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Preparative refolding of small monomeric outer membrane proteins

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ABSTRACT

The outer membrane of gram-negative bacteria constitutes an important hurdle for the transport of hydrophobic molecules into the cell. Mass flux is often facilitated by various outer membrane proteins. These proteins are of biotechnological importance because they could help to improve the performance of whole-cell biocatalysts or be incorporated into artificial cell-like systems. The characterization and understanding of their transport properties greatly benefits from the possibility to express and purify these proteins. We investigated folding parameters for the refolding of four small monomeric outer membrane proteins from *Escherichia coli* (OmpW) and different pseudomonads (AlkL, OprG and TodX). To this aim we screened a number of inexpensive detergents and detergent concentrations, folding additives as well as protein concentrations. Interestingly, detergents with a C_{12} chain were most effective in promoting the folding reaction, particularly the negatively charged *N*-Lauroylsarcosine for OmpW, OprG and TodX as well as the zwitterionic *N,N*-Dimethyl-*n*-dodecylamine *N*-oxide (LDAO) for AlkL. The addition of 1 M urea (AlkL, OmpW), 0.1 M glutamate (OprG) or 0.1 M glycine (TodX) could further improve the folding efficiency. In order to be able to reproducibly produce larger amounts of the proteins, we then established the folding in a miniaturized stirred-tank reactor system combined with a liquid handler. This approach led to a near-complete refolding of OprG (96%), a very good folding of AlkL (84%) and OmpW (71%), only TodX folding was more variable with a final folding efficiency of 52%, all obtained at a final protein concentration of 0.5 g/L.

1. Introduction

Whole-cell bioconversions of hydrophobic compounds are often limited by the barrier-function of the outer membrane of gram-negative bacteria [1]. This is due to the structure of the lipopolysaccharide layer which lowers the diffusion of hydrophobic molecules by about two orders of magnitude compared to simple lipid bilayers [2]. Accordingly, strategies for the permeabilization of whole-cell biocatalysts are often necessary. Popular methods include the use of detergents, organic solvents or EDTA [1]. Apart from these rather crude methods, cell envelope engineering represents another possibility to overcome mass transfer limitations. Recently, transport proteins for hydrophobic substrates have gained more attention. Overexpression of the *Escherichia coli* long-chain fatty acid transporter FadL was used to increase the microbial production of hydroxylated long-chain fatty acids [3]. The small outer membrane protein AlkL from the *Pseudomonas putida* OCT plasmid was found to increase oxygenation of alkanes [4,5], fatty acids or fatty acid methyl esters [5–7] and limonene [8]. In a membrane proteome study on phenylpropanoid producing *E. coli* strains, Zhou et al. [9] identified and functionally validated several outer membrane proteins involved in the import or export of these phytochemicals, and the engineering of outer membrane proteins was subsequently proposed to tailor better phenylpropanoid production strains.

Harnessing the full potential of suitable channel proteins to transport the desired substrates generally requires a better understanding of channel function and, possibly, channel engineering. To verify new candidates, *in vivo* data can be generated using classical genetical techniques such as gene overexpression or silencing [e.g. [9]]. The interpretation of such experiments is, however, complicated by the flexibility and functional redundancy of cellular networks and so the ultimate goal is often to obtain functional or even structural data *in vitro*. Classical tools to study

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channel proteins *in vitro* include electrophysiological measurements or the integration in liposomes. Alternatively, channel proteins can also be introduced into synthetic polymer membranes, e.g. into polymersomes. Due to their synthetic nature and increased stability, these structures could also be used as nanoreactors [e.g. [10,11]].

These functional assays, but also X-ray and NMR analyses, require large amounts of pure and stable protein. With membrane proteins this task is still far from trivial because both the expression and the subsequent purification in the presence of detergents present many hurdles not encountered when working with soluble proteins, consequently the yields are typically low [12].

An alternative approach to the native expression of the protein is the expression into inclusion bodies with subsequent refolding. This strategy is well suited for the synthesis of outer membrane proteins with their β-barrel architecture because the inclusion bodies are readily dissolved in urea or guanidinium hydrochloride. This facilitates the purification greatly and often yields hundreds of mg of pure but yet unfolded protein per liter culture even when cultivating in shake flasks. Besides, a number of these proteins are easily foldable in principle, provided that their basic requirements are met [13]. Reports about refolded membrane proteins have dramatically increased over the past 20 years and so far, at least 40 different β-barrel membrane proteins have been refolded successfully [14].

After initial attempts to laboriously express and solubilize four different outer membrane proteins (AlkL, OmpW, OprG and TodX) that are known or suspected to transport hydrophobic substrates [4,15–17] we sought to establish simple folding protocols for these proteins. Notably, purification of AlkL has not been attempted until now. AlkL is part of the alk-operon necessary for hydrocarbon utilization of this strain. As such, its role probably lies in the transport of hydrocarbon substrates across the outer membrane [e.g. [5,18–20]]. The homologous *Pseudomonas aeruginosa* outer membrane protein OprG was described to contribute to the pathogenicity of *P. aeruginosa* [21] and has recently been linked with the uptake of small amino acids such as glycine, alanine, valine, and serine [22]. OmpW was originally described as the receptor for colicin S4 [23] and forms a hydrophobic channel with a lateral opening into the hydrophobic membrane region, which could serve as a passageway for hydrophobic molecules across the outer membrane [16]. Hard evidence for its function is still scarce, but it has been described to aid in the efflux of quaternary cationic compounds [24] and to confer resistance to phagocytosis [25], while OmpW of *Pseudomonas fluorescens* mediates naphthalene uptake [26]. The three former proteins are structurally similar and belong to the OmpW family of 8-stranded β-barrel outer membrane proteins. TodX is a member of the FadL transporter family and forms a 14-stranded β-barrel with an N-terminal hatch domain blocking the barrel interior [15]. It is part of the toluene dioxygenase pathway in *Pseudomonas putida* and responsible for the transport of substrates such as toluene [27].

Of particular interest for the development of a refolding strategy was the ability to produce large amounts of the proteins to reconstitute them in functional form into polymersomes, but also to achieve decent protein concentrations in the first step. The rationale of this study was to first screen different standard detergents for their ability to promote folding of the four proteins and to combine these with a range of well-known folding additives. To achieve an efficient automated production process we then transferred the best conditions to parallel miniaturized stirred-tank reactors that were operated in conjunction with a liquid handler to produce mg amounts of the proteins.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and purchased from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Sigma Aldrich (Schnelldorf, Germany) or Serva (Heidelberg, Germany). Enzymes for DNA manipulations were purchased from New England Biolabs (Frankfurt, Germany) and oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany). Genomic DNA was obtained from the DSMZ (Braunschweig, Germany).

2.2. Cloning

The genes for OmpW from *Escherichia coli* MG1655 (DSM 18039; NCBI RefSeq: NC_000913.2; region 1314020 to 1314658), OprG from *Pseudomonas aeruginosa* PAO1 (DSM 22644; NCBI RefSeq: NC_002516.2; region 4544607 to 4545305) and TodX from *Pseudomonas putida* F1 (DSM 6899; GenBank: U18304.1; region 2147 to 3508) were amplified from genomic DNA by PCR using Phusion HF Polymerase. Oligonucleotide sequences are listed in Table 1. The alkL gene from the *Pseudomonas putida* OCT plasmid (GenBank: AJ245436.1; region 14924 to 15616) was synthesized with a standard ATG start codon by Eurofins Genomics (Ebersberg, Germany). Furthermore, the signal peptide was omitted to promote inclusion body formation (AlkL: ΔAA 2–27, OmpW: ΔAA 2–20, OprG: ΔAA 2–27, TodX: ΔAA 2–21). All genes were integrated separately into the expression vector pET21a using the restriction sites NdeI and HindIII to obtain constructs with a C-terminal His_6. His_8-tagged variants were subsequently created using the original forward primer and the generic primer pET28a_His8_rev (XhoI) on the subcloned genes. Quick Ligase was used for the ligation of constructs, which were subsequently transformed into competent *E. coli* DH5α (Invitrogen, Carlsbad, CA). After sequencing by Eurofins Genomics, plasmids were transformed into *E. coli* C43 (DE3) (Lucigen, Middleton, WI) for protein expression.

2.3. Recombinant protein expression and purification

A 5 mL preculture of Terrific Broth (TB) supplemented with 50 mg L^{-1} ampicillin was inoculated with a single colony, incubated at 30 °C over night and then subcultured into 5 × 200 mL TB in 1000 mL shake flasks without baffles. For the expression of TodX, the medium was also supplemented with 1 g L^{-1} glucose and 1 mM $MgSO_4$. Cells were grown at 37 °C and 250 rpm until a cell density (OD_{600}) of 0.6–0.8 was reached. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were harvested by centrifugation after another 4 h of expression. After a freeze-thaw cycle, cells were resuspended in 5 mL lysis buffer per g wet cells (50 mM Tris/HCl pH7, 50 mM NaCl, 5% glycerol, 0.25 g L^{-1} lysozyme, 1 U mL^{-1} DNAse I, 1 mM PMSF), incubated at 30 °C for 30 min and sonicated for 10 min (Bandelin Sonoplus, Berlin, Germany). After the addition of 1/3 volume of 4x membrane extraction buffer (6% Triton X-100, 1.5 M NaCl, 60 mM EDTA) and incubation for 1 h on ice, the lysate was centrifuged at 25,400g for 30 min at 4 °C. The resulting pellet was resuspended in washing buffer I (50 mM Tris/HCl pH 7.5, 50 mM NaCl, 1% Triton X-100, 1 M guanidine HCl) using an Ultra-Turrax (Ika, Staufen, Germany) and centrifuged again. This step was repeated twice with washing buffer II (50 mM Tris/HCl pH 7.5, 50 mM NaCl). The final pellet was dissolved in 18 mL solubilization buffer (50 mM Tris/HCl pH 8, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 6 M guanidine HCl) at 4 °C over night, centrifuged as before and applied to two serial 5 mL HisTrap FF crude columns (GE Healthcare, Uppsala,