C_{10}E_5$, decyl-penta-oxyethylenoxide; C_{10}M, decyl-maltoside; $C_{12}E_9$, dodecyl-nona-oxyethylenoxide; C_{12}G, dodecyl-glucoside; C_{12}M, dodecyl-maltoside; β-HG, β-heptyl-glucoside; HPT, 1,2,3-heptanetriol; LDAO, lauryl-dimethyl-aminoxide; MPD, 2-methyl-2,4-pentanediol; β-OG, β-octyl-glucoside; OHESO, octyl-hydroxyethyl-sulfoxide; Z312, 3-(dodecyldimethylammonio)-propanesulfonate or Zwittergent-3-12®

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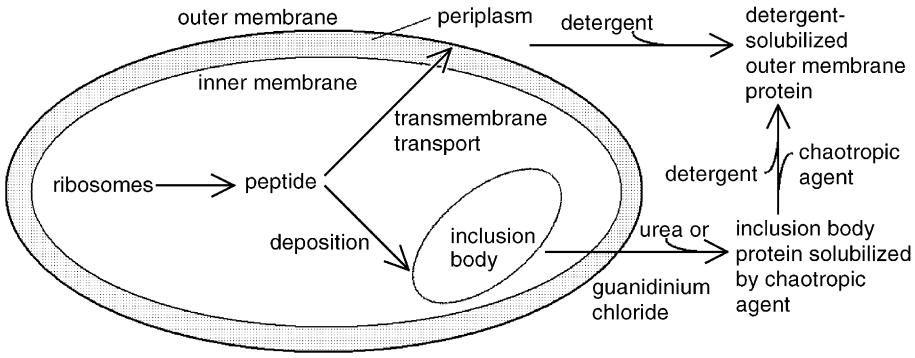

Fig. 1. Expression of prokaryotic outer membrane proteins.

native, protein yields were also enhanced by overexpression into cytoplasmic inclusion bodies, from which the polypeptide was then solubilized by the addition of denaturants and (re)folded by their removal. One section extends the review to the outer membrane proteins of eukaryotic organelles, where as yet little structural detail is known. The two major production methods are illustrated in Fig. 1. The general aim of the overexpression is to produce sufficient protein for crystallization followed by X-ray diffraction structure analysis. Here we restrict ourselves to the expression for crystallization although we are aware that the outer membrane proteins can also be applied to other purposes, for instance, to the development of vaccines for bacterial pathogens [3] or for protein engineering endeavors [4–7].

2. Functional expression of prokaryotic proteins in the outer membrane

Outer membrane proteins are produced with an N-terminal signal sequence which directs the nascent polypeptide through the translocon in the inner membrane to the periplasmic space. The signal sequence is removed during translocation and the native protein is folded and inserted into the outer membrane. Overexpressed outer membrane polypeptides without a signal sequence usually end up in inclusion bodies as illustrated in Fig. 1 and discussed in the next chapter.

The first X-ray grade crystals of outer membrane proteins were obtained with material extracted from the natural source, that is, by native expression [8–17]. For this purpose, the growth conditions were optimized to enhance the expression of the outer membrane proteins of interest, most notably of specific porins [15,18–20]. A higher degree of expression was obtained with plasmid-based systems. For example, the *Escherichia coli* maltoporin LamB was expressed using a plasmid carrying the *lamB* gene under control of the tac promoter [21]. Similarly, the sucrose porin ScrY of *Salmonella typhimurium* was produced in *E. coli* with a pBR322-derived plasmid that contained a combination of the natural promoter with the tac promoter [22]. To facilitate the purification process in all of these cases, an *E. coli* strain devoid of the abundant endogenous outer membrane proteins OmpF, OmpC and LamB was used.

An *E. coli* strain with similar deletions was used for a plasmid-dependent overexpression of FhuA [23]. Furthermore, the protein isolation procedure was improved by adding an internal hexa-histidine tag for binding to a Ni-NTA column [23] giving rise to a protein production of 8 mg/l culture (Table 1). In contrast, the homologous protein FepA overexpressed in a similar manner yielded only 0.25 mg/l culture [24]. The porin OmpF from *E. coli* was overexpressed using a plasmid that contained the *ompF* gene under control of the T7 promoter but was attenuated by a 2-bp deletion in the 'ori'-region, presumably to counteract toxicity problems [25]. Lethality on overexpression was also observed when inducing the expression of *scrY* too highly [22]. Sufficient pure material of TolC was obtained by expression in *E. coli* BL21 cells carrying a plasmid with the gene behind its own *E. coli* promoter [26,27]. The protein yields were 1–2 mg/l culture and the purified TolC crystallized in two and in three dimensions.

The toxic effects exerted by functionally expressed proteins on their host cells turned out to be a general problem limiting the expression level and also the range of proteins to be tackled [28]. The expression of the porin from *Rhodobacter blasticus* into the outer membrane of *E. coli*, for example, resulted in lysis of the cells before the desired protein was produced at high levels [29]. Presumably, the high pore activity of the *R. blasticus* porin, which shows roughly double the electric conductivity of the homologous OmpF from *E. coli*, seriously disturbed the host already at low expression levels [25]. With ScrY, the induction time was shortened to only 90 min to counteract its toxicity for *E. coli* [22]. In general, careful control of the expression level keeps the toxicity problem at bay, albeit at the expense of a high protein yield. The difficulties in keeping the bacteria alive are reflected in the large number of promoter and vector constructions used in various expression systems.

As mentioned above, low protein expression levels render the subsequent purification more difficult. This

Table 1
Outer membrane protein expression

Protein	Organism	Expression[a]	Yield[b]		Reference
			(mg/l)	(mg/g)	
OmpF	*Escherichia coli*	native	6.5		[84]
OmpF	*Escherichia coli*	functional		2–3	[25]
PhoE	*Escherichia coli*	functional	n.r.		[15]
Porin	*Rhodobacter capsulatus*	native		2	[9,71]
Porin	*Rhodobacter blasticus*	native		2	[71]
Porin	*Rhodobacter blasticus*	inclusion bodies	20–25		[29]
Porin	*Paracoccus denitrificans*	native	n.r.		[85]
OmpK36	*Klebsiella pneumoniae*	native	n.r.		[20]
Omp32	*Comamonas acidovorans*	native	n.r.		[82]
LamB	*Escherichia coli*	functional		1–1.2	[21]
LamB	*Salmonella typhimurium*	native		0.2	[19]
ScrY	*Salmonella typhimurium*	functional	8		[22]
OmpA	*Escherichia coli*	functional	7		[35]
OmpA	*Escherichia coli*	inclusion bodies	150		[35]
OmpX	*Escherichia coli*	inclusion bodies	150		[35]
OmpLA	*Escherichia coli*	inclusion bodies	35		[33]
OmpT	*Escherichia coli*	inclusion bodies	170		[36]
Omp21	*Comamonas acidovorans*	inclusion bodies	n.r.		[39]
OpcA	*Neisseria meningitidis*	inclusion bodies	n.r.		[37]
TolC	*Escherichia coli*	functional	1–2		[26]
FhuA	*Escherichia coli*	functional		0.5	[31]
FhuA	*Escherichia coli*	functional	8		[23]
FepA, FecA	*Escherichia coli*	functional	0.25		[30,86]
FepA	*Escherichia coli*	inclusion bodies	5–10		[24]
OEP16	Pea chloroplasts	inclusion bodies	n.r.		[47,52]
VDAC	Human mitochondria	inclusion bodies	n.r.		[50]
MAO-B	Human mitochondria	*Pichia pastoris* membranes	100		[49]

[a] *native* indicates an unmodified organism, *functional* means in all cases plasmid-driven in *E. coli*.

[b] l means liter of culture medium, g means gram of wet cells, n.r. means not reported.

procedure can be ameliorated by adding affinity tags to the protein. Such tags, however, are commonly considered crystallization hazards and therefore not popular. Improvements in purity can also be accomplished by using bacterial strains lacking the main outer membrane proteins [22,23,25]. Proteolysis is a further risk to the expressed protein. This problem was reported for FepA [30] in which case it was circumvented by using the host strain *E. coli* JM109 (Stratagene) although this strain is not considered protease deficient. The protease-deficient strain *E. coli* BL21, or derivatives of it, were used in the expression of OmpF [25], FhuA [31] and TolC [26]. The most developed strain lacked the *lamB*, *ompC* and *ompA* genes, had the genomic *ompF* gene inactivated by an insertion, and produced OmpF under the control of a T7 promoter [25]. The expression yield depends also on the cultivation temperature, which is usually 37 °C. The amount of the outer membrane protein OprM from *Pseudomonas aeruginosa*, however, increased 3-fold when the expression was performed at 28 °C instead [32].

3. Nonfunctional expression of prokaryotic outer membrane proteins into inclusion bodies

For bacterial outer membrane proteins, the expression into inclusion bodies with subsequent solubilization in denaturing buffer and in vitro (re)folding was surprisingly successful (Fig. 1). The outer membrane phospholipase A (OmpLA) accumulated into inclusion bodies when expressed without its signal sequence [33]. The inclusion bodies were then solubilized in urea and the protein (re)folded upon dilution into a buffer containing Triton X-100. The resulting fold was native as indicated by the enzymatic activity and by the gel electrophoresis migration behavior, which differed for the unfolded and folded states [33]. Moreover, the (re)folded protein yielded better crystals than the protein isolated from membranes [34]. The porin from *R. blasticus* was also expressed into inclusion bodies and solubilized by urea. In this instance, the protein was bound to an ion exchange column while in a buffer containing 4 M urea and 5% lauryl-dimethyl-aminoxide (LDAO). Subsequent removal of the denaturant gave rise to the (re)folded trimeric porin. The recovered protein formed large crystals which were analyzed by X-ray diffraction to 2.2 Å resolution, revealing that the (re)folded protein had assumed the previously established native conformation within fractions of an Angstrom [29].

In general, (re)folding from inclusion bodies is more difficult than the protein expression into the inclusion bodies itself. The procedure was successful for the small (ca. 17 kDa) outer membrane anchor proteins OmpA and OmpX [35]. Folding occurred upon dilution in an octyl-poly-oxyethylenoxide (C_8POE)-containing buffer from which the protein was purified in a single ion-exchange chromatography step. However, also the large 80-kDa outer membrane iron transporter FepA was produced in this way and then crystallized [24]. Here, the protocol involved a folding step in the presence of a zwitterionic detergent and SDS followed by two gel filtration and two ion exchange steps. Recently, the 20-kDa outer membrane protease OmpT [36] and the 19-kDa outer membrane adhesion protein OpcA from *Neisseria meningitidis* were expressed into

inclusion bodies, (re)folded and subsequently crystallized [37,38]. Two-dimensional crystalline arrays were obtained after the expression of *Comamonas acidovorans* outer membrane protein Omp21 into inclusion bodies [39]. In most cases of outer membrane protein expression into inclusion bodies, the T7-promoter system together with the strain *E. coli* BL21(DE3) in standard culture media was used [24,29,32,33,35,36,40,41]. For the porin from *R. blasticus*, the basal expression before induction was suppressed by using the BL21(DE3) pLysS strain (Novagen) [42].

4. Eukaryotic organellar proteins

In spite of the quite detailed knowledge about the structural properties of bacterial outer membrane proteins [43], no high-resolution structures of similar mitochondrial or plastid outer membrane proteins are known. The only structures of mitochondrial outer membrane proteins described so far are that of the human voltage-dependent anion-selective channel (VDAC) as a low-resolution projection derived from two-dimensional crystals [44,45] and that of human liver monoamine oxidase B (MAO-B) established by an X-ray analysis at atomic resolution [46]. However, MAO-B contains only a C-terminal α-helix anchor in the membrane while the major part of the protein is located in the cytosol, classifying it as an anchored cytosolic rather than a membrane protein. The outer membrane protein OEP16 from pea chloroplasts [47] yielded crystals that, however, were not suitable for an X-ray analysis. Taken together, the results with eukaryotic organellar proteins are much less spectacular than those with prokaryotic outer membrane proteins [48].

MAO-B was overexpressed in the membrane fraction of the yeast *Pichia pastoris* after expression systems based on *E. coli* and *Saccharomyces cerevisiae* failed [49]. Setting up the expression system for *P. pastoris* is much more complicated than for *E. coli*. To our knowledge, this is the first case where *P. pastoris* membrane expression was used for crystallization. It paid off yielding as much as 100 mg protein/l culture compared with a very low expression in an *E. coli* system [49] (Table 1). Because MAO-B is merely a membrane-anchored protein and not an integral membrane protein, the *P. pastoris* expression system may not be applicable for expressing other types of outer membrane proteins like, for instance, VDAC.

Until now, organellar outer membrane proteins were only expressed into inclusion bodies in *E. coli* [40,47,50–52]. In one case, the 'Walker' strain C41(DE3) [53] was used rather than BL21(DE3) [40]. RNAse-E-deficient strains may open a further pathway for increasing the expression of proteins [54]. The yeast mitochondrial outer membrane protein Tom40 was (re)folded by dilution and incorporation into liposomes [51]. In contrast, the plastid outer membrane protein Toc75 and the light harvesting complex II from pea could not be (re)folded by dilution. In these cases, the polypeptides were (re)folded while bound via His-tag to a Ni-NTA column used at high ionic strength, thus allowing the presence of some ionic detergents [40]. Both the immobilization on a column and the application of an ionic detergent are most suited to prevent aggregation, which is a major obstacle during the folding process [55]. Toc75 (re)folding was followed by tryptophan fluorescence [40]. The resulting protein was functional as shown by electric conduction measurement using planar lipid bilayers [56]. A similar assay confirmed the functionality of (re)folded Tom40 [51].

The purification of eukaryotic outer membrane proteins was facilitated by His-tag fusions [40,50,51] and also by a fusion with the maltose-binding protein (MBP) [57]. Using MBP fusions, the VDAC from yeast and the VDAC from *Neurospora crassa* were expressed in *E. coli* inclusion bodies with high yields. It was not possible, however, to isolate the MBP-fusion proteins by amylose affinity chromatography. The purification suffered from the fact that the (re)folded proteins were only soluble in buffers containing at least 1% LDAO. However, these proteins showed CD-spectra similar to those of porins and were active in single-channel electric conduction measurements.

Although outer membrane proteins are comparatively stable because they have to cope with the harsh external environment, stability is still one of the main considerations in crystallization attempts [58]. It is probably an even more pronounced problem for the organellar outer membrane proteins as these contact the milder cytosol. It should be noted that protein stability is a multifacetted parameter. The stability of membrane proteins should be differentiated in terms of heat, water-soluble denaturants and detergents [41,59]. Because a large number of detergents with a broad range of properties has become available, an appropriate detergent can usually be chosen for any given application.

Eukaryotic genes may differ dramatically from bacterial genes with respect to their codon usage, resulting in lower translation rates. This problem is mitigated by supplying rare tRNAs encoded on a plasmid that is co-transformed into the expression strain (Novagen, Stratagene). Moreover, it can be completely circumvented by constructing a synthetic gene showing the appropriate codon usage [59]. This can also be applied to large proteins [60] although the number of oligonucleotides required and the necessary correction of oligonucleotide aberrations increase the costs.

It is well known that the crystallization of prokaryotic inner membrane proteins was more successful for those derived from moderately [61] or highly thermophilic [62,63] bacteria. Unfortunately, eukaryotes do not reach the high thermophilicity that numerous prokaryotes show. The growth limit is around 60 °C as observed with some algae and fungi [64]. However, this 20 °C increase with respect to the majority of the eukaryotes should help, but it has not yet been exploited. The thermophilic eukaryotes are not well

understood. Almost no sequences are known, and it is probably difficult to establish an expression system.

5. Functional versus nonfunctional expression

Functional overexpression into the outer membrane of *E. coli* was most successful with outer membrane proteins from *E. coli* itself [21,23,25,26,30] and with ScrY of the closely related *S. typhimurium* [22]. It failed, however, with the porin from *R. blasticus*, which is an α-proteobacterium and thus more distantly related to the γ-proteobacterium *E. coli* [29]. Moreover, we are not aware of any case of the expression of an eukaryotic organellar outer membrane protein into the *E. coli* outer membrane. In general, the functional expression systems are difficult to set up and most of them suffer from comparatively low yields.

The main problem with nonfunctional expression into inclusion bodies is the in vitro (re)folding of the inactive polypeptide. A low yield in the folding process can easily abolish the advantage of a high expression level. The success of the (re)folding procedure will therefore determine

Table 2
Conditions for the three-dimensional crystallization of outer membrane proteins

	Reference	Salts (mM)	Organics (%)	Buffer (pH, mM)	Detergent (%)
OmpF	[15,87]	$MgCl_2$ (700)	PEG-2000 (6)	Tris (9.8, 20)	Octyl-hydroxyethyl-sulfoxide (OHESO) (0.8), C_8POE (0.1)
Porin *Rhodobacter capsulatus*	[11,12]	LiCl (300)	PEG-600 (8)	Tris (7.2, 20)	Octyl-tetra-oxyethylenoxide (C_8E_4) (0.6)
Porin *Rhodobacter blasticus*	[17]	LiCl (350)	PEG-600 (16)	Tris–HCl (6.8, 20)	C_8E_4 (0.6)
Porin *Rhodobacter blasticus* recombinant	[29,74]	LiCl (300)	PEG-600 (14)	Tris–HCl (7.2, 20)	C_8E_4 (0.6)
Porin *Paracoccus denitrificans*	[85,88]	KCl (200), $CaCl_2$ (10)	PEG-600 (16)	Tris–HCl (7.5, 20)	β-Octyl-glucoside (β-OG) (1.0)
OmpK36	[20]	$MgCl_2$ (500)	PEG-2000 (15)	Tris–HCl (9.8, 50)	OHESO (0.6), C_8POE (0.1)
Omp32	[82,89]	Li_2SO_4 (675)	–	HEPES (7.5, 50)	β-OG (1.0)
LamB *Escherichia coli*	[21,77]	$MgCl_2$ (100)	PEG-2000 (7.5)	HEPES (7.0, 20)	Dodecyl-nona-oxyethylenoxide ($C_{12}E_9$) (0.1), decyl-maltoside (C_{10}M) (0.4)
LamB *Salmonella typhimurium*	[18]	$MgCl_2$ (1), $CaCl_2$ (1)	PEG-1500 (16)	–	C_8E_4 (0.3), hexyl-dimethyl-aminoxide (C_6DAO) (0.8)
ScrY *Salmonella typhimurium*	[78]	LiCl (10), $MgSO_4$ (20)	PEG-2000 (7.5)	Tris (7.7, 20)	β-OG (1.2), β-heptyl-glucoside (β-HG) (1.0)
OmpA	[35,90]	–	PEG-8000 (6), 2-methyl-2,4-pentanediol (MPD) (5)	KH_2PO_4 (5.1, 25)	C_8E_4 (0.3)
OmpX	[35,91]	$CaCl_2$ (100)	2-propanol (10), glycerol (15)	Acetate (4.6, 50)	C_8E_4 (0.3)
OmpLA	[34,92]	$CaCl_2$ (1)	MPD (11)	bis-Tris (6.0, 100)	β-OG (0.9)
OmpT	[38]	NaCl (250), KCl (1.3)	MPD (14)	Citrate (5.5, 50)	β-OG (0.5)
OpcA	[37,93]	$Zn(OAc)_2$ (150), $ZnCl_2$ (50)	PEG-4000 (10)	Tris–HCl (7.5, 50)	Decyl-penta-oxyethylenoxide ($C_{10}E_5$) (0.5), β-HG (0.25)
TolC	[27]	NaCl (10), $MgCl_2$ (20)	1,2,3-Heptanetriol (HPT) (1.5), PEG-400 (10), PEG2000MME (7)	Tris (7.4, 20)	Hexyl-glucoside (C_6G), β-HG, β-OG, dodecyl-glucoside (C_{12}G), (0.6)
α-Haemolysin	[94]	$(NH_4)_2SO_4$ (–)	PEG5000MME (–)	Cacodylate (6.0, –)	β-OG (–)
FhuA	[73]	–	PEG2000MME (11), glycerol (20), PEG-200 (3)	Cacodylate (6.4, 100)	Decyl-dimethyl-aminoxide (C_{10}DAO) (0.8)
FhuA	[79]	NaCl (450)	PEG-2000 (33)	NaH_2PO_4 (6.2, 100)	OHESO (0.5)
FepA, FecA	[30,86,95]	NaCl (350)	PEG-1000 (14), HPT (1.8), glycerol (15)	Tricine (8.0, 50)	LDAO (0.055)
MAO-B	[46]	Li_2SO_4 (35)	PEG-4000 (6)	KH_2PO_4 (7.5, 25), *N*-(2-acetamido)-2-iminodiacetic acid (ADA) (6.5, 50)	3-(Dodecyldimethylammonio)-propanesulfonate or Zwittergent-3-12® (Z312) (0.14)

whether the inclusion body expression method finds a broader application [41,48]. The (re)folding attempts of outer membrane proteins resulted in the following observations: (i) the choice of the detergent is most important [33]; (ii) the in vitro (re)folding into liposomes yields generally less native protein than folding into detergent micelles [33,48]; (iii) the in vitro (re)folding requires the presence of micelles which means that the detergent concentration has to exceed the critical micelle concentration (CMC) [65]; (iv) the best results were obtained with zwitterionic detergents [29,36,37,50] and with polyoxyethylene detergents [33,35,39,47]; a mixture of a zwitterionic detergent with SDS was also applied [24].

A quantitative assessment of the (re)folding efficiency as a function of the applied detergent was performed with OmpLA by monitoring its enzymatic activity [33] and with OEP16 by following the CD-spectra [47]. If the outer membrane protein is not an enzyme and if its CD-spectrum cannot be easily determined, for instance, because of spectral interference of detergents, electrophoretic assays should be tried. At ambient temperatures, all bacterial outer membrane proteins examined assume two states with differing electrophoretic mobility in SDS-PAGE [29,33,35–37,65]. No such difference in migration behavior has been reported for eukaryotic organellar outer membrane proteins. The blue native PAGE method may be a further means of differentiating between 'folded' and 'unfolded' outer membrane proteins [51,66].

For inclusion body expression of less stable membrane proteins, the use of a mild detergent to quickly remove the denaturant, the addition of stabilizing agents [67], and working at low temperatures is recommended. The mildest detergents [68] used for this procedure were Triton X-100 [33,69], $C_{12}E_8$ and dodecyl-maltoside ($C_{12}M$) [47] (Table 2). The denaturant concentration is lowered very quickly if the protein is diluted into excess (re)folding buffer lacking the chaotropic agent. The dilution is most efficiently performed by adding the protein solution drop by drop to the (re)folding buffer using, for instance, a peristaltic pump [55]. In this way, the unfolded polypeptide is instantly dispersed and (re)folded, not leaving much time for aggregation. The temperature during protein (re)folding should always be considered to be an important variable [48,55,70].

6. Purification and crystallization

A desirable expression system produces a large amount of protein and allows its purification and subsequent crystallization. While fusion protein approaches are becoming increasingly popular, only three of the structurally established outer membrane proteins were produced as fusion proteins, and in all cases, as a fusion with a His-tag. These were FhuA [23], VDAC [50] and Omp21 [39]. Among them, only FhuA with an internal affinity-tag yielded three-dimensional crystals diffracting to high resolution (Table 2). Here, the insertion site was chosen at a surface-exposed residue identified by flow cytometry [23].

Outer membrane proteins are often difficult to solubilize. Therefore, most purification protocols for native proteins involve selective membrane extractions in which other outer membrane proteins are separated according to their solubilization properties in a given detergent. The target proteins were subsequently solubilized with LDAO [22,23,71], C_8POE [21] or Triton X-100 [26]. Obviously, they should be stable enough to maintain their native conformation during the extraction procedure. This was not the case during the isolation of MAO-B from *P. pastoris* membranes [49]. Therefore, the membrane was first digested with phospholipase A_2 so that the protein could be mildly solubilized using 0.5% Triton X-100.

All protocols for nonfunctional expression into inclusion bodies circumvent the membrane solubilization step. Here, however, mild detergents and suitable buffer conditions have to be established for the folding process in order not to harm the protein. In general, purity and the ease of impurity removal vary appreciably among different proteins expressed into inclusion bodies. In numerous cases, the purification of (re)folded protein was not easier than a differential extraction from an outer membrane. Most inclusion bodies contain other outer membrane proteins, ribosomal proteins and sometimes also truncated versions of the plasmid-encoded target protein [72]. Often these contaminants can be removed by washing, using a detergent-free buffer [33] or a buffer with detergents such as Triton X-100 [24,29,35,55] or LDAO [37]. It has been pointed out that extensive protein expression into inclusion bodies gives rise to morphological changes of the cell wall, which then calls for harsher procedures to isolate the inclusion bodies [55].

With nonfunctional expression systems involving a His-tag [39,50] the target protein can be purified in the fully or partially denatured state. Some proteins bind to an ion exchanger in the presence of high concentrations of urea [5,29,52]. In general, however, the protein was purified after (re)folding by dilution. Gel filtration columns can be used to remove misfolded and aggregated unfolded protein due to their different hydrodynamic radii [24,37,70]. For the separation of different conformational and aggregational states, ion exchange columns were applied for several outer membrane proteins, in particular, unfolded FepA eluted at lower ionic strength than native FepA [24], while unfolded OmpLA eluted at higher ionic strength [33] than its folded isoform.

In all structural analyses in which the full-length protein does not form suitable crystals, a truncation of the polypeptide chain should be considered. The outer membrane protein OmpA from *E. coli*, for instance, was known to consist of an integral membrane part (residues 1–171) and a periplasmic part (172–325) with very different properties. It was therefore soon decided to restrict the expression and

the structure analysis to the integral membrane moiety, and the corresponding DNA was cloned and expressed into inclusion bodies [35]. In contrast, TolC was functionally expressed and purified as the full-length protein. Subsequently, the C-terminal 43 residues were removed with V8 protease and the truncated protein was purified by anion-exchange chromatography [27].

Outer membrane proteins have been crystallized with lipids or lipopolysaccharides [73], but more often without them (Table 2). However, proteins isolated from membranes may require lipids for their stability, in particular, if they contain strong lipid-binding sites [17,31,74]. Lipopolysaccharides are necessary for the activity of the protease OmpT [75]. For the structure analysis, however, the protein was (re)folded from inclusion bodies and crystallized without a lipopolysaccharide. To prevent autoproteolysis over the long period of crystal growth, the protease activity of OmpT was completely inactivated by three point mutations [38]. Mutations are much more easily introduced into plasmid-driven than into genomic expression systems. Amino acid residue exchanges in external loops were actually necessary to produce high-quality crystals of the outer membrane proteins OmpA and OmpX [43,76]. The introduction of crystallization-enhancing mutations seems especially suited for proteins with a reasonably clear chain topology prediction [35].

Outer membrane proteins which cannot be sufficiently stabilized in an isolated state may form more stable complexes with other proteins and/or low molecular mass compounds. A number of bacterial outer membrane proteins have been characterized with their substrates [77–79], substrate analogues [16,18,80] or ligated lipids [81]. The structural analysis of a bacterial porin revealed, for instance, that it carried a periplasmic peptide ligand from its natural source [82]. A co-expression of outer membrane protein complexes for structural studies has not yet been reported. The stabilization of membrane proteins by designed mutations is being actively pursued [59,83].

7. Conclusion

Membrane protein structures were elucidated much later than those of water-soluble proteins. The late and limited success in membrane protein crystallization is most likely caused by the internal mobility of these proteins. Membrane proteins are strongly supported by their lipid bilayer environment, which is in contrast to water-soluble proteins that must contain a solid core to survive the dissolution forces of water. Moreover, in the crystal, the structured bilayer has to be replaced by a less structured detergent envelope, which fails to provide the accustomed stability contribution. Given these problems, the development of expression systems yielding large amounts of homogeneous material is certainly a necessary step toward the improvement of membrane protein crystallization.

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