Folding and Membrane Insertion of the Trimeric β-Barrel Protein OmpF

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ABSTRACT: We have studied folding and membrane insertion of the porin OmpF and compared it to OmpA. Both are β-barrel membrane proteins from the outer membrane of *Escherichia coli*, OmpF forming trimers and OmpA monomers. Each of them can be unfolded in solubilized form in a water/urea mixture. Refolding is initiated by dilution into a dispersion of lipid vesicles or lipid/detergent vesicles, whereupon OmpF and OmpA refold and insert into the membranes. Folding and insertion of the monomers proceed in a similar way for the two proteins, but native OmpF appears more slowly and with a lower yield than native OmpA because of trimerization of OmpF. The dependence of the yield of refolding, membrane insertion, and trimerization on pH, lipid concentration, and the presence of detergent was investigated. Trimerization of OmpF is shown to take place at or in the membrane and a membrane-inserted dimer is detected as an intermediate of this process.

Refolding of purified integral membrane proteins has been achieved *in vitro* for both some α-helix proteins (Huang et al., 1981; Paulsen et al., 1990) and some β-barrel proteins (Eisele & Rosenbusch, 1990; Dornmair et al., 1990). These first attempts to refold membrane proteins were successful in the sense that they folded into detergent micelles or mixed micelles of lipid and detergent. But refolding of an unfolded protein may also proceed by direct insertion of the protein into lipid vesicle membranes, which would reflect more closely the *in vivo* situation. This was shown for the first time for the β-barrel membrane protein OmpA[1] of *E. coli* (Surrey & Jähnig, 1992). It proved to be an ideal candidate for refolding and membrane insertion studies because of its weak tendency to aggregate in water. This property follows from its amphipathic β-structure (Vogel & Jähnig, 1986), raising the question whether other β-barrel membrane proteins refold and insert with similar ease into membranes starting from their unfolded state.

Here we report on the direct membrane insertion of another β-barrel membrane protein, the porin OmpF of *E. coli*. OmpF is a trimeric protein, each monomer forming a β-barrel of 16 β-strands and representing a water-filled pore through the membrane (Cowan et al., 1992). OmpF is one of the major pores in the outer membrane of *E. coli* (Benz & Bauer, 1988). By contrast, OmpA is monomeric and consists of two domains (Morona et al., 1984), a membrane-inserted small β-barrel, most probably formed by 8 amphipathic β-strands (Vogel & Jähnig, 1986), and a water-soluble domain residing in the periplasmic space of the bacterium. OmpA is supposed to be involved in fixing murein to the outer membrane (Leduc et al., 1992).

By comparing refolding, membrane insertion, and trimerization of OmpF with refolding and membrane insertion of monomeric OmpA, we intend to extract general characteristics of refolding and membrane insertion of β-barrel membrane proteins. For OmpA, the pathway of folding and membrane insertion was found to proceed via at least two intermediates (Surrey & Jähnig, 1995). When urea-unfolded OmpA was diluted into a dispersion of lipid vesicles, it underwent a fast transition to a partially folded state in water, followed by a moderately slow transition to a partially folded and membrane-inserted state, and finally, a slow transition to the fully folded, membrane-inserted state. An obvious question is whether or not OmpF would behave in a similar way and how trimerization would affect this pathway.

We addressed these questions by measuring the yield and kinetics of folding, membrane insertion, and trimerization of OmpF. As experimental techniques for the characterization of the conformational states and their transitions, we used circular dichroism (CD), tryptophan (Trp) fluorescence, protease digestion, SDS gel electrophoresis, centrifugation, and conductivity measurements.

MATERIALS AND METHODS

Chemicals. Stock solutions of urea (Serva) were deionized on a mixed-bed ion exchange column of BioRad AG 501-X8 and were used within one day. Chloroform and methanol (Merck) were "uvasol", acetone (Merck) was "lichrosolv". Dimyristoylphosphatidylcholine (DMPC) was from Avanti Polar Lipids.

Protein Purification. OmpF and OmpA were purified from the OmpC-free *E. coli* strain P400.6 (Morona et al., 1985), which was kindly provided by U. Henning. Cells were grown in 35 L of 1% bactotryptone, 0.5% yeast extract, 0.01% antifoaming agent (e.g., Fragol ucolub NWN 115) at 37 °C in a Bioengineering P 50 fermenter. Preextracted cell membranes were prepared as previously described (Surrey & Jähnig, 1992). They were resuspended in 200 mL of 8 M urea, 20 mM Tris, pH 8.5, 0.1% 2-mercaptoethanol (ME),

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[1] Abbreviations: OmpA, outer membrane protein A; OmpF, outer membrane protein F; DMPC, dimyristoylphosphatidylcholine; LPS, lipopolysaccharide; DM, dodecylmaltoside; ME, 2-mercaptoethanol; MOPS, morpholinopropane sulfonate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; CD, circular dichroism; CMC, critical micellar concentration.

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and the same volume of 2-propanol was added. The turbid suspension was incubated at 50 °C for 45 min and then centrifuged at 150000*g* for 90 min. This results in the separation of OmpA (in the supernatant) and OmpF (in the pellet). The supernatant containing unfolded OmpA was further purified by anion exchange chromatography (Q Sepharose Fast Flow, Pharmacia) in 8 M urea, 15 mM Tris, pH 8.5, 0.05% ME, followed by ultrafiltration and gelfiltration (Superdex 75 High Load 26/60, Pharmacia) in 8 M urea, 20 mM KP_i, pH 7.3. The OmpF-containing pellet was resuspended in 600 mL of 20 mM Tris, pH 8, 5 mM $MgCl_2$ and digested by 12 mg of trypsin at 37 °C for 1 h. OmpF was protected from protease degradation under these conditions (Rosenbusch, 1974). The digestion was stopped by addition of 25 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), followed by centrifugation at 20000*g* for 60 min in order to remove trypsin and peptides. After the pellet was washed with 400 mL of 20 mM morpholinopropane sulfonate (MOPS), pH 6.9, 25 μM TLCK (20000*g*, 60 min), it was resuspended in 6 M guanidinium chloride, 20 mM MOPS, pH 6.9, 25 μM TLCK, 0.2% ME. Addition of the same volume of 2-propanol and incubation at 75 °C for 30 min resulted in unfolding and solubilization of OmpF, which was then separated from insoluble material by centrifugation at 150000*g* for 1 h. In order for guanidinium chloride (which interferes with ion exchange chromatography) to be removed, OmpF was precipitated by addition of 300 mL of acetone to the supernatant and was incubated on ice for 2 h. After centrifugation at 20000*g* for 1 h, the precipitated OmpF was dissolved in 250 mL of 8 M urea, 15 mM MOPS, pH 6.9, 0.1% ME by sonication in a bath for 90 min, the temperature being kept at 15 °C. Dissolved OmpF was purified by anion exchange chromatography and gel filtration like OmpA, except that the pH was 6.9 instead of 8.5 during anion exchange chromatography. Stock solutions of unfolded OmpF and OmpA containing 1.2 mM protein, 8 M urea, 20 mM KP_i, pH 7.3, were stored at −20 °C. The yields of OmpF and OmpA were 86 and 88 mg, respectively. The purity of the proteins was 98−99% as determined by SDS gel electrophoresis. Purified protein was analyzed for LPS contamination according to Tsai and Frasch (1982), and no detectable amounts of LPS were found.

The concentrations of purified protein were determined by absorbance measurements at 280 nm using molar extinction coefficients of 43 300 M^{-1} cm^{-1} for OmpF and 43 800 M^{-1} cm^{-1} for OmpA. These coefficients were obtained upon weighing the purified proteins after precipitation in acetone and drying.

To determine the protein solubility in water, stock solutions of unfolded OmpF and OmpA in urea were diluted into water containing 20 mM buffer at various pH values, resulting in end concentrations of 3 μM protein and 20 mM urea. After incubation at 30 °C for 24 h and subsequent centrifugation at 150000*g* for 3 h, the solubility was obtained as the ratio of protein in the supernatant to total protein.

Preparation of Vesicles. Small preformed vesicles consisting of DMPC were prepared by sonication as described by Surrey and Jähnig (1992). Their diameter was 30−40 nm, as measured by quasielastic light scattering (Coulter N4/SD). Larger vesicles were prepared by resuspending a lipid film in buffer upon vortexing for 5 min, followed by extrusion through filters (Nuclepore). The resulting vesicles had diameters ranging from 180 to about 1000 nm, depending on the pore size of the filters. Vesicles or membrane sheets containing large amounts of dodecylmaltoside (DM) were prepared by adding DM to small or large vesicles. All vesicles were diluted to their final concentration after preparation and incubated at 30 °C for 24 h.

Conductance Measurements of Refolded OmpF. Conductance measurements were performed according to Benz et al. (1978). OmpF was refolded into vesicles of 7.4 mM DMPC/7.4 mM DM in 20 mM NaP_i, pH 6.5, and 100 ng of refolded protein was added on each side of a black lipid membrane consisting of diphytanoylphosphatidylcholine. Two hundred protein insertions were recorded and analyzed.

Fluorescence and Circular Dichroism Spectroscopy. OmpF and OmpA contain two and five Trp residues, respectively, which are all located in the membrane domain. Trp fluorescence measurements were performed (Perkin-Elmer LS 50B) using an excitation wavelength of 290 nm. For time drives, the excitation slit was set at the minimal value ("0 nm") to prevent photobleaching during the long measuring time, and the emission wavelength was 330 nm. Far-UV CD spectra were recorded (Jasco 720) using a 1 mm cuvette, or a 0.2 mm cuvette for solutions containing 8 M urea. All spectra and time drives were corrected for light scattering by subtracting the data of samples lacking protein.

SDS Gel Electrophoresis and Trypsin Digestion. SDS gel electrophoresis was performed according to Laemmli (1970) with two alterations: Samples were not heated prior to loading of the gel, and the gels were run at 7 °C with reduced voltage to prevent heating. The gels were stained with Coomassie blue. The yield and the time course of insertion can be followed by protease digestion (Schweizer et al., 1978; Eisele & Rosenbusch, 1990). Insertion was stopped by cooling samples of a refolding experiment on ice after different times, 1 μM trypsin was added, and the samples were incubated at 10 °C for 12 h and subjected to SDS gel electrophoresis.

RESULTS

Conformational States of OmpF. Possible conformational states of OmpF were investigated by CD, Trp fluorescence, and SDS gel electrophoresis of undigested and trypsin-digested samples. In addition, the solubility of OmpF in water was determined by centrifugation, and the activity in a membrane by conductivity measurements.

Purified OmpF in urea is unfolded as demonstrated by its CD spectrum (Figure 1A). The Trp fluorescence is weak with an emission maximum at 350 nm (Figure 1B). It is monomeric on the SDS gel with an apparent molecular mass of 37 kDa (Figure 2, lane 1), and it is completely degraded by trypsin (Figure 2, lane 5).

When the urea concentration was decreased to 20 mM by dilution of the stock solution of unfolded OmpF at pH 6.5, it adopted a partially folded state. The conformation is dominated by β-structure but does not reach the β-content of membrane-inserted OmpF (Figure 1A). The Trp fluorescence is still weak, but its emission maximum is shifted to 332 nm (Figure 1B). On the SDS gel, OmpF is still monomeric (Figure 2, lane 2) and completely accessible to trypsin (Figure 2, lane 6). Partially folded OmpF is insoluble around its theoretical isoelectric point of 4.5 and soluble at low or high pH (see below).

When urea-unfolded OmpF was diluted into a dispersion of vesicles, it folded into the native structure concomitant with insertion into the membrane and trimerization. In the

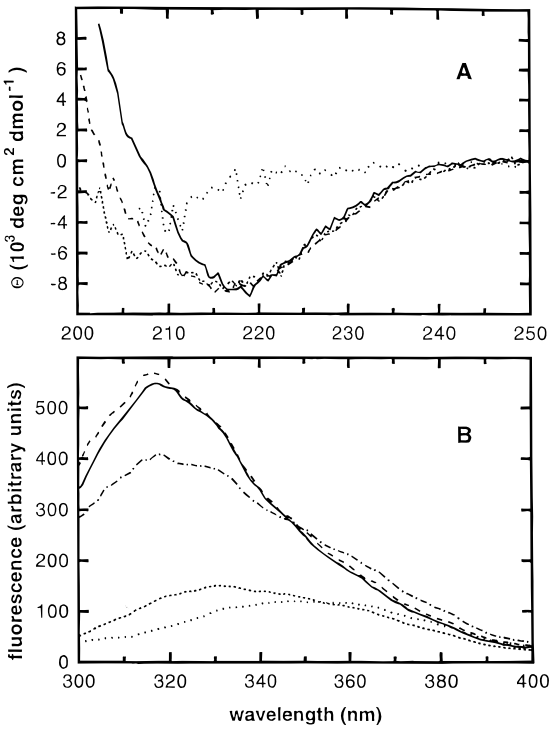

FIGURE 1: (A) Circular dichroism of 3.0 μM OmpF and (B) Trp fluorescence of 1.5 μM OmpF, under different conditions: (···) unfolded in 8 M urea at pH 7.3; (- - -) partially folded in water at pH 6.5; (— — —) folded and inserted in 7.4 mM DMPC/7.4 mM DM, pH 6.5; (——) folded and inserted in 1.5 mM DMPC, pH 6.5 at 30 °C; (—·—) adsorbed or partially inserted in 1.5 mM DMPC, pH 6.5 at 10 °C.

case of pure lipid vesicles, the yield of this process is low; it increases, however, when a detergent such as DM is mixed with the vesicles, and it becomes optimal for a molar ratio of DM/DMPC around 1 (see below). The CD spectrum of OmpF inserted in such mixed membranes (Figure 1A) is identical to the CD spectrum of OmpF reconstituted conventionally under preservation of the structure (Eisele & Rosenbusch, 1990) and, hence, is typical for β-structure. The Trp fluorescence is high, with an emission maximum at 318 nm (Figure 1B). On the SDS gel, 70% of the OmpF molecules appear as trimers (92 kDa), 25% as dimers (52 kDa), and 5% as monomers (37 kDa) (Figure 2, lane 4). Trimers and dimers are not digested by trypsin (Figure 2, lane 8), indicating membrane insertion, whereas monomers are degraded, indicating that stable membrane insertion of monomers does not occur. After uptake in SDS, trimers dissociate to monomers above 70 °C, whereas dimers already dissociate above 50 °C (data not shown). Thus, trimers are more stable than dimers.

Refolded and membrane-inserted OmpF is functionally active, as demonstrated by conductivity measurements (data not shown). The predominant conductivity was 2 nS, with a slight selectivity for cations, as indicated by the ratio of conductivities $p(K)/p(Cl) = 7$. The same values were found previously with conventionally reconstituted OmpF (Benz et al., 1978).

When urea-unfolded OmpF was diluted into a dispersion of DMPC vesicles devoid of DM at 30 °C, it also refolded and inserted but with a lower yield. Its CD spectrum is again characteristic for a high content of β-structure (Figure 1A), and its Trp fluorescence is high, with the emission maximum at 325 nm (Figure 1B). On the SDS gel, only about 12% of the OmpF molecules appear as trimers, about 8% as dimers,

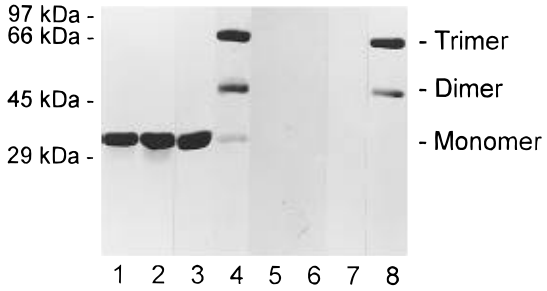

FIGURE 2: SDS gel electrophoresis of 3.0 μM OmpF under various conditions: (lane 1) unfolded in 8 M urea at pH 7.3; (lane 2) partially folded in water at pH 6.5; (lane 3) adsorbed or partially inserted in 1.5 mM DMPC, pH 6.5 at 10 °C; (lane 4) folded and inserted in 7.4 mM DMPC/7.4 mM DM, pH 6.5; (lanes 5−8) samples of lanes (1−4), but digested by trypsin.

while the majority of 80% appear as monomers. As in the case with DM, trimers and dimers are protected from trypsin digestion, whereas monomers are not.

When unfolded OmpF was diluted into a dispersion of DMPC vesicles at 10 °C, i.e., below the lipid phase transition temperature, OmpF did not insert and trimerize, but adsorbed to the membranes (Figures 1 and 2), as observed previously for OmpA (Surrey & Jähnig, 1992).

Yield of Refolding and Membrane Insertion of OmpF As Compared to OmpA. The influence of various parameters (pH, lipid concentration, amount of detergent present in the membranes, and size of the vesicles) on the yield of refolding and insertion of OmpF and OmpA was investigated. The yield was determined by protease digestion.

The dependence of the yield on pH is shown in Figure 3A. OmpF inserts with lower yield than OmpA over the complete pH range. Optimal insertion is found at pH 6.5 for OmpF (15%) and at pH 10 for OmpA (almost 100%). The solubilities of OmpF and OmpA in water (Figure 3A, inset) indicate that insertion is optimal under conditions where the proteins are soluble but at the same time not charged too much as would be the case at extreme pH values.

In Figure 3B, the dependence of the yield on lipid concentration is shown for the optimal value of pH 6.5 for OmpF and for OmpA at pH 7.3. For OmpF, the yields of dimers and trimers are shown; monomers are not included because they are digested by trypsin. The yield of trimers first increases with lipid concentration, but starts to decrease at 1.5 mM lipid. The yield of dimers increases with a delay as compared to that of trimers and does not decrease at higher lipid concentration but reaches even higher levels than the yield of trimers above a concentration of 2 mM lipid. For comparison, the yield of membrane insertion of OmpA simply increases with increasing lipid concentration, saturating around 4 mM lipid.

An increase in DM concentration led to an increased yield of refolding and membrane insertion of both OmpF and OmpA, as shown in Figure 3C. Especially, the yield of trimerization of OmpF could be improved from 15% to 73% at pH 6.5. Of the remaining OmpF molecules, 23% were detected as trypsin-resistent dimers; only about 4% were trypsin-accessible monomers. This indicates that membrane insertion was almost complete. The yield of refolding and membrane insertion of both proteins became optimal when the molar ratio DM/DMPC approaches 1. Detailed investigations indicated that this corresponds to the transition from membranes to micelles (data not shown). In these experiments, the concentration of detergent monomers in water

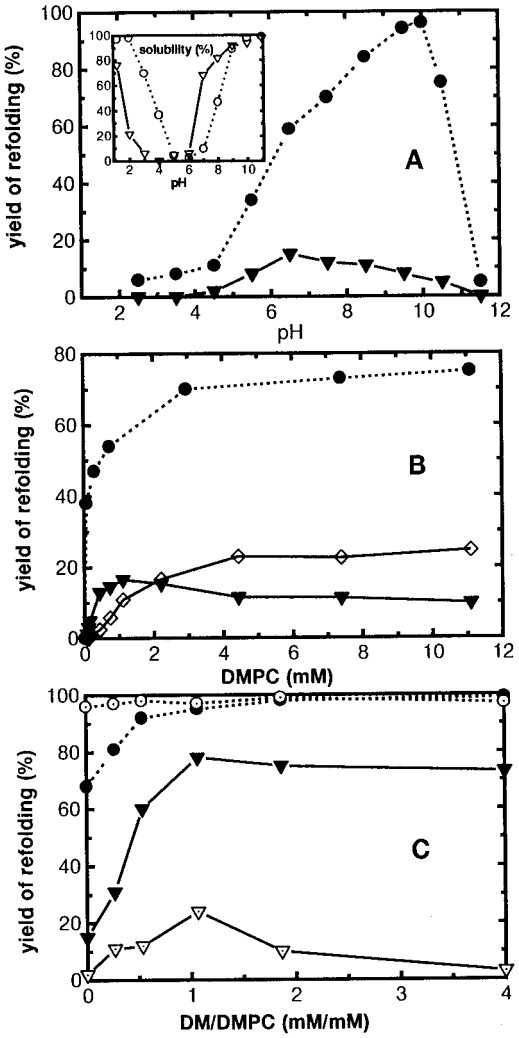

FIGURE 3: (A) Dependence on pH of the yield of insertion of OmpF (▼) as trimers into small vesicles of 1.5 mM DMPC and of OmpA (●) into small vesicles of 7.4 mM DMPC at 30 °C. Inset: pH dependence of the solubility of OmpF (▽) and OmpA (○) in water. (B) Dependence on lipid concentration of the yield of insertion of OmpF trimers (▼) and dimers (◇) at pH 6.5 and of OmpA at pH 7.3 (●) into small vesicles of DMPC at 30 °C. (C) Dependence on detergent concentration of the yield of insertion of OmpF trimers at pH 6.5 (▼) and pH 10.0 (▽) and of OmpA at pH 6.5 (●) and pH 10.0 (○) into small vesicles of 7.4 mM DMPC and varying amounts of DM. The protein concentration in all cases was 3 μM.

always remained below the critical micellar concentration (CMC), as determined by Lasch et al. (1990). Control experiments with DM below its CMC of 0.1 mM and without lipid revealed that detergent monomers do not induce refolding and trimerization (data not shown).

The yield of trimerization of OmpF was not dependent on vesicle size. OmpF inserted and trimerized into large vesicles of a diameter of 180 nm or larger to a similar extent as into small vesicles of 30−40 nm in diameter (data not shown). This is in contrast to OmpA, which inserts into small vesicles much more easily than into large vesicles (Surrey & Jähnig, 1995).

Kinetics of Folding and Membrane Insertion of OmpF As Compared to OmpA. The kinetics of refolding and insertion into pure DMPC vesicles were studied by CD, Trp fluorescence, and protease digestion, and were compared with the kinetics of OmpA.

CD measurements on OmpF could be performed over short times only, because of increasing turbidity of the samples, but they nonetheless reveal that within the mixing time

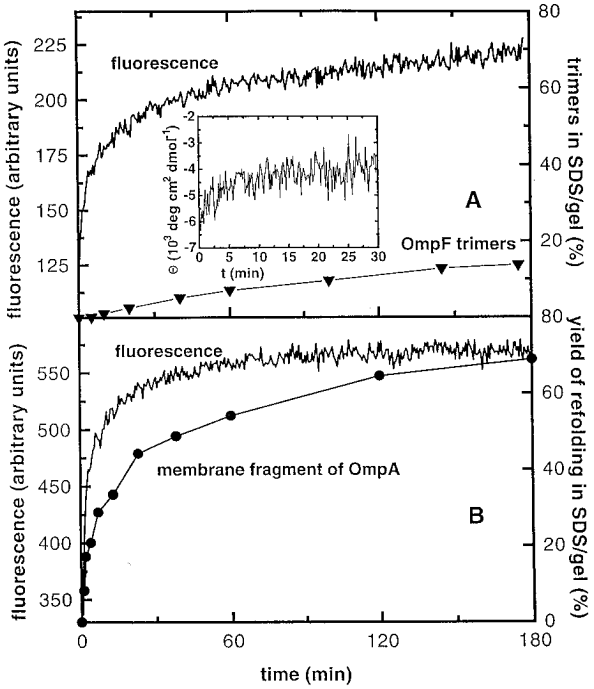

FIGURE 4: Kinetics of refolding and membrane insertion of 3 μM OmpF (A) at pH 6.5 and 3 μM OmpA (B) at pH 7.3 into 1.5 mM DMPC at 30 °C, as determined by Trp fluorescence and protease digestion with ensuing SDS gel electrophoresis. In panel A the fraction of OmpF trimers is shown (▼), and in panel B the fraction of membrane-inserted OmpA fragments (●). Inset in panel A: Circular dichroism of OmpF under the same conditions.

(below 1 s) the transition from the urea-unfolded state to the partially folded state in water takes place, followed by a biphasic transition toward the folded state (Figure 4A, inset). This biphasic transition is observed more clearly in the Trp fluorescence, the half-times lying around 5 min and 50 min (Figure 4A). The Trp fluorescence of OmpA exhibits a similar time course (Figure 4B). Such temporal behavior indicates the existence of a water-soluble and a membrane-bound intermediate. The time course of protection of the OmpF trimer against protease digestion is slower than the time course of Trp fluorescence. This kinetic difference between Trp fluorescence and protease digestion is much more pronounced for OmpF than for OmpA, with trimers appearing extremely slowly.

In order to study more closely the sequence of events in the course of trimerization of OmpF, insertion into DMPC membranes containing equimolar amounts of DM was investigated. Under these conditions, the yield of membrane insertion was nearly 100% (73% trimers and 23% dimers, Figure 3C). Trp fluorescence was not measured in this case because the samples were too turbid. Refolding and membrane insertion was followed by SDS gel electrophoresis of undigested and trypsin-digested OmpF. The densitometric analysis of the gels of digested OmpF indicates that the fraction of dimers increases within about 30 min and then decreases slightly to reach a value of 23% after 5 h (Figure 5). The fraction of trimers increases with a delay of a few minutes as compared to dimers and after 5 h reaches a value of 73%. The delay is indicative of a consecutive reaction, with dimers representing an intermediate state in trimer formation. Trimerization is accelerated compared to pure DMPC vesicles. The same should hold for dimerization; hence, the relatively rapid increase of fluorescence observed with pure DMPC vesicles cannot be caused solely by

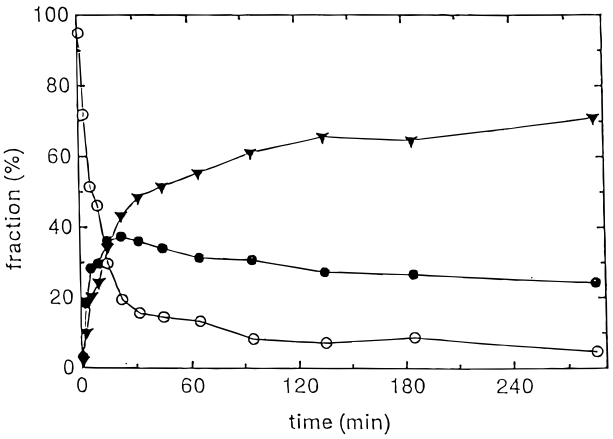

FIGURE 5: Kinetics of refolding and membrane insertion of 3 μM OmpF into 7.4 mM DMPC/7.4 mM DM, pH 6.5 at 30 °C, as determined by SDS gel electrophoresis of trypsin-digested samples and densitometric analysis showing the fractions of monomers (○), dimers (●), and trimers (▼).

insertion of dimers and trimers but must reflect to a considerable extent the association of monomers with membranes.

DISCUSSION

We have investigated refolding and membrane insertion of OmpF and compared it to OmpA. Hence, two β-barrel membrane proteins with different barrel size and oligomerization state in the membrane were studied: The trimeric OmpF consisting of three 16-stranded β-barrels, and the monomeric OmpA, whose membrane domain consists of a single 8-stranded β-barrel. If its periplasmic domain is cleaved off and only the membrane domain studied, the same results are obtained.

Conformational States. The states of OmpF in different environments resemble those of OmpA. Both proteins are soluble in their unfolded state in water/urea, and this appears to be a common property of β-barrel membrane proteins. Upon dilution of the urea-unfolded proteins into water, they adopt a partially folded state with a considerable content of β-structure, which is slightly higher for OmpF than for OmpA, but without any indication of trimerization of OmpF. The two proteins aggregate in this state at pH values around their isoelectric point, but can be kept soluble when charged at basic or acidic pH.

In the presence of vesicle membranes, the two proteins refold and insert. In the case of OmpA, monomers insert in a stable manner into the membrane, as indicated by their protease resistance. By contrast, OmpF monomers remain digestible, while dimers and trimers insert into the membrane, as indicated again by their protease resistance. The OmpF trimer was shown to be functionally active, i.e., it adopted the native structure. Trimerization of OmpF has been observed already in semipurified systems (Sen & Nikaido, 1991; de Cock et al., 1990). When the temperature is below the lipid phase transition temperature, both proteins adsorb to the membrane and adopt β-structure but remain monomeric and digestible.

Yield of Refolding and Insertion. OmpF inserts into membranes with a lower yield than does OmpA. This difference is caused mainly by oligomerization of OmpF. The yield of insertion of the two proteins depends on various parameters such as pH, concentration of lipid, and the presence of detergent. Optimal insertion is observed when the proteins are soluble in water, i.e., at basic pH up to a value of 10. Above pH 10, intramolecular charge repulsion could be the reason for the inability to refold and insert. No refolding and insertion occur in acidic solutions. A decreased stability of the proteins at acidic pH, as observed for OmpF in SDS below pH 4.5 (Markovic-Housley & Garavito, 1986), might be responsible for this behavior.

An increased content of detergent in the membranes increases the yield of insertion of the proteins, presumably due to perturbation of the membrane structure. Optimal conditions for trimerization of OmpF were found at the transition from membranes to micelles, indicating that regions of high flexibility are ideal for this process. Refolding is also possible into mixed micelles of lipid and detergent, as shown previously for OmpF (Eisele & Rosenbusch, 1990).

Of special relevance is the variation of the yield of insertion with lipid concentration. While the yield of inserted dimers increases monotonously with lipid concentration, the yield of inserted trimers first increases and then decreases. In the simplest model, one may consider inserted dimers and trimers as stable states that are in equilibrium with each other and with monomers in water. Increasing the lipid concentration would shift the equilibrium between inserted oligomers and water-dissolved monomers toward the membrane-inserted oligomers and leave the dimer−trimer equilibrium unaltered, so that the yields of dimers and trimers would both increase. This is not observed; hence the model has to be modified. One may assume that dimers are an intermediate state; they form irreversibly from water-dissolved monomers and transform irreversibly into trimers upon association with other monomers. An increase in lipid concentration would increase the yield of dimers, but the concomitant decrease in monomers would inhibit the formation of trimers at sufficiently high lipid/protein ratios. Inhibition would be avoided if the dimers could dissociate back into monomers. The observed decrease of the yield of trimers with lipid concentration thus indicates that membrane-inserted dimers are an intermediate state in trimerization and are relatively stable against dissociation into monomers.

It should be noted, however, that this conclusion was obtained under the assumption that the SDS gels reflect the actual state of oligomerization. If this assumption is waived, one may evoke other models. An attractive one would involve only trimers and no dimers as intermediate states. The monomers in the trimer would be protected or unprotected against protease digestion, e.g., all unprotected in the first intermediate, two protected and one unprotected in the second intermediate, and all protected in the final state. The second intermediate would be detected on the SDS gel as a protected dimer, which is indeed observed. However, in this model the yield of protected dimers as well as of trimers should increase monotonously with the lipid concentration, in contrast to the observation. Therefore, a model involving only trimers as intermediate states is not compatible with the experimental result. Especially, water-dissolved trimers are excluded, and trimerization must take place at or in the membrane. The sole possibility for an additional trimer is an intermediate state after the protected dimer, consisting of a protected dimer and an unprotected monomer.

Kinetics of Refolding and Insertion. The kinetics of refolding and membrane insertion of OmpA are reported elsewhere, together with a model for the folding pathway (Surrey & Jähnig, 1995). Upon dilution of urea-unfolded OmpA into water, a fast transition (below 1 s) from the

unfolded state U_W to a partially folded intermediate P_W was observed in CD measurements. This transition was proposed to correspond to the hydrophobic collapse of soluble proteins; hence the intermediate P_W would not be a partially correctly folded state but a completely misfolded state. In the presence of membranes, CD and Trp fluorescence revealed two slower transitions with half-times of about 5 and 30 min. They were interpreted as a transition from P_W to a partially correctly folded, membrane-bound intermediate P_M, and a transition from P_M to the native state F_M. The intermediate P_M was considered as the analogue of the molten globule of soluble proteins, implying that in P_M the global structure of OmpA would be established, but not yet the detailed structure. The time course of protection against trypsin digestion extended into the range of the transition from P_M to F_M, leading to the conclusion that in P_M the OmpA molecules are not fully inserted, thus a fraction of them is still accessible to the protease.

In the kinetic experiments on OmpF reported in the present article, the fast transition from the unfolded state in urea to the misfolded state in water was observed in CD measurements as previously for OmpA. The two slower transitions were again observed in CD and Trp fluorescence measurements and exhibited a similar time course as for OmpA. Therefore, the data will be interpreted in a similar way as for OmpA: Upon dilution of urea-unfolded OmpF into water, a fast transition from the unfolded state U_W to a misfolded intermediate P_W takes place. Upon association with membranes, P_W undergoes a moderately slow transition to a partially folded, membrane-bound monomeric intermediate $P1_M$, the analogue of the molten globule state of soluble proteins, from which it proceeds in a slow transition to the folded, membrane-inserted state. In this slow transition, however, OmpF differs from OmpA. In P_M, OmpA was at least partially protected against protease digestion, while OmpF monomers are not at all protected and must oligomerize to become protected. Dimers are already protected against protease digestion indicating insertion, and the delay with which trimers appear is compatible with the notion that dimers are an intermediate state in trimerization. Hence we propose the following model for the folding pathway of OmpF:

$$U_W \rightarrow P_W \rightarrow P1_M \rightarrow F2_M \rightarrow F3_M$$

This model is fully consistent with the conclusions drawn above from the variation of the yield of insertion with lipid concentration. As discussed there, a further intermediate $P1F2_M$ may exist between $F2_M$ and $F3_M$ consisting of a protected dimer and an unprotected monomer. Evidence for the existence of dimers in the folding pathway of OmpF has already been provided by Reid et al. (1988), and Misra et al. (1991) have proposed, based on genetic experiments, trimerization of the porin LamB to take place at the membrane.

A comparison with kinetic studies *in vivo* (Reid et al., 1988; Fourel et al., 1992) reveals that *in vitro* insertion and trimerization of OmpF into DMPC vesicles devoid of detergent is slower by several orders of magnitude than in the bacterium. Even the faster trimerization of OmpF in mixed membranes of DMPC and DM does not approach the rate of trimerization *in vivo*. This indicates that chaperones might catalyze membrane insertion and trimerization *in vivo*. Thus, apart from providing a model system to study folding and membrane insertion of a protein alone, OmpF may be used to investigate the influence of chaperones on folding, membrane insertion, and trimerization.

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