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Oligo-(*R*)-3-hydroxybutyrate modification of sorting signal enables pore formation by *Escherichia coli* OmpA

A. Negoda, E. Negoda, R.N. Reusch *

Department of Microbiol. and Mol. Gen., Michigan State University, East Lansing, MI 48824, USA

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ABSTRACT

The outer membrane protein A (OmpA) of *Escherichia coli* is a well-known model for protein targeting and protein folding. Wild-type OmpA, isolated either from cytoplasmic inclusion bodies or from outer membranes, forms narrow pores of ~80 pS in planar lipid bilayers at room temperature. The pores are well structured with narrow conductance range when OmpA is isolated using lithium dodecyl sulfate (LDS) or RapiGest surfactant but display irregular conductance when OmpA is isolated with urea or guanidine hydrochloride. Previous studies have shown that serine residues S163 and S167 of the sorting signal of OmpA (residues 163–169), i.e., the essential sequence for outer membrane incorporation, are covalently modified by oligomers of (*R*)-3-hydroxybutyrate (cOHB). Here we find that single-mutants S163 and S167 of OmpA, which still contain cOHB on one serine of the sorting signal, form narrow pores in planar lipid bilayers at room temperature with lower and more irregular conductance than wild-type OmpA, whereas double mutants S163:S167 and S163:V166 of OmpA, with no cOHB on the sorting signal, are unable to form stable pores in planar lipid bilayers. Our results indicate that modification of serines in the sorting signal of OmpA by cOHB in the cytoplasm enables OmpA to incorporate into lipid bilayers at room temperature as a narrow pore. They further suggest that cOHB modification may be an important factor in protein targeting and protein folding.

1. Introduction

Outer membrane protein A (OmpA) is a major outer membrane protein of *Escherichia coli*. The process by which OmpA and other outer membrane proteins are directed to and incorporated into the outer membrane is known as outer membrane sorting. OmpA precursors are synthesized in the cytoplasm with a 21-residue signal sequence at the amino terminal. They are guided by the Sec translocon into and through the cytoplasmic membrane into the periplasm in a largely unfolded state. The signal sequence is cleaved and the proteins, assisted by periplasmic chaperones and lipopolysaccharides, are folded and inserted into the outer membrane [1–4].

The mechanism by which periplasmic OmpA inserts into the outer membrane is not well understood. A segment of the 325 residue outer membrane protein OmpA, known as the sorting signal, has been shown to be critical to outer membrane incorporation. Klose et al. [5] used immunoelectron microscopy to examine a series of overlapping deletions of OmpA and defined a region between residues 154 and 180 as essential for outer membrane association; fragments missing this region were located in the periplasm. The critical region for outer membrane assembly was narrowed by Freudl et al. [6] to the eighth β-strand, residues 160–170. Klose et al. [7] found that the double mutant G160V;L162R was not defective in membrane assembly. Accordingly, these studies indicate that the putative sorting signal is contained within residues 163–170 (SLGVSYRF) of the mature protein.

Xian et al. [8] showed by Western blot immunoassay and chemical assay that the serine residues on fragment 162–174 of OmpA (LSLGVSYRFGQGE) are covalently modified by oligo-*R*-3-hydroxybutyrates (cOHB). The presence of cOHB on fragment 162–174 of wild-type OmpA and the absence of cOHB on the same fragment of double mutant S163G:S167G were confirmed by MALDI-MS. The sorting signal of OmpA is modified by cOHB in protein isolated either from inclusion bodies or from outer membranes which suggests that this modification occurs in the cytoplasm.

cOHB-modified proteins are found in prokaryotes and also in eukaryotes [9–11]. The physical properties of these oligomers—highly flexible, amphiphilic molecules that are insoluble in water but soluble in lipids [12–14]—suggest that they may act as chaperones to facilitate the incorporation of hydrophilic polypeptides into bilayers. Here we examine the effect of cOHB modification of the sorting signal on bilayer incorporation and pore formation by OmpA.

2. Materials and methods

2.1. Purification of OmpA from outer membranes

Whole OmpA (325 residues) was extracted from the outer membranes of JM109 by a modification of the method of Sugawara

* Corresponding author. Tel.: +1 517 884 5388; fax: +1 517 349 8957.
E-mail address: rnreusch@msu.edu (R.N. Reusch).

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and Nikaido [15,16]. Briefly, stationary-phase cells were collected by centrifugation and frozen at −20 °C overnight. The pellet was then suspended in 20 mM tris(hydroxymethyl) aminomethane (Tris)–HCl, pH 7.8, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.5 mg/ml lysozyme, and disintegrated by ultrasonication (Branson). Mg^{2+} (10 mM) and DNase (0.1 mg/ml) were added, and unbroken cells were removed by centrifugation at 5000 rpm for 20 min at 4 °C (Sorvall GSA rotor). Crude outer membrane fractions were recovered by centrifugation at 12,000 rpm for 40 min at 4 °C (Sorvall SS-34 rotor). Outer membranes were suspended in 0.3% lithium dodecyl sulfate (LDS), 5 mM EDTA in 20 mM HEPES, adjusted to pH 7.5 with KOH, to a final protein concentration of 2 mg/ml. After 1 hour with mild mixing at 4 °C, the suspension was centrifuged at 35,000 rpm (Beckman Type 50.2 TI rotor) for 45 min. The pellet was resuspended in 0.5% LDS in the same solvent, gently mixed for 1 hour at 4 °C, and centrifuged in the same manner. The pellet from the second extraction was resuspended in 2% LDS, 20 mM HEPES, pH 7.5, and gently mixed at 4 °C for > 1 hour. The pellet from this extraction was discarded and the supernatant containing soluble OmpA was loaded onto a column of Sephacryl S-300 (1.6×60 cm, HiPrep, Pharmacia), which had been equilibrated with 0.05% LDS, 0.4 M LiCl, 20 mM HEPES, pH 7.5. Fractions were eluted with the same solvent and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). OmpA-rich fractions were combined and concentrated using Centricon-10 (Amicon). Further purification when necessary was performed on a column of Superdex 75 (10/300) using the same solvent.

Alternatively, OmpA was extracted using 8 M urea as described by Hong et al. [17] or with 6 M guanidine hydrochloride (GuHCl) using the same procedure and then purified by Sephacryl S-300 chromatography as above.

2.2. Expression and purification of OmpA from inclusion bodies

His-OmpA was overexpressed in *E. coli* BL21(DE3)pLysS cells (Novagen) containing the pET-45b(+)-His-OmpA plasmid, and was grown in LB medium supplemented with 100 μg/ml ampicillin at 37 °C with aeration to an OD_{600} of ~0.6. Protein expression was induced by the addition of 0.5 mM isopropyl-1-thio-3-D-galactopyranoside (IPTG), and the cells were cultured at 37 °C for 2 hours before harvesting by centrifugation. Cells were disintegrated by ultrasonication as above and inclusion bodies were collected by centrifugation at 15,000 rpm for 30 min (Sorvall SS-34 rotor). His-OmpA was extracted from inclusion bodies with 0.1% RapiGest (Waters), pH 7.4, or alternatively with 8 M urea, pH 7.4 or with 6 M GuHCl, pH 7.4, and bound to Ni-agarose beads in the presence of 20 mM imidazole, pH 7.8. The beads were washed with 25 mM immidazole, pH 7.8, 1 mM dodecylmaltoside (DDM) and His-OmpA was eluted with 300 mM immidazole, pH 6.8, 1 mM DDM. His-OmpA was also extracted from inclusion bodies obtained by overexpression of the pET-45b(+)-His-OmpA plasmid in *E. coli* UH 203 OmpA-minus cells using the procedure described above for outer membranes, i.e., isolated with LDS and purified by Sephacryl S-300 chromatography.

2.3. Reconstitution of M-OmpA and I-OmpA

M-OmpA and I-OmpA preparations were concentrated to ~1 mg/ml by centrifugal filtration using 10K Centricon filters. Buffer substitution was then performed 5× with 20 mM *n*-octyl tetraethylene glycol monoether (C_8E_4) in 20 mM KHEPES, pH 7.4 using the same filters. The concentrate was then diluted with the C_8E_4 solution to 0.1 mg/ml. This solution (1 μl) was added to the *cis* side of a planar bilayer formed with synthetic diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar lipids).

2.4. Western blot assays for cOHB

In the Western blot assay for cOHB, 5 μg of OmpA was added to Laemmli loading buffer.

The sample was heated in a boiling water bath for 5 min, cooled, loaded on a 12% SDS–PAGE, and proteins were separated by electrophoresis. The gel was transferred to a supported nitrocellulose or PVDF membrane (Bio-Rad) in Tris glycine SDS buffer (Bio-Rad), 20% methanol, using a Mini Trans-Blot electrophoretic cell (Bio-Rad) or Gene Blotter (Idea Scientific Co.). The membrane was blocked with 1.25% gelatin (electrophoresis grade; Bio-Rad) in Tris-buffered saline, pH 7.5, 0.1% Tween-20. Primary incubation was with polyclonal anti-OHB IgG in blocking buffer produced in rabbits to a synthetic 8mer of OHB (courtesy of D. Seebach, ETH Zürich) conjugated to gelatin (Metabolix Inc.) and purified by protein A chromatography. The second antibody was goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) in the same buffer. Color development was performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Bio-Rad).

2.5. Planar lipid bilayer studies

Planar lipid bilayers were formed from a solution of DPhPC in *n*-decane (Aldrich) at a concentration of ~17 mg/ml. The solution was used to paint a bilayer in an aperture of ~150 μm diameter between aqueous solutions of 1 M KCl in 20 mM HEPES, pH 7.4 in a Delrin cup (Warner Instruments). All salts were ultrapure (Aldrich). After the bilayer was formed, a solution of OmpA in C_8E_4 (1 μl of 0.1 mg/ml) was added to the *cis* compartment.

Unitary currents were recorded with an integrating patch clamp amplifier (Axopatch 200A). The *trans* solution (voltage command side) was connected to a CV 201A head stage input and the *cis* solution was held at virtual ground via a pair of matched Ag–AgCl electrodes. Currents through the voltage-clamped bilayers were low-pass filtered at 10 kHz and recorded after digitization through an analog to digital converter (Digidata 1322A, Axon Instr.). Data were filtered through an 8 pole Bessel filter (9021 PF, Frequency Devices) and digitized at 1 kHz using pClamp 9.0 software (Axon Instr.). Single-channel conductance events were identified and analyzed by using Clampfit9 software (Axon Instr.). The data were averaged from > 10 independent recordings.

3. Results

3.1. Planar bilayer observations of wild-type OmpA isolated from outer membranes and from cytoplasmic inclusion bodies

Whole OmpA (325 residues) was extracted from outer membranes (M-OmpA) and His-tagged whole OmpA was extracted from inclusion bodies (I-OmpA) using several different solubilizing agents. M-OmpA was isolated using either lithium dodecyl sulfate (LDS) or urea or GuHCl and purified by size-exclusion chromatography using LDS (see Methods). I-OmpA was extracted using either RapiGest surfactant or urea or GuHCl and purified by Ni-agarose chromatography using DDM (see Methods). I-OmpA was also extracted with LDS but this preparation did not bind to Ni-agarose beads; consequently, I-OmpA extracted with LDS was purified using size-exclusion chromatography in the same manner as M-OmpA. The purified proteins all displayed the same electrophoretic migration on SDS–PAGE (Fig. 1, lanes 1–4). Unheated proteins migrated at ~30 kDa and proteins heated in 2% SDS before loading migrated at ~35 kDa.

The purified M-OmpA and I-OmpA proteins were reconstituted into C_8E_4 micelles, the solubilizing agent was removed by repeated filtration, and the proteins were then each incorporated into planar lipid bilayers of DPhPC at room temperature (see Methods). The