Non-lamellar Structure and Negative Charges of Lipopolysaccharides Required for Efficient Folding of Outer Membrane Protein PhoE of *Escherichia coli*

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Lipopolysaccharides (LPS) are amphiphilic molecules in the outer leaflet of the bacterial outer membrane. Recently, an early role for LPS in the folding of outer membrane porin PhoE was demonstrated in vitro. In order to elucidate the molecular mechanism of LPS-protein interactions, folding of PhoE protein was studied with a large set of well characterized LPS chemotypes. We demonstrate that negative charges in the inner core region contribute to the high efficiency of folding of PhoE protein. In addition, the supramolecular structure of the LPS aggregate seems to be important. LPS with a lipid A part that prefers a lamellar or a direct micellar structure and a high state of order of its acyl chains is much less efficient to support folding as compared with LPS with lipid A that prefers a non-lamellar structure and a low acyl chain order. These in vitro data indicate that extensive interactions between the core and lipid A region of LPS with the protein are required to support protein folding. The LPS-PhoE binding might be promoted by the presence of hydroxy fatty acids in the lipid A moiety of LPS.

Bacterial outer membrane proteins are synthesized as precursor proteins in the cytoplasm. After their translocation across the inner membrane via the Sec machinery (1) and processing to mature protein, they are assembled into the outer membrane (OM).1 Two proteins, such as the PhoE protein, are assembled as a trimer in the OM. How membrane proteins fold and assemble into a specific membrane is largely unknown. Kinetic and equilibrium folding studies of mostly soluble, hydrophilic proteins have indicated the existence of at least three main stages in protein folding, the formation of secondary structure, the folding pattern, and detailed tertiary structure (2). In vivo, the folding process is mediated by molecular chaperones that are proteins that will act primarily by preventing misfolding and aggregation of partially folded and unassembled protein subunits (3). In contrast to soluble proteins, membrane proteins will expose much more hydrophobic surfaces, exposed to the lipid phase, whereas the hydrophilic areas will be buried during folding. It can therefore be anticipated that membrane protein folding and assembly in vivo will require a membrane environment and molecular chaperones at or in this membrane. Indeed, refolding of bacterial outer membrane proteins can be accomplished in the presence of detergent and phospholipids (4–7). However, the kinetics of refolding of these membrane proteins were slow and, in the case of the trimeric porin OmpF, the yield was also low. Interestingly, phosphatidylethanolamine has recently been demonstrated to act as a non-protein molecular chaperone in the assembly of a bacterial cytoplasmic membrane transporter (8). Thus, certain lipids might also be required as molecular chaperone in the folding and assembly of bacterial outer membrane proteins.

Various in vivo and in vitro studies have implicated an important role for LPS in the folding and assembly of bacterial outer membrane proteins (9–14). LPS are the major amphiphilic components in the outer leaflet of the bacterial OM (15, 16). After synthesis in the inner membrane, LPS are translocated in an as yet unknown manner to the OM. Inhibition of fatty acid synthesis with the antibiotic cerulenin interferes with the assembly of pore proteins into the outer membrane (12, 17–19). Thus, there appears to be a direct relationship between de novo synthesis of lipids, LPS and/or phospholipids, and correct folding and assembly of pores proteins in vivo. Recently, an early role for LPS in folding of PhoE protein was demonstrated in vitro (14). Both, LPS and divalent cations were shown to be involved in the formation of an early intermediate, i.e. a folded monomer of PhoE protein. The subsequent assembly of monomers into trimers requires an additional incubation with outer membranes and Triton X-100 (0.08% w/v). The kinetics of the folding of PhoE with LPS is much higher (20) as compared with the kinetics observed in refolding studies with OmpA and OmpF in the presence of phospholipids (5–7). These in vitro results implicate a special role for LPS in outer membrane protein folding.

In order to understand how LPS can support outer membrane protein folding, it is important to elucidate the molecular mechanism of LPS-protein interactions. Both hydrophilic and hydrophobic interactions, involving the negatively charged phosphates in the core and at the C-1 and C-4’ positions of the lipid A backbone and the fatty acids, respectively, might be directly involved in LPS-protein interactions. In addition, the state of order of the acyl chains (described by the order parameter $S_n$) and the three-dimensional supramolecular structures of LPS might directly influence the capacity to support protein folding (for an overview see Refs. 21 and 22). The relationship between the molecular shape of the lipid molecule and the structural polymorphism of its aggregates is well documented for phospholipids (23). The same principles also apply for LPS (21, 22). Thus, the supramolecular structure of LPS aggregates...
can either be lamellar (L, M) or non-lamellar (cubic, Q or H). Factors like degree of saturation of acyl chains, temperature, head group size and ionization (pH, divalent cation concentration), and water content can influence the type of aggregate structures (lamellar and various non-lamellar phases).

We have used a large set of different LPS molecules, of which the chemical and biophysical properties were previously characterized, in the developed in vitro folding system to investigate which properties of LPS are involved in folding of PhoE protein into its folded monomeric, native-like state.

**MATERIALS AND METHODS**

**In Vitro Translation and Folding of PhoE Protein—Isolation of S135 cell extract from Escherichia coli strain MC4100 (24) and the in vitro transcription and translation reactions were performed as described previously (25). Plasmid pPS70 (26) was used to direct the synthesis of the mature form of PhoE protein that was radioactively labeled due to the incorporation of \(^{[35}S\)methionine during protein synthesis. Folding of PhoE protein was initiated by addition of purified LPS and 0.015% Triton X-100 after inhibition of protein synthesis with puromycin and was essentially performed as described previously (14). In short, 20 μl of a mixture of LPS with Triton X-100 (0.0338%, w/v) in buffer L (50 mM triethanolamine acetate, pH 7.5, 250 mM NaCl, 1 mM dithiothreitol) was mixed with 25 μl of a translation mixture and incubated for 30 min at 37 °C. Samples were treated with trypsin (45 μg/ml) for 15 min at 37 °C, phenylmethylsulfonyl fluoride (1 mM) was added, and the samples were transferred to ice. Prior to electrophoresis, sample buffer containing 2% SDS was added to the protein samples which were divided into two equal portions. One portion was incubated for 10 min at room temperature and the other portion at 100 °C. SDS-polyacrylamide gels (27) were run at 20 mA in a temperature-controlled room at 4 °C to prevent denaturation of the various folded structures of the PhoE protein during electrophoresis. The folded monomer (Figs. 2–4, lanes a and designated m) often runs as a smear originating from a species with a molecular mass of approximately 31 kDa up to the position of the denatured PhoE form (m; 38 kDa). Gels were incubated with Amplify, dried, and exposed to film (Fuji) at −70 °C. Data were quantified with a PhosphorImager (Molecular Dynamics). The amount of trypsin-resistant PhoE is calculated from the amount of denatured PhoE (m) present in lanes designated c (Figs. 2–4) as percentage of the total amount of full-length PhoE present in 25 μl of translation mixture.

**LPS Preparation and Chemical Modifications—**Bacteria from enterobacterial strains as well as the non-enterobacterial species Paracoccus denitrificans and Rhodobacter capsulatus were cultured as described (28, 29). The non-enterobacterial strain Chromobacterium violaceum was obtained from the Institute for Fermentation, Osaka, Japan (IFO number 12614), and was cultivated in polypeptone growth media. LPS was extracted from phenol-killed bacteria, and rough mutant strains were extracted according to a modified PCP (PCP I, phenol/chloroform/petroleum ether) buffer (Figs. 2–3, lanes TL) and was used for determining the total amount of full-length PhoE present in 25 μl of translation mixture. SDS-polyacrylamide gels (27) were run at 20 mA in a temperature-controlled room at 4 °C to prevent denaturation of the various folded forms of the PhoE protein during electrophoresis. The folded monomer (Figs. 2–4, lanes a and designated m) often runs as a smear originating from a species with a molecular mass of approximately 31 kDa up to the position of the denatured PhoE form (m; 38 kDa). Gels were incubated with Amplify, dried, and exposed to film (Fuji) at −70 °C. Data were quantified with a PhosphorImager (Molecular Dynamics). The amount of trypsin-resistant PhoE is calculated from the amount of denatured PhoE (m) present in lanes designated c (Figs. 2–4) as percentage of the total amount of full-length PhoE present in 25 μl of translation mixture.

**RESULTS**

**Folding of PhoE Protein with LPS of Wild Type, Mutant Forms, and of Lipid A Variants—**Folding of PhoE with LPS of a deep rough mutant, lacking the complete core region up to the 3-deoxy-D-manno-octulopyranosonic acid residues, was previously shown to be much less efficient as compared with wild-type LPS (14). In order to identify which region of the core was important to support protein folding, we made use of a well-characterized series of chemotypes of LPS derived from *S. minnesota* (Table I). The primary chemical structures of wild type LPS and of the LPS mutants down to the deep rough mutant (LPS Re) are largely known (Fig. 1A; 32, 46, and references therein) except for the precise substitution with phosphate groups, in particular for lipopolysaccharides with longer sugar chains (>LPS Rc). In addition to differences in total amount of negative charges, the type of supramolecular structure of the LPS aggregates (Refs. 40 and 41; aggregate structure of the lipid A part) and the state of order of the acyl chains at 37 °C (Ref. 46; order parameter for S is given in Table I).

Folding of in vitro synthesized PhoE protein with LPS of chemotype S, Ra, Rb1, and Rd1P was more efficient as compared with the folding efficiencies obtained with LPS of chemotypes RcP, Rd1P, Rd2, and Re (Table I and Fig. 2). The efficiency of folding is very reproducible and varies usually between 10% of the obtained average folding efficiency (as indicated in Tables I and II; e.g. 60 ± 6%). The reduction of the folding efficiencies with this latter group appears to be mainly due to the absence of a negative charge in the inner core region. The negative charge of the phosphate group at the first heptopyranose, designated Hep I, appears to be most critical in this respect, as has been suggested previously (13). A further reduction in folding efficiency, as observed with LPSs of chemotype Re, should be due to a further reduction of the net negative charge present. The folding efficiencies were not due to changes in the supramolecular structure and changes in the state of order of the acyl chains since the lipid A moiety of all LPS chemotypes prefers a nonlamellar cubic phase (Q) and the order parameter for S decreased gradually, due to the decrease of Tc from 37 to 30 °C accompanying the reduction of the size of the core structure (21, 46).
Folding of in vitro synthesized PhoE protein with LPS

LPS was purified from the indicated strains. Folding was performed at 37 °C with 0.015% Triton X-100 and 138 nmol/ml LPS (Kdo content). No folding was observed in the absence of LPS. The total negative charge, preferred aggregate structure of the lipid A part of LPS, and the order parameter (S37) of the lipid A acyl chains are indicated. The amount of trypsin-resistant PhoE (TrypP) as % of the total amount of full-length PhoE synthesized is an average of two independent experiments. Q, inverted cubic structure. L, lamellar structure, ND, not determined. NO, not observed.

All LPS chemotypes used above were in their natural salt form. The folding efficiencies did not change significantly when the counterions in the LPS of chemotype Re were exchanged by dialysis for Na+ or Li+ (Table I and Fig. 3A). Interestingly, the folding efficiency of Re LPS in the Mg2+ salt form was significantly reduced, whereas those of the Ba2+ and Ca2+ forms had lost the capacity to support protein folding completely (Table I and Fig. 3A). This reduced capacity to support folding seems to be correlated with a change in the supramolecular structure from a cubic, i.e. non-lamellar, to a lamellar phase and an increase in S37 (41). Parallel to the change in aggregate structure that is different to the basic chemical structure of enterobacterial LPS forms had a further reduction of the amount of free negative charges by removal of the counterions in the LPS of chemotype Re were exchanged by dialysis for Na+ or Li+ (Table I and Fig. 3A). Interestingly, the positions of further non-stoichiometric substituents like phosphates are not indicated. For example, the Rd1 mutant of strain Rz has two phosphate groups that are absent in LPS of the R7 strain. B, chemical structure of LPS of an Rc mutant of E. coli O111 (strain J5). The sites where the chemical modification occurs for the preparation of lipid A (HAc), de-O-acylated LPS (hydrazin, Hy), or dephosphorylated LPS (HF) are indicated. The lipid A part containing phosphates (P) and fatty acids (14O12, 14O14, and 14OH) and the phosphates in the core region are schematically indicated. Kdo, 3-deoxy-d-manno-octulopyranosonic acid.

FIG. 1. Schematic chemical structures of LPS S. minnesota and E. coli. For detailed structural information see Refs. 32–34. A, rough mutant lipopolysaccharide (R from LPS) from S. minnesota and its derivatives (Ra to Re). The positions of further non-stoichiometric substituents like phosphates are not indicated. For example, the Rd1 mutant of strain Rz has two phosphate groups that are absent in LPS of the R7 strain. B, chemical structure of LPS of an Rc mutant of E. coli O111 (strain J5). The sites where the chemical modification occurs for the preparation of lipid A (HAc), de-O-acylated LPS (hydrazin, Hy), or dephosphorylated LPS (HF) are indicated. The lipid A part containing phosphates (P) and fatty acids (14O12, 14O14, and 14OH) and the phosphates in the core region are schematically indicated. Kdo, 3-deoxy-d-manno-octulopyranosonic acid.

Folding of PhoE Protein with Chemically Modified LPS—In order to study the influence of phosphate groups and fatty acids on the protein folding further, a variety of chemically modified LPS preparations was used. The E. coli J-5 strain contains a well characterized LPS of chemotype Re (Fig. 1B; see Refs. 33 and 34). The lipid A portion, obtained from the LPS after mild acetic acid hydrolysis and centrifugation, showed low efficiency (Table II and Fig. 4) most likely due to removal of the negative charges from the core region (phosphates and 3-deoxy-d-manno-octulopyranosonic acid) and increase in the order parameter. Interestingly, removal of O-linked acyl chains with hydrazine drastically decreased the efficiency of folding (Table II and Fig. 4). This seems to be a direct consequence of the change in the aggregate structure from non-lamellar to direct micellar. A reduction of negative charges by removal of the phosphate groups from the de-O-acylated J-5 LPS additionally decreased the folding efficiency (Table II and Fig. 4). However, the de-O-acylated and de-phosphorylated J-5 LPS is still more efficient as compared with the lipid A of this LPS. These results suggest that the presence of negatively charged 3-deoxy-d-manno-octulopyranosonic acid residues are important for LPS-
protein interactions as well. Alternatively or in addition, the increased state of order in the acyl chains of this lipid A could directly affect LPS-protein interactions. Removal of all fatty acids from LPS of J-5 resulted in complete inactivation of the molecule with regard to protein folding (Fig. 4).

Similar results were obtained with rough mutant and chemically modified LPS derived from \textit{K. pneumoniae} rough strain R20 (Table II). Interestingly, the free lipid A of this LPS does not form a pure cubic phase as found for most enterobacterial strains, but x-ray diffraction patterns indicate a superposition of diffraction maxima typical for cubic and lamellar structures (Q/L). The chemical structure of this LPS has recently been determined (35). Removal of O-acyl chains results in a decreased efficiency of folding due to a transition of the aggregate state from non-lamellar to lamellar. The removal of the two phosphates at the lipid A part of de-O-acylated LPS of R20 or the complete removal of the core region (forming pure lipid A from) does not further affect the capacity to support folding.

Interestingly, this is the only LPS type known so far that lacks phosphate residues in the core region. Instead, the negative charges in the R20 core are derived from galacturonic acid residues. Apparently, if negative charges in the core region are important for LPS-protein interactions, they do not need to originate from phosphate groups.

\textbf{Folding of PhoE Protein with a Glycosphingolipid and Phospholipids—}

It is well established that Gram-negative bacteria contain LPS in the outer leaflet of the OM, and, at least, the presence of a deep rough LPS molecule appears to be essential in \textit{E. coli} since conditional lethal mutations have been obtained in genes encoding proteins that are involved in the early steps in lipid A biosynthesis (49). Interestingly, some exceptions of this rule have been described. The Gram-negative bacterium

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Strain and (LPS chemotype) & Tryp$^a$ & Negative charge & Aggregate structure & Order parameter $S_{22}$ ($\times 10^{-3}$) \\
\hline
\textit{E. coli} & & & & & & & & & \\
J-5 (Re) & 76 & $\geq 5$ & Q & 0.51 & & & & & \\
De-O-acyl LPS from J-5 & 35 & $\geq 5$ & M$^*$ & 0.37 & & & & & \\
De-O-acyl and dephospho & 26 & 3 & M$^*$ & 0.07 & & & & & \\
LPS from J-5 & & & & & & & & & \\
Lipid A from LPS J-5 & 11 & 2 & Q & 0.83 & & & & & \\
\hline
\textit{K. pneumoniae} & & & & & & & & & \\
R20 (R form) & 86 & 7 & Q$^*$ & 0.38 & & & & & \\
De-O-acyl LPS from R20 & 12 & 7 & M$^*$ & 0.23 & & & & & \\
De-O-acyl and dephospho & 12 & 5 & M$^*$ & 0.27 & & & & & \\
LPS from R20 & & & & & & & & & \\
Lipid A from LPS R20 & 8 & 2 & Q/L & 0.80 & & & & & \\
\hline
\end{tabular}
\caption{Folding of \textit{in vitro} synthesized PhoE protein with chemically modified LPS}
\end{table}

* Prediction according to data from Ref. 42.
Sphingomonas paucimobilis contains a glycosphingolipid in the outer leaflet (50). Furthermore, an lpxA mutant of Neisseria meningitidis has been described recently which completely lacks LPS due to an early block in lipid A biosynthesis (51), whereas it still contains outer membrane proteins. We therefore investigated whether folding of the E. coli outer membrane protein PhoE can proceed in an LPS-independent manner. Folding of PhoE with GSL-1 was relatively inefficient (10% trypsin-resistant PhoE with 427 µg/ml GSL-1). This could either be due to its different chemical structure compared with that of LPS or could be correlated with the preferred lamellar state of GSL-1. Furthermore, folding of PhoE into a monomer is hardly achieved with purified phospholipids from E. coli (2% trypsin-resistant PhoE with 62 nmol/ml of phospholipid), whereas similar amounts of LPS would support the folding of nearly all synthesized PhoE protein. Interestingly, a synthetic non-E. coli phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) could support folding of PhoE albeit inefficiently (11% trypsin-resistant PhoE with 62 nmol/ml DOPC). Possibly, certain phospholipid species might take over the role of LPS partially and promote outer membrane protein folding, e.g. in the Neisseria lpxA mutant. This process might be less efficient as in the wild type Neisseria strain which contains LPS, and the observed reduced growth rate of the lpxA mutant strain might be a consequence of this.

DISCUSSION

Lipids play many different roles in cells. Clearly, their organization in membranes provides the correct environment for many different enzymes and membrane-associated processes to occur (52). However, it is only recently that lipids have been recognized as important components that can be involved in the process of folding and assembly of membrane proteins. In this study we describe for the first time which properties of LPS are required in order to support LPS-dependent folding of PhoE protein into its folded monomeric state. We demonstrate that specific negative charges in the inner core region of LPS and a non-lamellar structure of the lipid contribute to the efficiency of LPS-dependent protein folding. The results explain the long-standing observation that deep rough mutants of E. coli and Salmonella contain reduced amounts of outer membrane proteins (9–11). We propose that important LPS-protein interactions are mediated by salt bridges formed between divalent cations and the negative charges in inner core and certain negative charges in the pore protein. These negative charges are likely to participate in the strong and tight network of divalent cation and carboxylate groups within the layer of LPS-core units in such a way that the interface between these core units and protein would become as tight as the LPS layer itself (53). Furthermore, it may be expected that besides the negative charges the hydroxy fatty acids of the lipid A moiety also are of importance for the similar PhoE binding as has been proposed recently for the binding of LPS to the OmpF protein (54).

Evidence was obtained that the three-dimensional supramolecular structure of LPS is important in order to support the folding of PhoE protein in vitro. LPS with lipid A that prefers the lamellar or even the direct micellar state was much less capable to support folding as compared with LPS with lipid A that preferred a non-lamellar inverted state. LPS in the lamellar state could be less available or not available for LPS-protein interactions and/or the specific molecular shape of the LPS molecule is not compatible for LPS-protein interaction. Thus, extensive hydrophilic and hydrophobic interactions between LPS and protein, involving the negative charges in the inner core and lipid A, respectively, seem to be required for efficient protein folding. The lipid A moiety of LPS in the outer leaflet of the outer membrane is likely to be in a lamellar state and, thus, might not be suitable for folding of the porin, although one could argue that some mechanism exists that allows the exposure of this residential LPS to the outer membrane protein during its biogenesis. Furthermore, LPS could adopt a different structure in contact with a porin than in a porin-free bilayer. Alternatively, de novo synthesized LPS is required for assembly of pore proteins in vivo as was previously suggested (12, 17–19). This de novo synthesized LPS might be preferentially in a monovalent cation form (or at least not in its full divalent cation form) since biosynthesis of LPS occurs at the site of the inner membrane that is facing the cytoplasm and that is known to contain especially free K+ as available cation to compensate for negative charges in the cytoplasm (55), whereas Mg2+ and Ca2+ concentrations are very low (56, 57). Thus, de novo synthesized LPS might be transported from its site of synthesis to the OM in a salt form (K+ salt) that is optimal to support protein folding. At later stages in the biogenesis, the LPS are most likely converted to (partial) Mg2+ and/or Ca2+ salt forms. New research into the molecular mechanism of LPS biogenesis is required to investigate its possible connection with porin biogenesis, and this research has to take into account the specific chemical and biophysical properties of LPS.

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