Biochimica et Biophysica Acta 1808 (2011) 2206–2213

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Folding studies of purified LamB protein, the maltoporin from the *Escherichia coli* outer membrane: Trimer dissociation can be separated from unfolding

Valerie Baldwin, Mandeep Bhatia, Mary Luckey *

Department of Chemistry and Biochemistry, San Francisco State University, 1600 Holloway Ave., San Francisco, CA 94132, USA

ARTICLE INFO

Article history:
Received 1 February 2011
Received in revised form 29 April 2011
Accepted 16 May 2011
Available online 24 May 2011

Keywords:
Folding β-barrel protein
Outer membrane
LamB protein
Maltoporin
Disulfide bond
Oligomerization

ABSTRACT

The folding mechanisms for β-barrel membrane proteins present unique challenges because acquisition of both secondary and tertiary structure is coupled with insertion into the bilayer. For the porins in *Escherichia coli* outer membrane, the assembly pathway also includes association into homotrimers. We study the folding pathway for purified LamB protein in detergent and observe extreme hysteresis in unfolding and refolding, as indicated by the shift in intrinsic fluorescence. The strong hysteresis is not seen in unfolding and refolding a mutant LamB protein lacking the disulfide bond, as it unfolds at much lower denaturant concentrations than wild type LamB protein. The disulfide bond is proposed to stabilize the structure of LamB protein by clasping together the two sides of Loop 1 as it lines the inner cavity of the barrel. In addition we find that low pH promotes dissociation of the LamB trimer to folded monomers, which run at about one third the size of the native trimer during SDS PAGE and are much more resistant to trypsin than the unfolded protein. We postulate the loss at low pH of two salt bridges between Loop 2 of the neighboring subunit and the inner wall of the monomer barrel destabilizes the quaternary structure.

1. Introduction

The outer membranes of Gram negative bacteria provide important barriers that protect cells from harmful agents while allowing uptake of nutrients by transport through β-barrel proteins [1]. In addition to transporters, outer membrane β-barrel proteins function in adhesion and virulence and act as enzymes. In Gram negative bacteria the known β-barrel proteins are found exclusively in the outer membranes, yet β-barrels are predicted to make up 2–3% of the Gram negative proteome [2]. They are also found in *Mycobacteria* and in the mitochondria and chloroplasts of eukaryotes [1].

Porins are a class of β-barrel proteins that provide solute diffusion channels of varying selectivity. They are homotrimers, with a channel in each monomer. The most abundant porins in *Escherichia coli*, the OmpF and OmpC porins, are called general porins: they allow passage of hydrophilic solutes up to a size limit that is due to constrictions of their channels evident in the crystal structures [3,4]. The LamB protein is a well-characterized example of a specific porin: it is called maltoporin because it is required for growth on limiting concentrations of maltose and it facilitates passage of larger maltodextrins [5,6]. Enhanced uptake of starch breakdown products through the LamB channel is likely to give the enteric bacteria a competitive advantage. A large assortment of *lamB* mutants has been used to probe the roles of different parts of the protein [7–9]. Once the X-ray structure provided details of its β-barrel topology and the residues lining the "greasy slide" that confer specificity to the pore [10–12], genetic analysis of the architecture of LamB protein could be targeted to determining structure–function relationships of various loops and pore residues [13,14].

Biogenesis of outer membrane porins is complicated by their location and their structure. Because of their destined location in the outer membrane, newly synthesized porins must cross the cytoplasmic membrane and periplasmic space, thus their biogenesis involves several stages [15]. Recognition of their signal sequences as they emerge from the ribosome targets the nascent peptides for export across the cytoplasmic membrane via a complex called the SecYEG/SecA translocon in *E. coli* [16]. Upon entry to the periplasm, nascent porins interact with chaperones such as Skp and DegP, which maintain their unfolded state [17], and SurA, which may begin their folding process [18,19]. Incorporation into the outer membrane utilizes a second transport complex called Bam (for β-barrel assembly machinery) that consists of a major pore in the outer membrane formed by the β-barrel BamA (or YaeT, a homolog of Omp85) and several accessory lipoproteins [20]. Just how the Bam complex facilitates outer membrane insertion is an active field of research [21].

Biogenesis of porins is also complicated by their structure: unlike α-helical membrane proteins, the β-barrel cannot assemble from previously inserted transmembrane segments because the cost of insertion into the hydrophobic core of the bilayer of individual β-strands

Abbreviations: octylPOE, n-octyl (polydisperse)oligo-oxyethylene; SDS, sodium dodecyl sulfate; DM, dodecylmaltoside; GdmCl, guanidinium chloride; DTT, dithiothreitol; Hepes, N-2-Hydroxypiperazine-N-ethanesulfonic acid; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; DMPC, dimyristoylphosphatidylcholine

* Corresponding author. Tel.: +1 415 338 1288; fax: +1 415 338 2384.
E-mail address: luckey@sfsu.edu (M. Luckey).

0005-2736/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2011.05.013

lacking hydrogen bonds is prohibitive [22]. Rather, membrane insertion must be coupled with acquisition of both secondary and tertiary structure to produce the contiguous membrane-spanning barrel held by interstrand hydrogen bonds. Furthermore, the porins must additionally attain their quaternary structure, presumably by associations formed in the membrane. Therefore the β-barrel membrane proteins present interesting challenges to the protein folding problem. Fortunately, folding and assembly of these β-barrel proteins can be probed in vitro because they are capable of assembling directly into lipid bilayers with little or no assistance.

The most extensive folding studies of outer membrane β-barrel proteins have examined the OmpA protein, in particular the barrel domain of OmpA consisting of residues 172–325 of the peptide chain [reviewed in 23,24]. Unfolded OmpA barrel domain that has been purified in urea or SDS folds when diluted into a variety of detergents or phospholipids. The native state of refolded OmpA has been demonstrated with various spectroscopic methods as well as conductance measurements and phage binding. In early studies, acquisition of tertiary structure inferred from faster mobility on SDS PAGE was found to be synchronous with formation of secondary structure observed by CD. Kinetic assays based on the shift in electrophoretic mobility showed that OmpA folding follows a single-step second-order rate law (pseudo-first order at high lipid concentrations) and depends on the nature of the amphiphile (head group and chain length of phospholipid) as well as the size of the lipid vesicles. The use of depth-dependent fluorescence quenchers as well as single-Tryptophan mutants allowed characterization of intermediates along the folding pathway as four Trp residues move across the bilayer and the fifth stays near the insertion site. Recently, intramolecular quenching of the individual Trp residues by nitroxides on engineered Cys residues determined when pairs of β-strands converged and demonstrated that they form the OmpA barrel concurrent with insertion into the membrane [25].

How much more complex is the assembly pathway of an oligomeric β-barrel, such as the OmpF and LamB porins of *E. coli*? In an early study, OmpF purified in a nonionic detergent, n-octyl (polydisperse)oligo-oxyethylene (octylPOE), was denatured by heating in 6 M guanidinium chloride (GdmCl) and resolubilized in SDS [26]. Unfolded OmpF lost the characteristic minimum at 217 nm in its CD spectrum that typifies β structure, appeared as a monomer on SDS PAGE, and was digested by trypsin. Once refolded by either dialysis or dilution into octylPOE-lecithin mixed micelles in the presence of SDS, from 40 to 80% appeared as trimer on SDS PAGE, the trimer band was resistant to trypsin, and the reconstituted refolded OmpF gave conductance in lipid bilayers characteristic of native OmpF pores.

For a direct comparison of the in vitro assembly of OmpA and OmpF, OmpF was purified using chaotropic agents (both GdmCl and urea) and alcohol precipitation with no detergent [27]. This procedure produced protein in 8 M urea that was unfolded according to its CD and fluorescence spectra. Refolding by dilution to 20 mM urea produced a very low yield of OmpF trimer with addition of DMPC alone, but with a 1:1 mixture of DMPC:dodecylmaltoside (DM). Over 70% of the OmpF was trimers and another 25% dimers. Both trimers and dimers were resistant to trypsin, and the refolded OmpF had characteristic CD and fluorescence spectra as well as conductance properties of native OmpF. The kinetics of assembly from aqueous solutions of OmpA or OmpF in urea exhibited a striking difference. The appearance of trypsin-resistant OmpF trimer was much slower (15% after 3 h in DMPC and 73% after 5 h in DMPC + DM) than appearance of folded OmpA (70% after three hours in DMPC). The slower assembly of OmpF was attributed to the oligomerization step.

Using the ability to refold β-barrel membrane proteins from aqueous solutions, a recent study expanded this approach to compare the folding efficiencies of nine outer membrane proteins that were overexpressed and solubilized in urea [28]. The "heat modifiability" of these proteins produces different bands of folded and unfolded proteins on SDS PAGE and thus is used to quantitate their folding. This allowed determination of the effects of many different bilayer compositions on the folding process. Although the outer membrane proteins tested vary in their ability to fold in any single bilayer environment, they generally folded better in thinner bilayers, in bilayers with increased curvatures, and at higher pH values. Interestingly, only four of the nine outer membrane proteins could fold into small vesicles prepared with *E. coli* lipid extracts. The one trimeric porin included, OmpF, folded only with phosphatidyl choline with unnaturally short acyl chains. OmpF also does not conform to the "heat modifiability" that is the basis of the SDS-PAGE assay for folding. Rather than two bands corresponding to folded and unfolded species, OmpF exhibits three bands on SDS PAGE: folded trimers, unfolded monomers, and presumed dimers.

The folding and assembly of porins is a significant aspect of outer membrane biogenesis, given their critical role in nutrient transport. Folding studies can also reveal new information about the effects of mutations on porin structure. While numerous studies have been reported on other outer membrane proteins, there has been no previous information about the in vitro folding of LamB protein. Such investigations can take advantage of the wealth of *lamB* mutants to ultimately probe many aspects of its assembly. In the approach we have taken, intrinsic fluorescence of purified LamB protein in detergent is used to monitor unfolding and refolding of both the wild type protein and a mutant protein lacking the disulfide bond. We have also tested the folding state of a compact monomer observed at low pH on SDS PAGE as a means to assay trimer dissociation separate from denaturation.

2. Materials and methods

2.1. Materials

N-octyl (polydisperse)oligo-oxyethylene (octylPOE) was purchased from Alexis and guanidium chloride (8 M solution) purchased from Pierce. Highly-purified SDS was purchased from Gallard-Schlesinger. Trypsin from bovine pancreas and soybean trypsin inhibitor were obtained from Calbiochem. Low molecular weight protein standards, including prestained standards, were purchased from BioRad. Phosphorylase A was purchased from Boehringer Mannheim, catalase, fumarase and glycerolaldehydephosphatase from Boehringer, and cytochrome C from Fluka. Carboanhydrase B, dioleophosphatidyl choline and egg phosphatidyl choline were purchased from Sigma. All chemicals were reagent grade.

2.2. Protein purification

LamB protein was purified from octylPOE extracts of whole envelopes of *E. coli* strain MCR106 harboring high expression plasmids for wild type or for the C22S mutant [29] using starch affinity chromatography as described by Keller, et al. [30]. Purified protein in 20 mM Hepes (N-2-Hydroxypiperazine-N-ethanesulfonic acid), 0.5% octylPOE and 0.02% NaN_3 was stored in glass vials at 4 °C. Protein concentration was determined by the Lowry assay [31] with 0.1% SDS added to samples containing octylPOE [32].

2.3. Gel electrophoresis

SDS PAGE was carried out on an 11% resolving gel as previously described [29] with BioRad low molecular weight standards, except where indicated on a 10% resolving gel with a noncommercial mixture of standards composed of phosphorylase A (95 kDa), catalase (57.5 kDa), fumarase (49 kDa), glycerolaldehydephosphatase (36 kDa), carboanhydrase B (29 kDa), and cytochrome C (11.7 kDa). Every gel included additional standards containing 2 μg samples of LamB protein with and without heat treatment (100 °C for 10 min).