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Folding and Insertion of the Outer Membrane Protein OmpA Is Assisted by the Chaperone Skp and by Lipopolysaccharide*

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Paula V. Bulieris‡, Susanne Behrens§, Otto Holst¶, and Jörg H. Kleinschmidt‡‖

From the ‡Fachbereich Biologie, Fach M 694, Universität Konstanz, D-78457 Konstanz, Germany, the §Abteilung Molekulare Genetik und Präparative Molekularbiologie, Institut für Mikrobiologie und Genetik, Georg August Universität Göttingen, Grisebachstrasse 8, D-37077 Göttingen, Germany, and the ¶Strukturbiochemie, Forschungszentrum Borstel, Parkallee 22, D-23845 Borstel, Germany

We have studied the folding pathway of a β-barrel membrane protein using outer membrane protein A (OmpA) of *Escherichia coli* as an example. The deletion of the gene of periplasmic Skp impairs the assembly of outer membrane proteins of bacteria. We investigated how Skp facilitates the insertion and folding of completely unfolded OmpA into phospholipid membranes and which are the biochemical and biophysical requirements of a possible Skp-assisted folding pathway. In refolding experiments, Skp alone was not sufficient to facilitate membrane insertion and folding of OmpA. In addition, lipopolysaccharide (LPS) was required. OmpA remained unfolded when bound to Skp and LPS in solution. From this complex, OmpA folded spontaneously into lipid bilayers as determined by electrophoretic mobility measurements, fluorescence spectroscopy, and circular dichroism spectroscopy. The folding of OmpA into lipid bilayers was inhibited when one of the periplasmic components, either Skp or LPS, was absent. Membrane insertion and folding of OmpA was most efficient at specific molar ratios of OmpA, Skp, and LPS. Unfolded OmpA in complex with Skp and LPS folded faster into phospholipid bilayers than urea-unfolded OmpA. Together, these results describe a first assisted folding pathway of an integral membrane protein on the example of OmpA.

Studies on the mechanistic principles of insertion and folding of integral membrane proteins have been mostly performed *in vitro* with two model proteins, the α-helical bundle protein bacteriorhodopsin of *Halobium salinarium* (1–4) and the 8-stranded β-barrel outer membrane protein A (OmpA)[1] of *Escherichia coli* (5–12) (for recent reviews, see Refs. 1 and 13–15). In case of bacteriorhodopsin, the folding kinetics into lipid membranes were studied after mixing denatured bacteriorhodopsin in SDS micelles with preformed lipid bilayers (1). Surrey and Jähnig (11, 16) demonstrated the oriented folding and insertion of completely unfolded OmpA into dimyristoylphosphatidylcholine bilayers upon dilution of the denaturant urea in absence of detergent. It has been observed that OmpA folds relatively fast into micelles but with rather slow kinetics into phospholipid membranes (7, 11, 12). Previous studies have also shown that urea-unfolded OmpA inserts and folds into phospholipid bilayers by a highly concerted mechanism that takes place via at least three structurally distinct membrane-bound folding intermediates (8–10). The folding kinetics and the yields of folded OmpA depended strongly on the properties of the lipid bilayer (6, 11, 16), on temperature (8–10), and on pH (11). It was further demonstrated that secondary and tertiary structure formation in OmpA are synchronized and coupled to membrane insertion (6). However, in these previous studies, the OmpA folding kinetics were relatively slow, suggesting that folding *in vivo* might be facilitated by folding catalysts.

The folding of soluble proteins in the cytoplasm is assisted by molecular chaperones (for recent reviews, see Refs. 17–20). It is not known how molecular chaperones facilitate the folding process of membrane proteins. Periplasmic and OMPs of Gram-negative bacteria are translocated across the inner membrane in a mostly unfolded form by the SecA/E/Y/G export system (see Refs. 21, 22). In the periplasm, a signal peptidase cleaves off the signal sequence. It has been suggested that periplasmic proteins assist in the assembly of OMPs, because reduced concentrations of some OMPs in the outer membrane of *E. coli* were observed after deletion of the genes for the periplasmic proteins Skp or SurA (23–25). Skp binds to the NH_2-terminal part of OmpA and is required for the release of incompletely folded OmpA into the periplasm (26, 27). The conditions that OMPs require for subsequent folding and membrane insertion have not yet been described.

The *skp* gene maps at the 4-min region on the chromosome and is located upstream of genes that encode proteins involved in lipid A biosynthesis (28–30). For example, the gene *firA*, which codes for UDP-3-*O*-[3-hydroxymyristoyl]glucosamine-*N*-acyltransferase is located only four bases downstream of the stop codon of *skp* (31). Lipid A is an essential component of lipopolysaccharide (LPS) that is present in the periplasm after biogenesis (32) and the major component of the outer leaflet of the outer membrane. Pulse-labeling and biochemical reconstitution experiments suggested that LPS is required for efficient assembly of OMPs such as trimeric PhoE (33) and monomeric OmpA (34) into outer membranes. In these previous studies, refolding was performed with micelles of LPS and Triton X-100 instead of phospholipid bilayers. It is not clear whether the LPS concentration in the periplasm is above the CMC, but OMPs would not fold in the presence of monomers (7). Also, the prefolding of OMPs into LPS micelles and subsequent fusion of

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‖ To whom correspondence should be addressed. Tel.: 49-7531-88-3360; Fax: 49-7531-88-3183; E-mail: joerg.helmut.kleinschmidt@uni-konstanz.de.

[1] The abbreviations used are: OmpA, outer membrane protein A; OMPs, outer membrane proteins; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; Mcps, million counts per second, mdeg, millidegrees; LPS, lipopolysaccharide; Skp, 17-kDa protein; SUV, small unilamellar vesicle.

the micelles with the outer membrane can lead to two different orientations of OMPs in the lipid bilayer, but only one direction of OMPs is observed in cells. The long β-strand-connecting loops are facing the extracellular space.

In this study, we sought to explore possible assisted folding pathways of OmpA. We have addressed the following questions. 1) How do Skp and LPS (either separately or in combination) affect unfolded OmpA in solution? 2) Are there effects of Skp (or LPS) on the folding of OmpA into lipid bilayers? 3) At which stoichiometries and how strong does Skp (or LPS) bind to OmpA? 4) Does Skp (or LPS) lead to secondary structure formation of OmpA in solution?

EXPERIMENTAL PROCEDURES

Purification of Skp—The Skp protein was purified from the periplasmic fraction of *E. coli* CAG16037 (35) harboring the plasmids pSkp (pQE60 from Qiagen carrying the *skp* gene) and pPLT13 (mini-F carrying *lacIq*, see Ref. 36). Cells were grown in LB medium at 37 °C and were induced at an A_{600} of 0.3 with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3–4 h. The cells were harvested at an A_{600} of ~1.5, resuspended in sucrose buffer (100 mM Tris-HCl, pH 8.0, 20% sucrose) and equilibrated on ice for 10 min. After centrifugation, cells were resuspended in sucrose buffer with 10 mM EDTA and incubated with lysozyme (10 μg/ml) for 30 min on ice. Spheroplasts were sedimented at low speed after the addition of $MgSO_4$ (20 mM). The resulting periplasmic fraction was dialyzed overnight at 4 °C against buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl), run through a Poros® DEAE column and then loaded onto a Poros CM column. The DEAE column was removed, and the CM column was washed with 3 volumes of buffer A. Skp was eluted from the column by a NaCl gradient (100–750 mM NaCl in buffer A), dialyzed overnight at 4 °C against buffer A, and concentrated in Centriprep YM-3 concentrator units (Amicon). The Skp concentration was determined photometrically at 280 nm with a molar absorption coefficient of 1280 M^{-1} cm^{-1} (37) and using the method of Lowry *et al.* (38) with bovine serum albumin as a standard.

Purification of OmpA—OmpA was purified from *E. coli* as described previously (16). OmpA concentrations of stock solutions were determined using the method of Lowry *et al.* (38).

Purification of R-LPS—E. coli rough mutant F576 was cultivated as described previously (39), and its LPS (R2 core type, M ≈ 3900 g/mol) was isolated as reported previously (40).

Preparation of Lipid Bilayers—1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform and mixed to a molar ratio of 50% DOPC, 30% DOPE, and 20% DOPG. The lipid mixtures were first dried under a stream of nitrogen and then in the desiccator under high vacuum for at least 3 h. Lipids were then hydrated in 10 mM HEPES buffer, pH 7.0, with 2 mM EDTA and dispersed by vortexing. Small unilamellar vesicles (SUV) were prepared by sonicating the lipid dispersions with a Branson W450D ultrasonicator for 40 min with a 50% pulse cycle.

Kinetics of OmpA Insertion and Folding into Membranes Detected by Electrophoresis—When isolated from bacteria using the method by Surrey and Jähnig (16), OmpA is completely unfolded in 8 M urea. The folding of OmpA was initiated by rapidly mixing 5 μl of denatured OmpA first with 46 μl of HEPES buffer (10 mM, pH 7.0, with 2 mM EDTA) containing Skp followed by the immediate addition of 10 μl of LPS and then by the addition of 35 μl of preformed lipid bilayers. The final concentrations were 7.1 μM OmpA, 28.4 μM Skp, 1.4 mM lipid, and from 7.1 to 280 μM LPS. When experiments were performed in the absence of Skp, LPS, or lipid vesicles, the corresponding solutions were replaced by the same volume of buffer. Unless indicated otherwise, all of the mixing steps were performed in this sequence very rapidly.

Samples of the reaction mixture were taken at different times after the initiation of folding, and an equal volume of 0.125 M Tris buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol was added. SDS binds to both folded and unfolded OmpA and inhibits further folding (10, 11). SDS-PAGE was performed as described previously (41, 42) but without heat denaturation of the samples. The different electrophoretic mobilities of folded (apparent molecular mass = 30 kDa) and unfolded OmpA (apparent molecular mass = 35 kDa) were used to determine the fraction of folded OmpA by densitometry as described previously (10). Time courses of folding were monitored over 180 min. Experimental errors were estimated from three to six experiments.

Folding Monitored by CD Spectroscopy—5.3 μl of a stock solution of OmpA (3 mg/ml) were diluted first into buffer. The buffer volume was calculated for each experiment to obtain a final volume of 150 μl. In experiments with Skp, LPS, or lipid vesicles, 5.9 μl of a stock solution of Skp (1.8 mg/ml), 16.6 μl of a stock solution of LPS (0.5 mg/ml), or 23.5 μl of a stock solution of lipid vesicles (3.9 mM) were added in this sequence. The mixing of all of the reactants was done rapidly in an Eppendorf vial. Samples were incubated for at least 3 h and then transferred into a CD cuvette. Far-UV CD spectra of 3.0 μM OmpA were recorded at 20 °C on a Jasco 715 CD spectrometer using a 0.5-mm thermostated cuvette. Five scans were accumulated from 190 to 250 nm (205–250 nm in presence of 8 M urea) with a response time of 16 s, a bandwidth of 1 nm, and a scan speed of 10 nm/min. Background spectra without OmpA and Skp were subtracted. The recorded CD spectra were normalized to obtain the mean residue molar ellipticity [Θ](λ) as shown in Equation 1,

$$[\Theta](\lambda) = 100\,\frac{\Theta(\lambda)}{(c_1 \times n_1 + c_2 \times n_2) \times l} \qquad \text{(Eq. 1)}$$

where l is the path length of the thermostated cuvette and Θ(λ) is the recorded ellipticity at wavelength λ. c_1 and c_2 are the concentrations, and n_1 and n_2 are the numbers of the amino acid residues of OmpA and Skp, respectively.

Skp and LPS Binding to OmpA Monitored by Fluorescence Spectroscopy—LPS (M = 3900 g/mol) binding to unfolded OmpA was determined by first recording background spectra of LPS at different concentrations in 490 μl of glycine buffer (10 mM, pH 8.5). The LPS concentration ranged from 0 to 0.13 mg/ml. After the addition of 10 μl of urea-denatured OmpA (1 mg/ml), the fluorescence spectra of OmpA were recorded at each LPS concentration. The background spectra exhibited only very weak intensity because of light scattering and were subtracted. Skp binding to OmpA was determined analogously in 500 μl of glycine buffer at Skp concentrations ranging from 0 to 0.08 mg/ml and at an OmpA concentration of 0.02 mg/ml. Background spectra of Skp were subtracted. Skp does not contain tryptophan and is only weakly fluorescent when excited at 290 nm.

Fluorescence spectra were recorded as described previously (10) on a Spex Fluorolog-3 spectrofluorometer with double monochromators in the excitation and emission pathways. The excitation wavelength was 290 nm, and the bandwidths of the excitation monochromators were 3 nm. The bandwidths of the emission monochromators were 3.5 nm. The integration time was 0.1 s, and an increment of 0.5 nm was used to scan spectra in the range of 310–370 nm.

The association constant K for the binding of the ligand L (*i.e.* LPS or Skp) to a protein binding site P_x ($P_x + L \rightarrow P_xL$) as defined by the mass action law, *i.e.* $K = [P_xL]/([P_x][L])$ was determined by fitting the fluorescence intensities as a function of molar ratio of ligand added to the protein. It was assumed that all of the binding sites on the protein are equivalent for all of the molecules of the selected ligand (either LPS or Skp).

RESULTS

Skp and LPS Affect the Folding of OmpA—To investigate how Skp and LPS affect the folding kinetics of OmpA, we used an electrophoretic mobility assay as described previously (6, 10, 11, 16). In SDS-PAGE, folded OmpA migrates at 30 kDa, whereas unfolded OmpA migrates at 35 kDa if not heat-denatured prior to electrophoresis (43). The 30-kDa form has been shown by Raman, Fourier Transform Infrared, and CD spectroscopy (7, 11, 16, 44–46) by phage inactivation assays (43) and by single channel conductivity measurements (47) to correspond to completely folded and active OmpA. In the experiments shown in Fig. 1, the folding of OmpA upon dilution of the denaturant urea was monitored at pH 7.0 over a time course of 180 min. In presence of Skp in aqueous solution, OmpA did not fold (Fig. 1*A*, *gel 1*). When OmpA was incubated with a 5-fold molar excess of LPS (Fig. 1*A*, *gel 2*), approximately 45% OmpA folded as analyzed by densitometry (Fig. 1*B*, ▲). OmpA folding quickly leveled off within 1 h after initiation of folding. However, folding into LPS was strongly inhibited when OmpA was first reacted with Skp prior to the addition of LPS (Fig. 1, *A*, *gel 3*, and *B*, ◆).

Consistent with previous reports (11), OmpA refolded to 75%

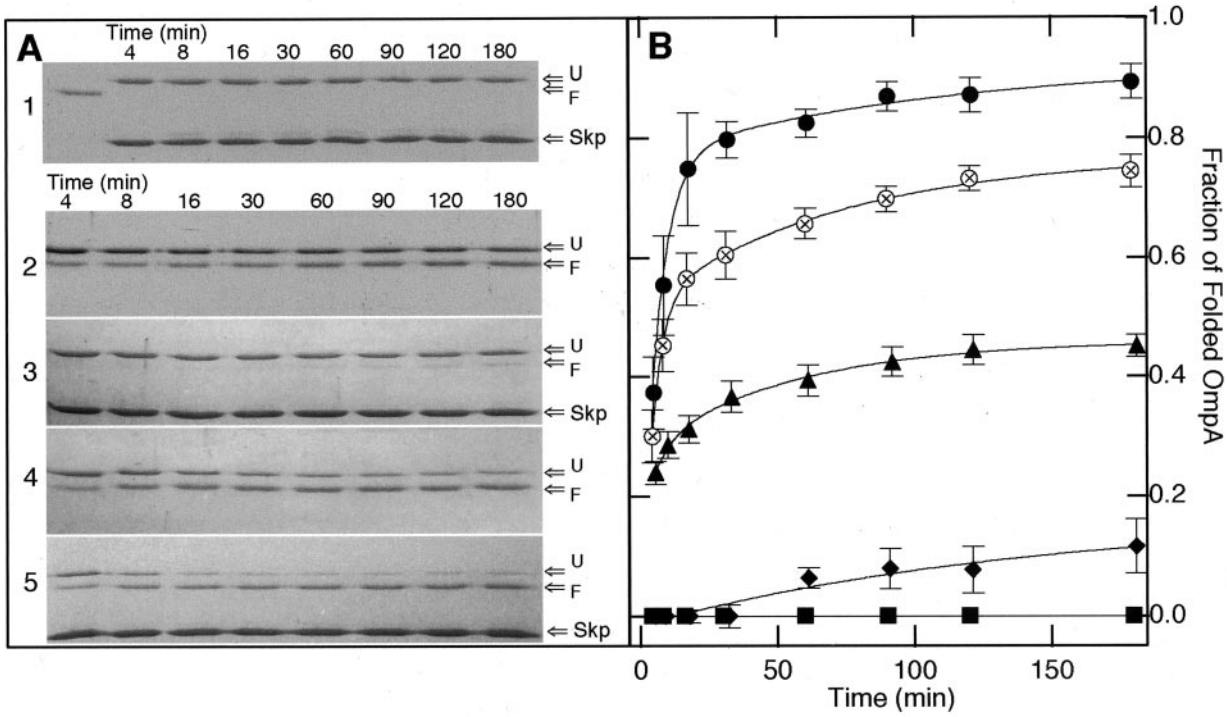

FIG. 1. **Membrane insertion and folding of OmpA is affected by LPS and Skp.** *A*, SDS-polyacrylamide gels showing the time courses of OmpA folding at 30 °C. The *arrows* indicate the migration of unfolded (*U*) and folded (*F*) OmpA. The folding of OmpA was monitored after dilution of the denaturant in the presence of a 4-fold molar excess of Skp (*gel 1*), a 5-fold molar excess of LPS (*gel 2*), a 4-fold molar excess of Skp followed by a 5-fold molar excess of LPS (*gel 3*), a 200-fold molar excess of phospholipids (*gel 4*), and a 4-fold molar excess of Skp followed by a 5-fold molar excess of LPS followed by the immediate addition of a 200-fold molar excess of phospholipids (*gel 5*). For comparison, the migration of native OmpA (*F*) at 30 kDa is shown in the *first lane* of *gel 1*. Where present, the migration of Skp (17 kDa) is also indicated. The experiments 2–5 were done in parallel. In all of the five experiments, OmpA, LPS, Skp, and lipid vesicles were from the same stock solutions. *B*, densitometric analysis of the fraction of folded OmpA as determined from SDS gels as shown *panel A*: Skp/OmpA = 4:1 mol/mol (■); LPS/OmpA = 5:1 mol/mol (▲); Skp/LPS/OmpA = 4/5/1 mol/mol/mol (♦); phospholipid bilayers (⊗); and Skp/LPS/OmpA = 4/5/1 mol/mol/mol in presence of phospholipid bilayers (●).

when reacted with preformed phospholipid bilayers (Fig. 1*A*, *gel 4*, and *B*, ⊗). When OmpA was preincubated first with Skp and then with LPS before vesicles were added, up to 90% folding of OmpA into phospholipid bilayers was observed (Fig. 1*A*, *gel 5*). Also, the SDS gels and their densitometric analysis indicated much faster folding kinetics (Fig. 1*B*, ●) in comparison with OmpA refolding into lipid bilayers in the absence of Skp and LPS. Before preincubation with Skp and LPS, the denaturant urea was diluted 10-fold (see "Experimental Procedures"), *i.e.* to a concentration lower then necessary to keep OmpA unfolded. Because the same vesicle preparations were used and the reactions analyzed in gels 4 and 5 were performed in parallel, we concluded that the interaction with Skp and LPS prevented a hydrophobic collapse of OmpA after urea dilution.

Skp and LPS Are Jointly Required for Efficient Membrane Insertion and Folding of OmpA—The improved folding kinetics of OmpA into phospholipid bilayers after preincubation with Skp and LPS raised the question of whether these periplasmic components were mutually required to assist the folding of OmpA into lipid bilayers or whether one component, either Skp or LPS, would suffice. Fig. 2 shows the time dependence of OmpA folding into lipid bilayers as analyzed by SDS-PAGE and subsequent densitometry. In *panel A*, preformed lipid bilayers were added to OmpA immediately after urea dilution in the presence of Skp (♦), in the presence of LPS (▲), in the presence of Skp and LPS (●), and in the absence of Skp and LPS (⊗). The four experiments were performed in parallel with the same stock solutions. Folding kinetics into lipid bilayers were fastest and folding was most complete when Skp and LPS were both present. When Skp and LPS were both absent, the folding into lipid bilayers was less efficient. However, when only one of the periplasmic components, either Skp or LPS, was added, the efficiency of OmpA folding into lipid bilayers was further reduced.

In a second set of experiments (Fig. 2, *panel B*), LPS and lipid were added 30 min after dilution of the denaturant with buffer in the presence or absence of Skp (*panel B*). The introduction of this delay led to much stronger dependencies of the folding kinetics and yields of folded OmpA in the presence of Skp and LPS. When LPS was added to Skp·OmpA complexes prior to lipid addition (●), the largest folding yields were obtained (>90%). When only Skp was present (♦), OmpA folding into lipid bilayers was slowest and the lowest folding yields were observed. Slow folding and low yields were also observed when LPS and lipid bilayers were added 30 min after dilution of urea by addition of Skp-free buffer to OmpA (▲). When OmpA was incubated in the presence of LPS but in the absence of Skp for 30 min prior to the addition of preformed lipid bilayers (△), OmpA partially folded as observed previously (Fig. 1, ▲). In this experiment, the folding yields were lower than observed for OmpA folding into lipid bilayers in the absence of LPS (⊗).

With one exception, even slower folding kinetics and lower yields of folded OmpA were observed when the delay time was increased to 1 h for all of the folding reactions (data not shown). Still, up to 90% OmpA folded after 180 min when OmpA was preincubated with Skp for 1 h before LPS and lipid were added. In contrast, a 1-h preincubation of OmpA in buffer in the absence of LPS and Skp led to <60% folding after the addition of lipid bilayers.

Based on these results, we concluded the following. 1) Skp alone prevents the aggregation or misfolding of OmpA in solution, because the addition of LPS and preformed lipid bilayers

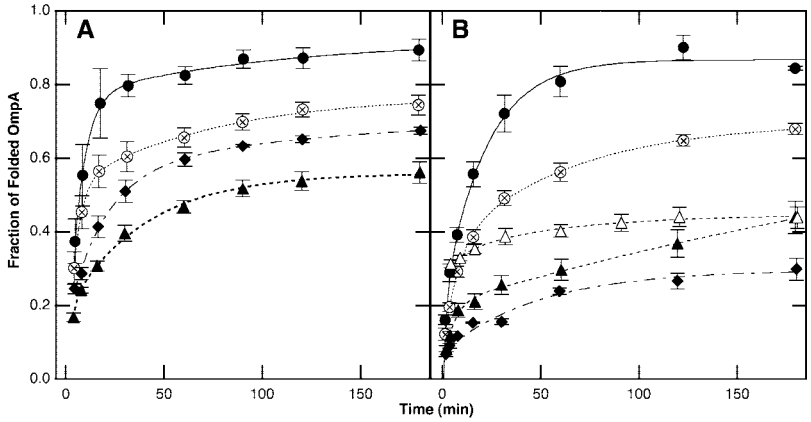

FIG. 2. **Efficient folding of OmpA into lipid bilayers requires the simultaneous presence of Skp and LPS.** The OmpA-folding kinetics were determined by electrophoresis and densitometry. OmpA, Skp, LPS, and lipid were reacted as described under "Experimental Procedures" at 30 °C. The Skp/OmpA ratio was 4 mol/mol. The LPS/OmpA ratio was 5 mol/mol, and the lipid/OmpA ratio was 200 mol/mol. *A*, folding was initiated after urea dilution by immediate addition of lipid bilayers in absence of Skp and LPS (⊗), in presence of Skp (♦), in presence of LPS (▲), and in presence of both Skp and LPS (●). *B*, the corresponding experiments were performed but with a 30-min delay between urea dilution in presence or absence of Skp and the addition of lipid or addition of first LPS and then lipid. In an additional experiment, only LPS added was to OmpA 30 min before lipid addition (△).

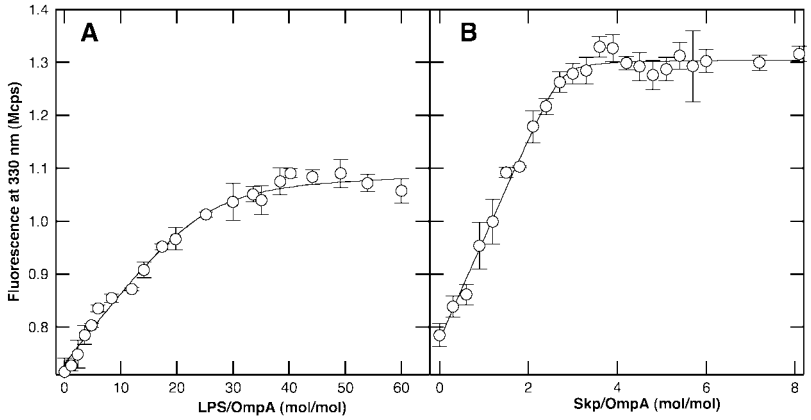

FIG. 3. **Binding of LPS and Skp to unfolded OmpA in solution.** The intrinsic fluorescence intensity of OmpA at 330 nm is shown as a function of the molar LPS/OmpA ratio (*A*) and the molar Skp/OmpA ratio (*B*) at an OmpA concentration of 0.6 μM. Each data point is the average of three separate measurements at the same ligand/OmpA ratio. The binding data were fitted (*solid lines*) to the corresponding mass action laws of ligand binding assuming the equivalent binding sites. Background intensities of LPS or Skp in absence of OmpA were subtracted. All of the experiments were performed at 20 °C.

after >30 min still leads to efficient folding. 2) LPS is required for efficient folding of OmpA into lipid bilayers from Skp·OmpA complexes. In the absence of LPS, Skp strongly inhibits insertion and folding of OmpA.

Skp and LPS Bind to OmpA in Solution with Different Stoichiometries and Affinities—To determine the stoichiometry and the strength of the binding of Skp and LPS to unfolded OmpA in solution, we used intrinsic fluorescence spectroscopy (Fig. 3).

First, LPS binding to unfolded OmpA in aqueous solution was investigated. We observed an increasing Trp fluorescence of OmpA at 330 nm with increasing LPS concentration until a saturation was reached (Fig. 3*A*). When these fluorescence intensities were fitted as a function of the LPS concentration to a simple mass action law for LPS binding (assuming equivalent LPS binding sites), a stoichiometry of 25 ± 2 LPS molecules bound to unfolded OmpA was obtained. The association constant for LPS·OmpA complex formation was K_{LPS} = 1.2 ± 0.7 μM^{-1}, corresponding to a free energy of ΔG^{o}_{LPS} = −34.7 ± 1.5 kJ/mol for LPS binding to OmpA.

We then investigated the binding of Skp to unfolded OmpA. Small fluorescence contributions of Skp were measured at each Skp concentration and subtracted from the OmpA fluorescence intensity after mixing. The fluorescence intensity of OmpA increased strongly as a function of the molar Skp/OmpA ratio (Fig. 3*B*). We fitted our data to the mass action law for Skp binding and found a binding stoichiometry of 2.8 ± 0.1 ≈ 3 Skp molecules bound per OmpA molecule. The binding curve sharply levels off at this stoichiometry, indicating strong binding of Skp to unfolded OmpA. From the fit to the mass action law of Skp binding, we estimated K_{Skp} = 46 ± 30 μM^{-1}, corresponding to a free energy of ΔG^{o}_{Skp} = −43 ± 2 kJ/mol. This binding constant is between 8- and 150-fold higher than the binding constant of LPS and demonstrates a preferred binding of Skp to OmpA over LPS.

OmpA Folding into Lipid Bilayers Is Optimal at Specific Concentrations of LPS and Skp—To examine how different LPS/OmpA ratios affect the folding of OmpA into a 200-fold molar excess of lipid, we performed folding experiments in the absence (Fig. 4*A*) and presence (Fig. 4*B*) of a 4-fold molar excess of Skp at molar LPS/OmpA ratios ranging from 0 to 40. Experiments with and without Skp at the same LPS/OmpA ratio were performed in parallel using the same lipid vesicle preparation (Fig. 4).

Representative gels shown in Fig. 4*A* demonstrate the time courses of OmpA folding into lipid bilayers at molar LPS/OmpA ratios of 0, 1, 5, 7, and 40 in absence of Skp. The corresponding mole fractions of folded OmpA are shown in Fig. 4*C*. In absence

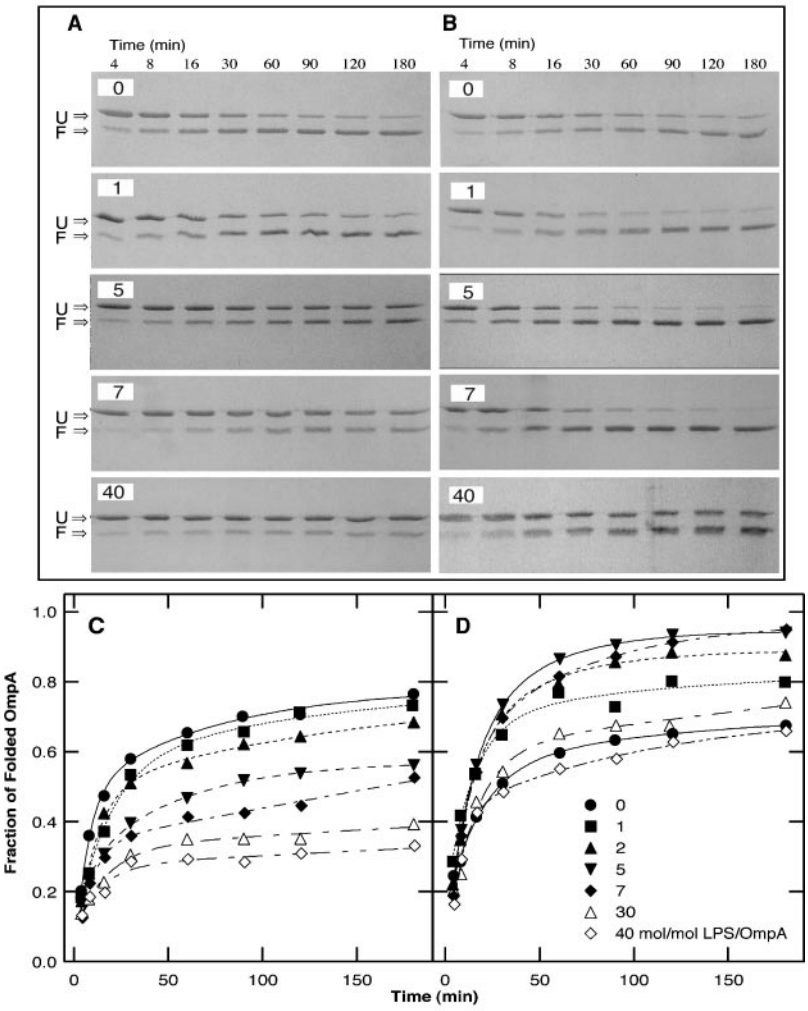

FIG. 4. **Dependence of OmpA folding into lipid bilayers on the concentration of LPS.** Insertion and folding of 7.1 μM OmpA into a 200-fold molar excess of lipid at LPS/OmpA ratios of 0, 1, 5, 7, and 40 mol/mol (from *top* to *bottom*) as a function of time after initiation of folding. *A*, folding kinetics monitored in absence of Skp. *B*, folding kinetics monitored in presence of 4 mol/mol Skp/OmpA. Skp migrated at 17 kDa in the gels shown in *B*, but only the bands of unfolded (U) and folded (*F*) OmpA are shown. *C* and *D*, densitometric analysis of OmpA folding as a function of the LPS concentration obtained from SDS gels including those shown in *panels A* and *B*. Folding experiments were performed either in the absence (*C*) or presence (*D*) of a 4-fold molar excess of Skp over OmpA. The LPS to OmpA ratios were 0 (●), 1 (■), 2 (▲), 5 (▼), 7 (♦), 30 (△), and 40 (◇).

of LPS, ~70% OmpA folded within 180 min (as observed in our previous experiment) (Fig. 1*A*, *gel 4*, and *B*, ⊗). When LPS was present, the rates of folding and the yields of folded OmpA decreased with increasing LPS/OmpA ratios. At a 40-fold molar excess of LPS (Fig. 4, *A* and *C*), only ~30% OmpA folded within 180 min, indicating that insertion and folding of OmpA into phospholipid bilayers are inhibited by LPS in the absence of Skp.

In presence of Skp, LPS increased the folding rates and yields of folded OmpA as indicated by the gels (Fig. 4*B*) and the densitometric analysis (Fig. 4*D*). When LPS was added to complexes of Skp and OmpA (at 4 Skp/OmpA) prior to the addition of preformed phospholipid bilayers, the highest yields of folded OmpA were obtained at molar LPS/OmpA ratios between 2 and 7 and >90% OmpA folded. When we further increased the amounts of LPS added to OmpA·Skp complexes, LPS inhibited OmpA folding into lipid bilayers at LPS/OmpA molar ratios of >10, similar to the LPS effect on the folding of OmpA in the absence of Skp (*panel A*). In comparison with the set of experiments in the absence of Skp (*panel A*), 50% OmpA still folded even at 40 LPS/OmpA. In the absence of LPS, Skp slightly inhibited the folding of OmpA into lipid bilayers (Fig. 4, *A* and *B*, *gels 0*) as determined by densitometry (Fig. 4, *C* and *D*, ●). This inhibitory effect of Skp in the absence of LPS was stronger at a higher Skp/OmpA ratio of 8 mol/mol (gel not shown) or in experiments with delayed lipid addition (Fig. 2*B*, ♦). The yields of OmpA folding after 180 min are summarized in Fig. 5.

OmpA Remains in an Unfolded State When Bound to Skp in Solution—To obtain more information on the folding of OmpA

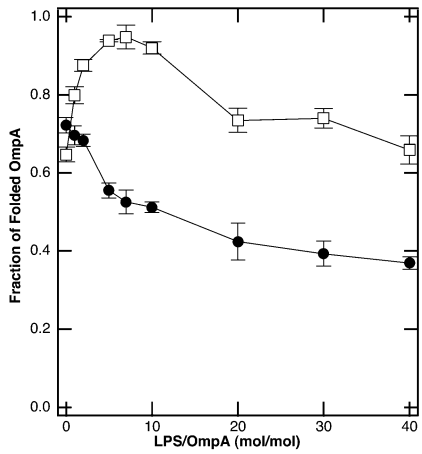

FIG. 5. **Final yields of folded OmpA, both in the presence (□) and absence (●) of Skp as a function of the LPS/OmpA molar ratio.**

in the presence of equimolar amounts of Skp, we recorded circular dichroism spectra (Fig. 6) of OmpA·Skp complexes (—) in aqueous solution (*panel A*) in the presence of a 5-fold molar excess of LPS (*panel B*) and in presence of both LPS and preformed phospholipid bilayers (*panel C*). Furthermore, the spectra of Skp (– –) were recorded in the absence of OmpA under otherwise identical conditions. At last, we recorded the spectra of OmpA (·····) in absence of Skp under these conditions. All of the spectra were normalized to the mean molar ellipticity

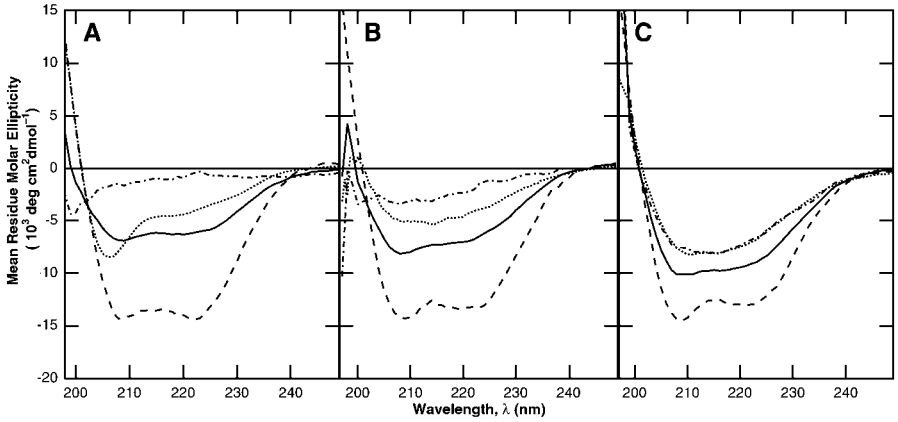

FIG. 6. **Secondary structure of OmpA-Skp complexes by circular dichroism spectroscopy.** CD spectra of Skp (– –), OmpA (····), and OmpA bound to Skp (—) in aqueous buffer (*A*), in presence of LPS (*B*), and in presence of LPS and lipid bilayers (*C*). The component spectrum of OmpA in Skp-OmpA complexes (– · – ·) is also shown in *panels A–C*. This spectrum was obtained by subtracting the spectrum of Skp from the spectrum of the complex of OmpA and Skp. The mean residue molar ellipticity was calculated for all spectra according to Eq. 1.

per residue (see "Experimental Procedures") and are described in three separate paragraphs below for *panels A–C*. The secondary structure of OmpA in OmpA·Skp complexes can be obtained by subtraction of the spectrum of Skp from the spectrum of Skp·OmpA complexes if the secondary structure of Skp remains largely unaffected in the presence of folded or unfolded OmpA. The line shapes and relative amplitudes of the spectra indicated that changes in Skp secondary structure in presence of OmpA are comparably small. Therefore, we also calculated the component spectra of OmpA (– · – ·), which are shown in *panels A–C* for comparison.

In aqueous solution (*panel A*), the amplitude of the spectrum of OmpA·Skp complexes was only 45% of the amplitude of the CD spectrum of Skp at a wavelength of 215 nm. Despite the strongly reduced amplitude, the spectral line shape of the complex was characteristic for relatively large contents of α-helix secondary structure, similar to the spectrum of Skp in solution in the absence of OmpA. Therefore, we concluded that the secondary structures of Skp and OmpA remained largely unaffected by the binding of unfolded OmpA to Skp. The subtraction of the spectrum of Skp (– –) from the spectrum of the Skp·OmpA complex (—) resulted in a spectrum (– · – ·) indicating the random coil structure of OmpA, because this spectrum and the spectrum of urea-denatured OmpA (see Refs. 7, 11, and 16) were very similar. The spectrum of OmpA in aqueous solution in the absence of LPS and Skp (Fig. 6*A*, ·····) is shown for comparison. The relatively large amplitude of this spectrum suggests more secondary structure in the hydrophobically collapsed state of OmpA than in complex with Skp. Skp apparently prevents the misfolding of OmpA in solution.

When OmpA·Skp complexes or Skp were incubated in presence of a 5-fold molar excess of LPS (*panel B*), the amplitude of the spectrum of the complex (—) increased to 56% of the amplitude of the spectrum of Skp (– –). Therefore, minor amounts of secondary structure may have formed in OmpA. The OmpA spectrum (– · – ·) obtained by subtraction of the Skp spectrum from the spectrum of Skp·OmpA complexes (—) was characterized by a minimum located at 208 nm. The amplitude of this minimum was still relatively small, also suggesting that only minor amounts of secondary structure are formed in OmpA in complex with Skp and LPS. In absence of Skp, the incubation of OmpA with a 5-fold molar excess of LPS led to spectra that were characterized by a minimum at 215 nm and an amplitude that indicated larger amounts of β-sheet secondary structure. This partial formation of β-sheet structure was consistent with our previous observation of partial folding of OmpA into LPS in the absence of Skp as shown by SDS-PAGE (see Fig. 1, *A*, *gels 2* and *3*, and *B*, ♦ and ▲).

After the addition of lipid bilayers (Fig. 6*C*), the amplitude of the circular dichroism spectra of Skp·OmpA complexes increased further to 78% of the amplitude of Skp. This increase was stronger than the increase observed upon the addition of LPS, and it demonstrated that completion of secondary structure formation in OmpA required the presence of membranes. Because our SDS-PAGE results indicated efficient OmpA folding in the presence of both Skp and LPS and bilayers, we calculated the spectrum of OmpA in Skp·OmpA complexes by subtraction of the spectrum of Skp from the spectrum of the complexes. This calculated spectrum (– · – ·) had indeed the same line shape as OmpA that was completely refolded into lipid bilayers in the absence of Skp and LPS (·····).

DISCUSSION

Upon investigating the roles of the periplasmic components Skp and LPS on the insertion and folding of OmpA into phospholipid bilayers in detail, we found a first assisted folding pathway for the integral membrane protein OmpA that is described by three major stages. First, although Skp binding to OmpA alone is sufficient to keep OmpA unfolded in solution, thus effectively replacing the denaturant urea, it is inhibiting the membrane insertion and folding of OmpA. Second, the interaction of Skp·OmpA complexes with LPS does not lead to OmpA folding but facilitates OmpA insertion and folding into phospholipid bilayers in the third stage. Membrane insertion and folding of OmpA is optimal at specific molar ratios of Skp, LPS, and OmpA.

Skp Solubilizes Unfolded OmpA by Forming a Positively Charged Complex—Previous studies have described OmpA insertion and folding into phospholipid bilayers in the absence of any folding catalysts (6–11, 16). It was previously reported that the rates and yields of OmpA folding are higher when deprotonated at pH 10, *i.e.* at a negative net charge of the protein that leads to increased solubility of OmpA (11). At pH 7.0, the binding of the highly basic Skp to OmpA at a 3:1 stoichiometry leads to a positively charged protein complex and, therefore, also increases the solubility of OmpA. To prevent the aggregation of OmpA in solution, Skp binding must shield the more hydrophobic regions of unfolded OmpA from the aqueous space. The observed increase in Trp fluorescence upon binding of Skp (Fig. 3*B*) indicates that in solution, Skp specifically recognizes and binds to OmpA segments that will later form the transmembrane region of the β-barrel, because all of Trps of OmpA are located in the transmembrane β-strands. *In vivo*, Skp binds to OmpA close to the periplasmic surface of the inner membrane and is required for the release of OmpA into the periplasm (26). This is consistent with our observation that the folding kinetics of Skp-bound OmpA into phospholipid bilayers were inhibited when compared with the folding kinetics of urea-unfolded OmpA (Fig. 2, *panel B*, ♦ and ⊗).

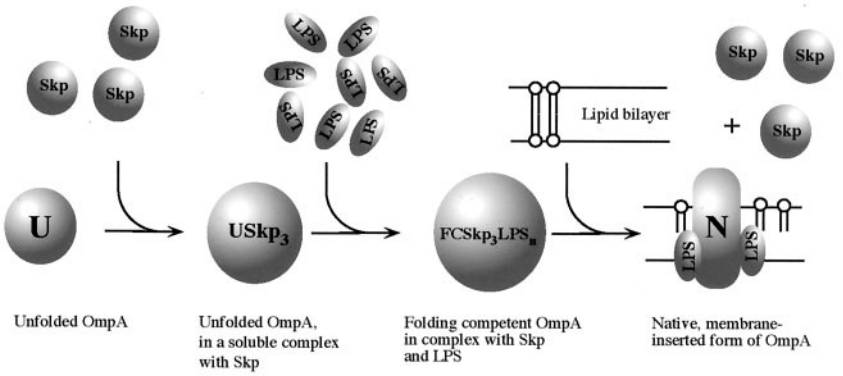

FIG. 7. **Scheme for an assisted folding pathway of a bacterial outer membrane protein.** OmpA is translocated through the cytoplasma membrane in an unfolded form (*U*) and binds to a small number of molecules of the periplasmic chaperone Skp, which solubilizes OmpA in the unfolded state ($USkp_3$). The complex of unfolded OmpA and Skp interacts with a small number of LPS molecules to form a folding competent intermediate of OmpA ($FCSkp_3LPS_n$). In the final step, folding competent OmpA inserts and folds into the lipid bilayer.

Skp Prevents Partial Folding and Unspecific Interactions of Unfolded OmpA with LPS—We observed that OmpA partially folds into LPS micelles when Skp is absent (we estimated a critical micelle concentration of $CMC_{LPS} \approx 8\ \mu M$ as described in Ref. 48). Folding into LPS micelles was incomplete at LPS/OmpA ratios between 5 and 40 (*cf.* Fig. 4*A*), indicating that OmpA interacts unspecifically with LPS micelles. In our previous study (7), OmpA folded into micelles of neutral (*i.e.* uncharged or zwitterionic) detergents, independent of the hydrophobic chain length and chemical structure of the headgroup of the amphiphile, but never into negatively charged SDS micelles as determined by CD spectroscopy (data not shown) and SDS-PAGE (6, 7, 10, 11, 16). LPS is negatively charged with two monophosphate and two diphosphate groups in the inner core region (49). In the absence of Skp in solution, the different phosphate groups may interact in different modes with unfolded OmpA partially inhibiting OmpA folding into LPS micelles. Consistent with unspecific OmpA-LPS interactions, the folding of OmpA into phospholipid bilayers was also inhibited in the absence of Skp when LPS was present (Fig. 4, *A* and *C*). When both LPS and Skp were present (Fig. 4, *B* and *D*), an inhibition of folding into lipid bilayers was only observed at LPS/OmpA ratios of >10 mol/mol. Skp binding to OmpA was much stronger than the binding of LPS to OmpA, indicating that LPS cannot easily replace Skp bound to OmpA. Only at very high LPS concentrations and low Skp concentrations, the thermodynamic binding equilibria would favor LPS binding over Skp binding. For this reason, the folding of OmpA into lipid bilayers may be partially inhibited even in presence of Skp if the LPS concentration is high enough (Fig. 4, *B* and *D*). Therefore, in cells, it may be important to keep a balance between the concentrations of Skp and LPS in the periplasm. At balanced LPS/Skp ratios, Skp suppresses a partial folding of OmpA into LPS-micelles (Fig. 1, *gels 2* and *3*). Schäfer *et al.* (26) show that Skp binds immediately after translocation of OmpA across the inner membrane. Our present study indicates that this early binding of Skp also prevents a partial folding and unspecific interactions of unfolded OmpA with LPS in the periplasm (Figs. 1, *gel 3*, and 6*B*).

Structure Formation in OmpA Requires the Presence of the Phospholipid Bilayer—OmpA neither developed the native tertiary structure (Fig. 1*A*, *gel 3*) nor the large amounts of β-sheet secondary structure (Fig. 6*B*) when in complex with Skp and LPS in solution at Skp/OmpA ratios of ≥1. The formation of native secondary (Fig. 6*C*) and tertiary (Fig. 1*A*, *gel 5*) structure required the insertion of OmpA from the complex with Skp and LPS into preformed lipid bilayers. This finding is consistent with our previous reports (6, 8, 9) that OmpA folds and inserts from a urea-unfolded form into phospholipid bilayers by a concerted mechanism in which the formation of secondary and tertiary structures are strictly coupled and synchronized to insertion into the bilayer.

Insertion of OmpA into Lipid Bilayers from the Complexes with Skp and LPS—OmpA folding into phospholipid bilayers was most efficient in the presence of 4 mol of Skp and 2–7 mol of LPS/mol OmpA. Because we determined a binding stoichiometry of 25 LPS/unfolded OmpA in the absence of Skp in solution (Fig. 3*B*), only a small number of LPS molecules (10–25%) apparently bind to Skp·OmpA complexes and are required for efficient OmpA folding. From the crystal structures of LPS (49) and from the structure of OmpA (50, 51), we found that ~5 LPS molecules can form a first shell around folded OmpA in the outer leaflet of the outer membrane, which is consistent with the stoichiometry of 2–7 mol/mol LPS/OmpA found for the fastest folding kinetics of OmpA in presence of Skp. To the best of our knowledge, there are no enzymes in the outer membrane that actively generate a trans-bilayer asymmetry. We propose that this asymmetry may be a direct result of specific binding of Skp and LPS to OMPs prior to OMP insertion and folding into the phospholipid bilayer. Interestingly, such interactions were observed in the crystal structure of LPS bound to another outer membrane protein, FhuA, of *E. coli* (49).

A First Pathway for OmpA Membrane Insertion and Folding into Lipid Membranes—The results of our present *in vitro* study are evidence for a first pathway of assisted OmpA folding and membrane insertion that is summarized in Fig. 7. In an unfolded form that has been trans-located across the inner membrane, OmpA binds up to 3 molecules of Skp and forms a soluble complex, $USkp_3$, in which OmpA is kept largely unfolded as indicated by circular dichroism spectroscopy. This complex then binds a small number (n = 2–7) of LPS per OmpA in solution to form a folding and insertion-competent form of OmpA bound to Skp and LPS ($FCSkp_3LPS_n$). In this complex, OmpA develops minor amounts of secondary structure (Fig. 6*B*). When this insertion-competent intermediate of OmpA is reacted with preformed phospholipid bilayers, OmpA rapidly inserts and folds to its native state N as indicated by the formation of native secondary and tertiary structures (see Figs. 1, *gel 5*, and 6*C*). The functions of Skp and LPS as "co-chaperones" may be directly reflected in the close neighborhood of the genes *skp* and *firA* in the 4-min region on the chromosome, in particular, as LPS and Skp are required at specific molar ratios of ~0.5–1.7 mol LPS/mol Skp for optimal folding rates and yields of membrane-inserted OmpA. This co-assisted folding of OmpA by Skp and LPS very likely is a general folding pathway of outer membrane proteins because Skp was found to bind several OMPs (23), because deletion of the *skp* gene resulted in reduced expression levels of several OMPs in the outer membrane (23), and because specific LPS binding motifs were reported for FhuA (49) and, more recently, also for OmpT (52).

The Skp-LPS-assisted folding pathway that we found in this study may not be the only mechanism by which OMPs like OmpA insert and fold into the outer membrane, because the deletion of the *skp* gene only decreases the concentration of

OMPs in the outer membrane but does not entirely eliminate their presence. However, our experiments demonstrate for the first time the successful *in vitro* folding of a membrane protein into lipid bilayers from an unfolded soluble state that only involves components from the cell and that does not require urea as a denaturant. The strategy that we used here to investigate the Skp/LPS-assisted folding pathway will also prove useful to discover other parallel pathways in future studies.

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