β-Barrel proteins from bacterial outer membranes: structure, function and refolding

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Recently solved outer membrane protein structures include the smallest and largest known β-barrel structures, with functions distinct from the general and specific porins. Both protein expressed in outer membranes and protein deposited as cytoplasmic aggregates have been used for the structure determinations. As most β-barrel proteins can be overexpressed in an aggregated form (inclusion bodies) and refolded to the native state, this provides an alternative to membrane-targeted expression strategies and yields sufficient quantities of protein for future structural studies.

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Abbreviations

cmc	critical micelle concentration
FepA	ferric enterobactin receptor
FhuA	ferrichrome receptor
LPS	lipopolysaccharide
OmpA	outer membrane protein A
OMPLA	outer membrane phospholipase A

Introduction

Integral outer membrane proteins from Gram-negative bacteria use amphipathic β-strands to traverse the membrane. In all known structures of this type, the β-sheet has a simple meander topology. It is twisted to form a closed barrel, in which the last β-strand is hydrogen bonded to the first. The versatility of transmembrane β-barrels is illustrated by the variety of structures that has been solved. The general porins, represented by porin from *Rhodobacter capsulatus* [1] and OmpF [2], use 16-stranded β-barrels that associate to form homotrimers. These channels carry out the passive diffusion of small (~600 Da) molecules. In comparison, the specific porins, such as maltoporin (LamB) [3] and sucrose porin (ScrY) [4], are composed of 18 β-strands and also form homotrimers. They recognise specific sugars and utilise facilitated diffusion for uptake. Both classes of porins have been recently reviewed [5]. Yet another type of β-barrel is seen in α-hemolysin, a staphylococcal toxin that assembles as a heptamer and inserts a 14-stranded β-barrel into the targeted cell membrane [6]. In the past year, several new types of β-barrel structures have been solved by X-ray crystallography. These proteins differ both in architecture and in function from the previous examples: outer membrane protein A (OmpA) is the smallest known β-barrel structure and functions as a solid transmembrane anchor. Outer membrane phospholipase A (OMPLA) is an enzyme that hydrolyses endogenous phospholipids and is regulated by reversible dimerisation. Two active transporters of iron chelates, ferric enterobactin receptor (FepA) and ferrichrome receptor (FhuA), are the largest known β-barrel structures. They both contain a globular domain folded into the barrel lumen, which functions in ligand binding and transport.

Of the four new structures discussed in here, two were solved using proteins isolated from *Escherichia coli* outer membranes (FepA and FhuA) and two were solved using proteins expressed in aggregated forms that were refolded to the native state (OmpA and OMPLA). To show that refolded protein is virtually identical to the native species, structures of the *Rhodopseudomonas blastica* porin were solved with both the native (membrane-inserted) protein and the refolded material [7]. The root mean square deviation of the Cα atoms for the best superposition was within the limits of error for the structure determinations.

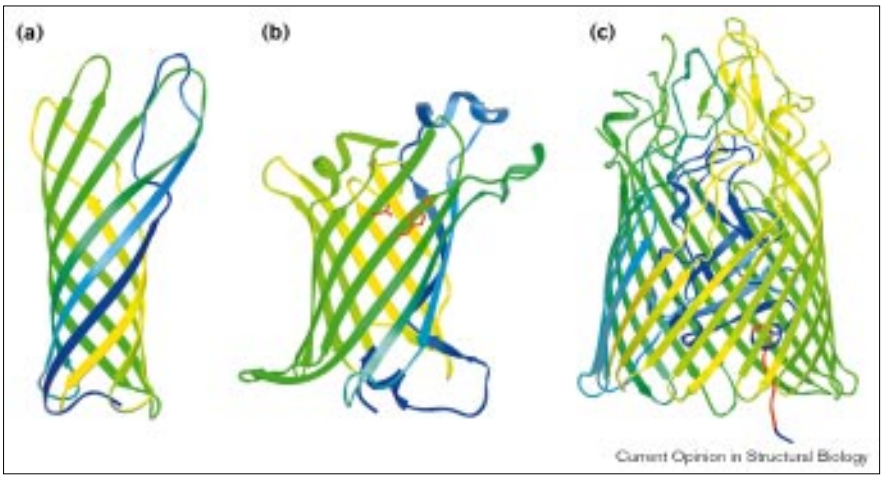
This review describes the structures and functions of OmpA, OMPLA, FepA and FhuA. In addition, as more functional protein can be potentially obtained by refolding cytoplasmic aggregates than by solubilising membrane-inserted proteins (as a result of limitations on membrane-targeted expression), refolding methods that aim to provide large quantities of correctly folded protein for structural studies will be discussed.

Outer membrane protein A

OmpA from *E. coli* is one of the most abundant proteins in the outer membrane and has been used to extensively study membrane protein folding [8,9]. It is composed of 325 amino acids: residues 1–171 form the smallest membrane-spanning β-barrel so far observed and residues 172–325 form a periplasmic domain whose structure remains unknown. The periplasmic domain has been proposed to bind the peptidoglycan layer, such that OmpA physically links the peptidoglycan layer to the outer membrane [10]. The extracellular loops of the barrel facilitate F-mediated bacterial conjugation [11] and recognise specific colicins [12] and bacteriophages [13].

Pautsch and Schulz [14••] have solved the structure of the membrane-spanning domain of OmpA (residues 1–171) at 2.5 Å resolution (Figure 1a), starting from aggregated protein that was subsequently refolded [15•]. The β-barrel consists of eight antiparallel strands with an average length of 13 residues. The strands are tilted approximately 45° with respect to the barrel axis. The shape of the barrel is almost circular, with an elliptical axis ratio of 5:4. The four extracellular loops consist of 7 to 17 residues and are highly mobile.

Figure 1



Ribbon diagrams of OmpA [14••], OMPLA [24] and FepA [30••], gradient-coloured from blue at the N termini, through green, to yellow at the C termini. **(a)** The eight-stranded transmembrane β-barrel of OmpA. The periplasmic domain, not present in the construct used for the crystal structure determination, would extend from the end of the yellow strand. **(b)** Monomeric OMPLA, a 12-stranded β-barrel. Residues S144, H142 and N156 comprising the catalytic triad are shown in red. **(c)** FepA, a 22-stranded β-barrel with a globular domain (blue) inserted into the barrel lumen. The TonB box is shown in red. This figure was drawn using SETOR [59].

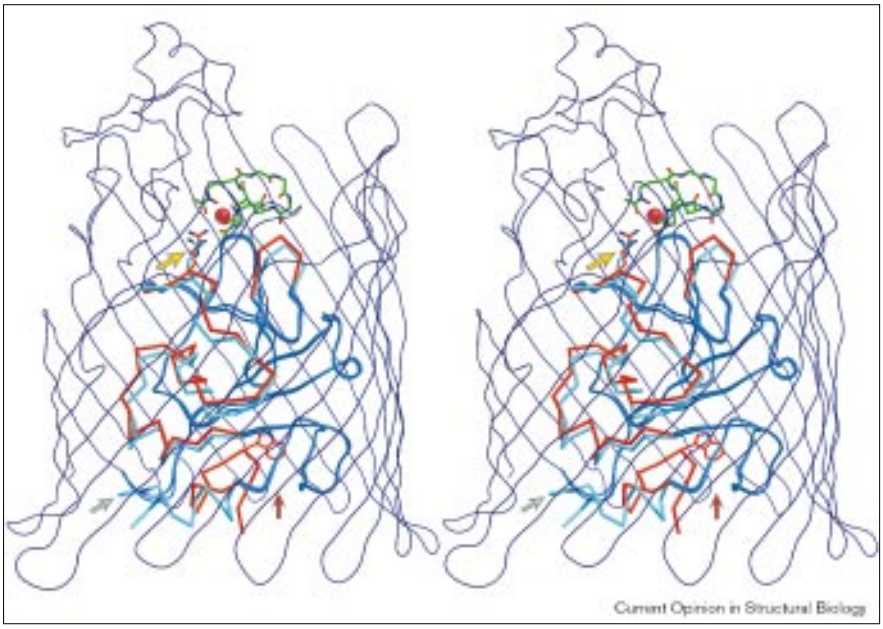
Although ionophore activity for OmpA has been reported [16,17], the barrel interior contains no channel and has been described by Pautsch and Schulz as a "solid inverse micelle" [14••]. The barrel interior contains an extensive hydrogen-bonding network that creates several large aqueous cavities, but no passage that is large enough for the transport of water or small ions. Sequence alignment with five bacterial homologues shows that the residues that form the internal polar network are highly conserved; the authors speculate that these residues may either be involved in protein folding or have some unknown function. The structure of OmpA provides the first example of the β-barrel scaffold being used primarily as a solid transmembrane anchor for the separate functions of the extracellular loops and the periplasmic domain.

Outer membrane phospholipase A

OMPLA is one of the few enzymes found in the outer membrane. It hydrolyses endogenous phospholipids and is activated upon cell rupture [18–20]. OMPLA is involved in colicin secretion in *E. coli* and has been implicated in bacterial virulence [21,22]. As this enzyme resides in its own substrate, its activity must be tightly regulated to prevent unwanted cell lysis. OMPLA is a 269-residue protein that displays broad substrate specificity and shows no sequence similarity to soluble phospholipases. It requires calcium for activity and is regulated by reversible dimerisation [23].

The structure of *E. coli* OMPLA in monomeric (2.2 Å resolution) and dimeric (2.1 Å resolution) forms is presented in a soon-to-be published paper from Dijkstra's group

Figure 2



Conformational changes induced in the N-terminal domain of FhuA upon ferrichrome binding [28••]. The barrel is drawn in dark blue, portions of the N-terminal domain that do not move are in mid blue and portions of the N-terminal domain that show movement are drawn in cyan (no ligand) and red (ferrichrome-bound). Ferrichrome is drawn in green, with the iron atom represented by a red sphere. Residue R81 (indicated by the yellow arrow), located on the extracellular side of the N-terminal domain, shows a small shift upon ligand binding, while residue W22 (cyan and red arrows for the unbound and ligand-bound structures, respectively), located on the periplasmic side, undergoes a movement of 17 Å when ferrichrome is bound. This figure was kindly provided by K Locher and was made using the program DINO [60].

Table 1

Refolding procedures for β-barrel membrane proteins.

Protein	Number of strands/ oligomeric state	Expression system	Host	IB	Unfolding conditions	Refolding conditions	Scale	Crystals obtained	References
OmpF	16/3	Chromosomal	*E. coli*	No	6 M guanidine plus 95°C, then exchange into 2% SDS	Dilution into mixed micelles (asolectin plus C8-POE), dialysis	40–160 μg from 200 μg unfolded protein	No	[42]
OmpF	16/3	Chromosomal	*E. coli*	No	8 M urea	Dilution into mixed micelles (DMPC–LM), dialysis	ND	No	[50]
OmpA	8/1	Chromosomal	*E. coli*	No	4 M urea	Dilution into OG, reconstitution into DMPC vesicles	ND	No	[61]
OmpA	8/1	Chromosomal	*E. coli*	No	0.05% SDS, 100°C, 10 min	Addition of OG, then reconstitution into DMPC vesicles	ND	No	[51]
PhoE	16/3	*In vitro* synthesis	NA	No	Protein obtained unfolded	Addition of Triton X-100	ND	No	[55]
Rps. blastica porin	16/3	pET3b	*E. coli* BL21(DE3)–pLysS	Yes	8 M urea	Dilution into 10% LDAO, refolding on anion exchange column	25 mg/l	Yes	[7]
OMPLA	12/2	pJP29	*E. coli* BL21(DE3)	Yes	8 M urea	Dilution into 10 mM Triton X-100, 0.87 M urea, 16 hr RT	35 mg/l	Yes	[25]
OmpA and OmpX	8/1	pET3b	*E. coli* BL21(DE3)	Yes	6 M guanidine	Dilution into 5% C8-POE plus 0.6 M arginine	150 mg/l	Yes	[15•]
H. influenzae type-b porin	16?/3	pET11a	*E. coli* BL21(DE3)	Yes	8 M urea	1 M NaCl plus 10% SB3-14, gel filtration chromatography to reduce detergent concentration to 0.05%, storage at 4°C for 3 weeks	60 mg/l	No	[44]
H. influenzae porin	16?/3	pKTH	*B. subtilis*	Yes	8 M urea	1% SDS, 100°C, added 0.1% SB3-14	21 mg/l	No	[56]
Neisserial porins	16?/3	pET17b	*E. coli* BL21(DE3)	Yes	8 M urea	5% SB3-14 plus 4M urea, gel filtration chromatography to reduce detergent concentration to 0.05%	50 mg/l	No	[45]
FepA	22/1	pET17b	*E. coli* BL21(DE3)	Yes	7 M urea	Removal of urea by dialysis, then addition of 1% SDS plus 5% SB3-14, gel filtration chromatography to reduce detergent concentration to 0.05%	5 mg/l	Yes	[31]
S. cerevisiae Tom40	Unknown	pET11a	*E. coli* BL21(DE3)	Yes	8 M urea	Dilution into Mega-9 or mixed micelles (asolectin plus Mega-9), dialysis	ND	No	[47•]
N. crassa and *S. cerevisiae* VDAC	Unknown	pET15b	*E. coli* BL21(DE3)	Yes	6 M guanidine	Addition of 2% LDAO, dialysis	40 mg/l	No	[46•]
Chloroplast Toc75	Unknown	pET24	*E. coli* c41	Yes	7 M urea plus 1.2% sarkosyl	Dilution to 3.2 M urea plus 0.6% sarkosyl, refolding on Ni-column in presence of Triton X-100	2 mg from 25 mg IBs	No	[48•]
Pea root porin plastid	Unknown	pQE60	*E. coli* TG1	Yes	1% SDS	Dilution into 2% Genapol X-80, addition of sterols, reconstitution into DOPC vesicles	ND	No	[49]

C8-POE, octylpolyoxyethylene; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; IB, insoluble aggregate (inclusion bodies); LDAO, dodecyldimethylamine oxide; LM, dodecyl-β-D-maltoside; Mega-9, nonanoyl-*N*-methyl glucamide; NA, not applicable; ND, not determined; OG, octyl-β-D-glucopyranoside; RT, room temperature; sarkosyl, lauroylsarcosine; SB3-14, 3-(tetradecyldimethylammonio)propane-1-sulfonate; VDAC, voltage-dependent anion-selective channel.

(HJ Snijder, BW Dijkstra and co-workers, personal communication; see also [24]). Crystals were grown from the refolded protein [25]. This provided a source of lipopolysaccharide (LPS)-free material, which was necessary for reproducible crystallisation [26]. The protein folds as a 12-stranded β-barrel, displaying a curved side and a flat side within the membrane bilayer (Figure 1b). The six extracellular loops contain between 9 and 19 residues. Three of these loops, together with the N and C termini, close the barrel on both sides of the membrane and prevent ion transport. The interior of the barrel is hydrophilic and extensively hydrogen bonded, conferring rigidity on the barrel scaffold.

The enzyme's active site is formed by a catalytic triad of residues, S144, H142 and N156, located on the exterior of the barrel wall, just above the extracellular side of the lipid bilayer (Figure 1b). The arrangement of these residues resembles the catalytic triads found in serine proteases, but with opposite chirality (as observed in other esterases). OMPLA is activated by dimerisation via the parallel association of the flat sides of two β-barrels. Calcium is necessary for both dimerisation and activity; a single calcium ion is found at each active site within the dimer interface. The dimer interface shields the active sites from the membrane environment, while forming complete substrate-binding pockets and a functional oxyanion hole (which stabilises the transition-state intermediate). Whereas soluble lipases undergo conformational changes in order to create a functional oxyanion hole and substrate-binding pocket, OMPLA relies on reversible oligomerisation, with little conformational change in the monomers, to achieve the same result.

Ferric enterobactin and ferrichrome receptors

In addition to the two classes of porins that carry out general (OmpF, *Rb. capsulatus* porin and *Rps. blastica* porin) and specific (LamB and ScrY) diffusion, Gram-negative bacteria express active transporters for the uptake of ferric chelates and vitamin B_{12}. These transporters exhibit high ligand specificity and can recognise and bind ligand in the absence of other factors. Transport into the periplasm, however, requires transmembrane protein motive force and interaction with the integral inner membrane protein complex TonB–ExbB–ExbD (reviewed by Braun *et al.* [27•]). Three structures have been determined that show that members of this family share a common fold. Structures of the 714-residue ferrichrome receptor (FhuA) from *E. coli* have been determined at 2.7 Å resolution in the presence and absence of ferrichrome by Locher *et al.* [28••] and Ferguson *et al.* [29••]. The structure of the 724-residue ferric enterobactin receptor (FepA) from *E. coli* has been solved at 2.4 Å in a partially ligand-bound state by Buchanan *et al.* [30••] (Figure 1c). Although each of these structures was solved from crystals of protein expressed in *E. coli* outer membranes, FepA has also been expressed in an aggregated form in the cytoplasm and refolded to the native state [31].

The iron transporters use a 22-stranded β-barrel to span the outer membrane, with strands tilted approximately 45° to the barrel axis. The shape of the barrel is elliptical, with a cross-section approximately 35 Å by 45 Å. The extracellular loops are generally longer than those observed in OmpA and OMPLA, containing 7 to 37 residues in FepA. In contrast to the general and specific porins, they do not use an extracellular loop to constrict the barrel lumen. Instead, both receptors contain a 150-residue globular domain at the N terminus that folds into the barrel pore, completely blocking access to the periplasm. A seven-residue sequence, the TonB box, is known to interact with the periplasmic portion of its inner membrane transport partner, TonB. The TonB box is located at the beginning of the N-terminal domain (residue 12 in FepA) and is visible in the FepA structure, although it is disordered in the FhuA structures. In the FepA structure, it protrudes into the periplasm, providing easy access for coupling to TonB.

The N-terminal domain is not only accessible from the periplasmic side of the membrane, but also spans the length of the barrel pore and protrudes to the extracellular side. It contains two (FepA) or three (FhuA) long loops that extend beyond the outer membrane and are accessible to the unbound ligand. The two FhuA structures with bound ferrichrome show that these loops participate in ligand binding. Extracellular loops L3 and L11 of the β-barrel also contribute to the ferrichrome-binding site. Although the binding sites for ferrichrome and ferric enterobactin will differ in detail (ferrichrome is neutral at physiological pH, whereas ferric enterobactin carries a net charge of –3), the major position of the iron atom in FepA suggests that the two loops of the N-terminal domain could form part of the ligand-binding site.

The FhuA structures show that ligand binding propagates a conformational change through the N-terminal domain. The ligand-binding loops on the extracellular side of the domain show small (1–2 Å), but significant, displacements upon the binding of ferrichrome. These changes are amplified towards the periplasmic side of the domain by the unwinding of an α helix, causing the polypeptide chain to shift to the opposite side of the barrel wall. This results in large movements of the polypeptide chain; for example, residue W22 is displaced by 17 Å (Figure 2). It is unclear how the conformational changes in the N-terminal domain, induced by ligand binding, lead to TonB recognition. As the channel remains blocked in the ligand-bound structures, the transport mechanism, including its energy requirement, also remains to be elucidated.

In vivo folding of outer membrane proteins

The folding and insertion processes of outer membrane proteins are less well understood than those of inner membrane proteins [32•]. Nevertheless, a brief discussion of *in vivo* folding is useful for comparison with *in vitro* procedures. Bacterial outer membrane proteins are synthesised in the cytoplasm with N-terminal cleavable signal peptides. Translocation across the inner membrane is dependent on the Sec machinery, with the signal peptide being cleaved off

by signal peptidase I. Several models for protein insertion into the outer membrane have been proposed. The first model suggests that an outer membrane protein is inserted directly into the membrane at contact sites between the inner and outer membranes called adhesion zones [33]. In this model, the protein would never enter the periplasm. As periplasmic intermediates have been identified [34,35], however, a second model postulates that the protein must pass through the periplasm en route to the outer membrane. This suggests at least partial folding of a soluble intermediate before insertion into the outer membrane. A third model proposes that the intermediate remains associated with the periplasmic side of the inner membrane and folds by interacting with newly synthesised LPS, prior to insertion into the outer membrane [36,37]. Folding catalysts, such as DsbA [38] and SurA [39], and putative periplasmic chaperones [40,41] may also participate in the folding process. Experiments on phosphoporin (PhoE) suggest that once monomers have folded, they can assemble into trimers prior to insertion into the outer membrane [36].

Refolding of outer membrane proteins

The following discussion explores the practical aspects of large-scale refolding experiments. Refolding studies on OmpA [8,9] and OmpF [42] have relied on the natural abundance of both of these proteins in *E. coli* and, therefore, have used membrane-inserted proteins as the starting material. Most outer membrane proteins do not occur naturally in large quantities, however, and, therefore, they have to be overexpressed. Unfortunately, some proteins are toxic to *E. coli* cells when they are targeted to the outer membrane. In contrast, deposition of the protein as cytoplasmic inclusion bodies is generally well tolerated by the expression host and, therefore, this technique is used in many laboratories. By far the most popular method of producing cytoplasmic aggregates from outer membrane proteins uses the pET vector system [43], which employs a T7 promoter for expression in *E. coli* strains containing a chromosomal copy of the T7 RNA polymerase gene (Table 1). The gene encoding the target outer membrane protein is modified to remove its signal sequence, preventing protein translocation. Expression results in the accumulation of the misfolded protein in the cytoplasm, at levels up to 1 gram of protein per litre of cell culture. This method has been used successfully to overexpress (in *E. coli*) outer membrane proteins from bacteria [7,15•,22,31,44,45], fungi [46•], yeast [47•] and plants [48•,49].

After collecting the protein aggregates by differential centrifugation, they are generally solubilised in 8 M urea or 6 M guanidinium–HCl, yielding the disaggregated, unfolded material for renaturation. Some proteins exhibit a preference for urea in order to refold correctly: OMPLA refolds from guanidine much less efficiently than from urea [25] and FepA could only be refolded from urea-solubilised inclusion bodies [31]. This may be as a result of the high ionic strength of guanidine solutions, which could inhibit the ionic interactions necessary for correct folding.

Once inclusion bodies have been solubilised, refolding is induced by adding lipids, mixed lipid–detergent micelles or detergents, followed by dilution, dialysis or chromatography to remove the denaturant. Lipid vesicles have been used to refold OmpA [8,9] and OmpF [50], although the yields were low. In these examples, the protein spontaneously refolds and inserts into lipid vesicles, without any requirement for detergent. LPS, found in the outer leaflet of the outer membrane, is not required for refolding OmpA [51], but improves the efficiency of the refolding of PhoE [52,53•]. The yields of refolded OmpA and OmpF improve when the unfolded material is added to mixed lipid–detergent micelles of dimyristoylphosphatidylcholine–dodecylmaltoside [50], presumably as a result of the perturbation of the lipid bilayer structure by the detergent. The importance of bilayer curvature and rigidity for membrane protein folding has been investigated using the (α-helical) inner membrane protein bacteriorhodopsin [54] and it is possible that characteristics of the lipid environment are also important for β-barrel protein folding.

The most common method for refolding outer membrane proteins uses detergent, but no lipid, to induce the native conformation; all protocols that have produced crystals fall into this category (Table 1). Generally, detergent is added to unfolded protein in denaturant, the denaturant is removed by dialysis or column chromatography (anion exchange, immobilised metal affinity or gel filtration chromatography), usually with a concurrent decrease in the detergent concentration; however, the methods differ widely in detail. The unfolded protein may be diluted 15-fold [25], 10-fold [47•], sixfold [15•] or twofold [7,44,45] into a detergent-containing buffer or detergent may be added to a concentrated protein solution [31,46•]. The optimal concentration of detergent added varies from a concentration equivalent to four times the critical micelle concentration (cmc) [47•] up to 500 times the cmc [31,44,45]. In addition, the choice of detergent is critical to obtaining high yields of refolded protein. *In vitro* synthesised PhoE absolutely requires Triton X-100 or a similar phenyl-substituted detergent [55], whereas Triton X-100 will not induce refolding of FepA [31]. Several protocols rely on a combination of SDS plus another detergent [31,42,49,51,56]. The role of SDS in refolding is not clearly understood — it is possible that the anionic detergent mimics the negatively charged LPS in the outer membrane [55]. Another explanation is that the α-helical secondary structure induced by SDS [57], although non-native, may provide a better starting point for the refolding process [56].

A number of additional parameters have been found to influence refolding yields. High ionic strength [44] and the retention of low concentrations of denaturant [25] have improved refolding results. The protein concentration has been found to be critical in several cases [7,25,45] and must be determined for individual proteins. In one instance, the addition of a polar additive increased the yield of the refolded protein [15•]. Interestingly, temperature plays an important role — most of

the proteins listed in Table 1 have been refolded at room temperature. The single exception is *Haemophilus influenzae* type-b porin, for which folding was initiated at room temperature, with subsequent storage of the protein at 4°C for three weeks, to improve the yield of the refolded material [44]. Clearly, a number of conditions influence protein refolding, but a sparse matrix approach for parameter sampling has recently been described [58] that should simplify the refolding of both soluble and membrane proteins.

Conclusions

The crystal structures described here show that the transmembrane β-barrel is a versatile structural element, used by proteins with diverse functions. β-Barrels consisting of 8, 12, 14, 16, 18 and 22 strands are known and other types are likely to follow [46•–48•,49]. Oligomerisation states include monomers, dimers and trimers, with functions of membrane localisation, enzyme activity, transport and cell lysis. Many all-β membrane proteins can be deposited in *E. coli* as cytoplasmic inclusion bodies and a number of successful refolding procedures have been documented. This approach to providing large quantities of homogeneous protein should result in an abundance of new β-barrel structures in the near future.

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