Refolding of *Escherichia coli* outer membrane protein F in detergent creates LPS-free trimers and asymmetric dimers

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The *Escherichia coli* OmpF (outer-membrane protein F; matrix porin) is a homotrimeric β -barrel and a member of the bacterial porin superfamily. It is the best characterized porin protein, but has resisted attempts to refold it efficiently *in vitro*. In the present paper, we report the discovery of detergent-based folding conditions, including dodecylglucoside, which can create pure samples of trimeric OmpF. Whereas outer membrane LPS (lipopolysaccharide) is clearly required for *in vivo* folding, the artificially refolded and LPS-free trimer has properties identical with those of the outer-membrane-derived form. Thus LPS is not required either for *in vitro* folding or for structural integrity. Dimeric forms of OmpF have been observed *in vivo* and are proposed to be folding intermediates. *In vitro*, dimers occur transiently in refolding of trimeric OmpF and, in the presence of dodecylmaltoide, pure dimer can be prepared. This form has less β -

structure by CD and shows lower thermal stability than the trimer. Study of these proteins at the single-molecule level is possible because each OmpF subunit forms a distinct ion channel. Whereas each trimer contains three channels of equal conductance, each dimer always contains two distinct channel sizes. This provides clear evidence that the two otherwise identical monomers adopt different structures in the dimer and indicates that the asymmetric interaction, characteristic of C3 symmetry, is formed at the dimer stage. This asymmetric dimer may be generally relevant to the folding of oligomeric proteins with odd numbers of subunits such as aspartate transcarbamoylase.

Key words: folding, ion channel, lipopolysaccharide, oligomer, outer-membrane protein F (OmpF), single-molecule technique.

INTRODUCTION

Recent analysis of single protein molecules has extended our understanding of protein folding and may allow us to examine previously hidden processes in protein folding [1–4]. Normal protein oligomers are essential for life, whereas unwanted oligomers are the basis of amyloid and prion diseases. Furthermore, the folding of membrane and oligomeric proteins has important implications for both biotechnology and medicine [5]. All protein oligomerization must begin by the association of two polypeptides, and these seed dimers lack the molecular context which will eventually stabilize them in the native state. This ensures that the seed dimer is a particularly difficult species to study. Some structures, including chaperones and toxins, form precise odd-numbered oligomers and here the progression from the dimer state is especially interesting.

OmpF (outer-membrane protein F), a trimeric outer-membrane protein from *Escherichia coli*, is a member of the non-specific diffusion porin family of Gram-negative bacteria. These proteins, which naturally transport small metabolites, form the uptake pathway for many antibiotics. They are extremely abundant on the cell surface and are the targets of protective antibodies and phages. They all consist of β -barrel trimers [6–8] which fold in the periplasm and outer membrane after being secreted as monomers across the cytoplasmic membrane. Studies of the closely related PhoE porin have shown the clearest picture of maturation of these proteins *in vivo*. After signal-sequence-dependent secretion across the inner membrane, the unfolded monomers bind to the periplasmic chaperone Skp [9] and then associate with phospholipids [10] or LPS (lipopolysaccharide) [10–12] before assembling into trimers. PhoE trimer formation precedes outer-

membrane insertion [13], and this step may be conducted by the Omp85 protein which belongs to a family of proteins that assist outer-membrane protein assembly in bacteria, chloroplasts and mitochondria [14]. In its X-ray crystallographic three-dimensional structure, OmpF exhibits intricate intermolecular contacts in which each subunit interacts strongly with the other two (Figure 1) [15]. In particular, the latching loop, which binds one monomer to the next (clockwise when viewed from the extracellular surface), means that each monomer interacts highly asymmetrically with its neighbours [15]. The dimeric form cannot make the same detailed contacts and would be assumed to be short lived, but, surprisingly, stable dimers occur on the *in vivo* [16] and *in vitro* [17–19] folding pathways. On the other hand, folded monomers may be created *in vivo* in the presence of LPS [20] or may result from trimer breakdown [13], but have not been observed *in vitro* when detergents only are used.

Although some porins can easily be refolded *in vitro* [21–25], preparation of large amounts of refolded OmpF has not previously been possible [17–19, 26, 27]. This may be due to the particularly intricate nature of the monomer interactions. In the present paper, we describe a new method which can produce amounts of pure dimer and trimer sufficient for biophysical study. LPS is important for efficient assembly of OmpF and related PhoE *in vivo* [28–30], for ion channel function [11] and it assists folding *in vitro* [20], but its role in the stability of the trimer has been long obscured by the lack of truly LPS-free samples. In the present paper, we show results which finally prove that LPS is not a required structural component of the OmpF trimer. More surprisingly, we are further able to show that the dimer consists of two differently folded monomers, therefore addressing a fundamental question on the folding of homotrimeric proteins [31].

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; BLM, bilayer lipid membrane; DG, n-dodecyl- β -D-glucopyranoside; DM, n-dodecyl- β -D-maltoide; DSC, differential scanning calorimetry; DTT, dithiothreitol; LPS, lipopolysaccharide; octyl-POE, n-octyl-oligo-oxyethylene; OG, n-octyl- β -D-glucopyranoside; Omp, outer membrane protein; V_{app} , applied transmembrane voltage; Z, zwittergent.

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Figure 1 OmpF trimer showing complex interfaces between monomers

Top left: cross-section of OmpF trimer indicating the interaction between two subunits. Note the intricate surface contacts. Top right: ribbon diagram of monomer showing protrusion of the latching loop. Bottom left: top view showing line of cross section and the different contacts made by each monomer with its right- and left-hand partner. Bottom right: ribbon diagram of the same view with one latching loop shown (see also the Supplementary Data at <http://www.BiochemJ.org/bj/392/bj3920375add.htm>).

MATERIALS AND METHODS

Bacterial strains and plasmids

OmpF inclusion bodies were expressed from *E. coli* BZB1107 using the pMS119 vector expression system [32]. The *ompF* signal sequence (residues 1–22) plus the initial alanine residue were replaced by a single methionine residue by the QuikChange method (Stratagene). Amplification of the QuikChange product was carried out in *E. coli* XL1-Blue cells before the plasmid was purified and transformed into *E. coli* BZB1107 cells for protein expression.

Protein expression and purification

Transformed cells were grown at 37°C in LB (Luria–Bertani) medium containing 0.3% (w/v) glucose, 50 µg/ml ampicillin and 30 µg/ml kanamycin. At a D_{600} of 0.6, the culture was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside), grown for a further 3 h, and then cells were harvested by centrifugation at 3000 *g* for 30 min. The cell pellet was resuspended in 20 mM Tris/HCl (pH 8.0), 0.5 mg of DNase, 0.5 mg of RNase and 0.102 mM benzamidine, and lysed using a French press. The suspension was then incubated on ice with 0.024 mg/ml AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride]/HCl. After removal of the remaining unbroken cells by centrifugation at 3000 *g* for 15 min, the inclusion bodies were pelleted by centrifugation at 15000 rev./min for 1 h in a 45-Ti rotor on a Beckman L7-80 ultracentrifuge. The membrane fraction was removed from the

inclusion body pellet by homogenization with 20 mM Tris/HCl, 1% (v/v) Triton X-100, pH 8.0, at 37°C for 30 min, followed by centrifugation as above. This washing step was repeated twice before the washed inclusion bodies were solubilized in a denaturation buffer (20 mM Tris/HCl and 6 M guanidinium chloride, pH 8.0) at 55°C for 30 min. Any undissolved material was removed by centrifugation at 38000 rev./min for 1.5 h. Denaturation buffer was replaced with 6 M urea and 50 mM Tris/HCl, pH 8.0, on a PD10 column (Amersham Biosciences) and the sample was purified by a 1-ml HiTrap Q Sepharose column (Amersham Biosciences) equilibrated with 6 M urea and 50 mM Bis-Tris (pH 7.0). Proteins bound to the column were eluted out using a linear gradient of NaCl from 0 to 0.5 M.

Protein refolding

The purified fractions containing the pure inclusion bodies were pooled and used for refolding. Refolding buffer without detergents contained 50 mM Tris/HCl, pH 8.0, 1 mM DTT (dithiothreitol) and 0.1 mM EDTA. Refolding was performed by a 20× dilution with thorough mixing of the pooled sample, whose concentration had been adjusted to 1.22 mg/ml, into the refolding buffer containing the desired detergents, followed by a stationary incubation at 37°C. The efficiencies of refolding were investigated for a variety of single and mixed detergents in various amounts: DG (n-dodecyl-β-D-glucopyranoside), DM (n-dodecyl-β-D-maltoside), OG (n-octyl-β-D-glucopyranoside), and a range of zwittergents (Calbiochem) (Z): Z8, Z10, Z12, Z14 and Z16.

Sample preparation for analyses

The refolded trimer was concentrated by ethanol precipitation. The precipitate was resuspended in 0.5 % (v/v) octyl-POE (n-octyl-oligo-oxyethylene), 20 mM Tris/HCl and 300 mM NaCl (pH 8.0). This resuspension buffer was exchanged with 1 % (w/v) DM and 1 % (w/v) OG in the refolding buffer (pH 8.0) on a PD-10 column.

The refolded dimer was concentrated by Centriprep YM-30 (Millipore). To standardize the detergent concentration, the concentrated sample was dialysed in a 50 kDa cut-off membrane against 1 % (w/v) DM and 1 % (w/v) OG in the refolding buffer (pH 8). Unless specified, all the refolded samples used in the analyses were in 1 % (w/v) DM, 1 % (w/v) OG, 50 mM Tris/HCl, 1 mM DTT and 0.1 mM EDTA (pH 8).

CD

CD measurements were performed on a Jasco J-810 spectropolarimeter. For far-UV CD spectra of secondary structure, samples in 0.2-mm path length cuvettes were scanned in the wavelength range 250–190 nm, using a 1 nm nominal bandwidth with ten accumulations. Spectra were then corrected for background by subtraction of a buffer and detergent blank.

To study protein thermal unfolding, samples were heated in 1-mm pathlength cuvettes from 20 to 95 °C at a rate of 1 °C/min. Secondary-structural changes were detected from the signal at wavelengths where the signals had the most abrupt change (208 nm for dimers, and 218 nm for trimers). The bandwidth of the wavelength was 2 nm. To determine the T_m (midpoint temperature), the data were then analysed using the method described in [33] and fitted to the Boltzman model using Origin 5.0.

Gel filtration

Gel-filtration chromatography was carried out using a Superose 12 column on a FPLC system (Amersham Biosciences). The column was pre-equilibrated with a filtered and degassed running buffer [0.1 % (w/v) DM, 150 mM NaCl and 25 mM Tris/HCl (pH 7.5)]. The experiment was performed at a flow rate of 0.5 ml/min.

Dimer cross-linking

Dimers in 50 mM sodium phosphate, 1 % (w/v) OG and 1 % (w/v) DM (pH 8) were cross-linked by glutaraldehyde: 40 μ l of 25 % (w/v) glutaraldehyde (grade 1, Sigma) was mixed with 1 ml of the dimer sample, followed by a 2 min incubation at room temperature (22 °C). To stabilize the cross-linkage, 50 μ l of freshly prepared 2 M NaBH₄ was added and incubated for 20 min at room temperature. Then, the sample was incubated at > 95 °C in SDS/PAGE sample buffer for 10 min before analysis by SDS/PAGE. This cross-linking procedure was as described in [34].

Proteolysis

Proteolysis studies were performed using 5 μ g/ml of proteinase K. Dimers were in 1 % (w/v) DM, 1 % (w/v) OG and 50 mM Tris/HCl (pH 8), while trimers were in 0.5 % (v/v) octyl-POE, 20 mM Tris/HCl and 300 mM NaCl (pH 8.0). Samples with the enzyme were incubated at 37 °C for the indicated time with a subsequent incubation with 10 mM AEBSF for 10 min at room temperature to terminate the enzyme activity. Samples mixed with loading buffer were finally analysed by SDS/PAGE.

DSC (differential scanning calorimetry)

Samples for the DSC analyses were filtered through 0.2- μ m diameter filters and degassed before loading. The experiment was

Figure 2 Refolded dimer and trimer states of OmpF

(A) SDS/PAGE analyses of the trimer refolding over time in 0.5 % (w/v) DG/0.2 % (w/v) DM in 50 mM Tris/HCl, 1 mM DTT and 0.1 mM EDTA (pH 8). Numbers above the lanes indicate the incubation time in min. Monomers only are present at zero time. (B) Pure preparations of dimer and trimer after refolding. Left-hand lane, trimer prepared as in (A), and right-hand lane, dimer [buffer as trimer but with 1 % (w/v) DM/1 % (w/v) OG]. (C) SDS/12% PAGE assays of the heat denatured (D) and native (N) cross-linked dimers. The boiled sample runs at the expected molecular mass (74 kDa) of the dimer. Molecular-mass sizes are indicated in kDa. For more detergent conditions, see also the Supplementary Data at <http://www.BiochemJ.org/bj/392/bj3920375add.htm>.

conducted at a heating rate of 1 °C/min from 25 to 100 °C, and an approximate pressure of 200 kPa. The data were fitted using the Origin 5.0 program.

Bilayer recordings

Planar bilayer experiments were conducted in two 1-ml chambers separated by a 100- μ m thick Teflon wall with a 150- μ m diameter aperture. The orifice was pre-treated with a hexadecane/pentane (1:10, v/v) solution. Both chambers were filled with 1 M NaCl, 5 mM CaCl₂, 50 mM Tris/HCl (pH 7.4) and a few microlitres of asolectin (10 mg/ml in hexane) added to the surface. To form the bilayer, the level of the solution in each chamber was raised above the pre-treated hole. Protein samples were added to the *cis* compartment where the voltage was applied through an Ag/AgCl wire. The *trans* side was held at virtual earth by the amplifier (Biologic BLM120). The exported signal was digitized and analysed by Strathclyde electrophysiology data recorder (version 2.3.3) software [35].

RESULTS AND DISCUSSION

The *in vitro* refolding of OmpF (from the urea-denatured state) can be controlled by altering the detergent conditions and has been well characterized [18,19]. By refolding in 0.5 % (w/v) DG/0.2 % (w/v) DM, we have shown for the first time that pure trimer preparations can be made (Figure 2A). Monomers detected at the beginning then disappear as initially SDS-resistant dimers, then trimers, are created (see also [18]). DM had been proved to promote refolding of OmpF in DMPC (dimyristoyl phosphatidylcholine) [17] to a mixture of dimers and trimers in approximately equal amounts; however, we stopped trimerization and stabilized the dimers in 1 % (w/v) DM with 1 % (w/v) OG (Figure 2B). In contrast, when the more hydrophobic DG was used with DM, trimerization was promoted such that 0.5 % (w/v) DG/0.2 % (w/v) DM gave the best yield of trimers (Figure 2B, and Supplementary Data at <http://www.BiochemJ.org/bj/392/bj3920375add.htm>). DM/OG solutions were poorly soluble and were maintained at 37 °C; nevertheless the mixture was not stable and formed two phases. This proved to be advantageous, since

the viscous lower layer contained concentrated OmpF protein trimers. The unusual detergent conditions required for efficient trimerization may recreate the specific conditions that may be provided by LPS [28], but there is no negative charge on these detergents. Alternatively, the phase behaviour of insoluble DG mixtures may contribute to a local concentration of the subunits, thus accelerating trimer formation. We cannot draw from this any strong conclusions regarding the folding *in vivo*, but, since LPS is not required for folding, it is important to determine whether the LPS-free dimers and trimers are similar to their *in vivo* folded counterparts.

Since OmpF remains folded in SDS, it migrates further, and somewhat variably, on SDS/PAGE compared with marker proteins of the same molecular mass. In order to confirm that the 58 kDa band on SDS/PAGE contained the 74 kDa dimer, the sample was cross-linked using glutaraldehyde and boiled before electrophoresis. This product runs at approx. 75 kDa, confirming that this form was the dimer (Figure 2C). To show that dimers exist in solution and not just on SDS/PAGE, samples of dimer and trimer were analysed by size-exclusion chromatography calibrated with samples of native LamB, PhoE, OmpF and OmpA. This again confirmed that dimers and trimers observed on SDS/PAGE were also present in 0.1 % (w/v) DM (Figure 3A).

To compare the secondary structure of dimers with that of trimers, CD of protein samples in 1 % (v/v) DM/1 % (v/v) OG was used (Figure 3B). The refolded trimer spectrum is similar to that of purified native OmpF (results not shown) and the published native OmpF spectrum [36] (Figure 3B). The spectrum of the dimer suggests a less folded β -structure. Unlike the trimer, the dimer was found to be destabilized by long exposure to SDS. CD revealed that the secondary structure of the dimer sample in 1 % (w/v) SDS became more α -helical after 3 days at -20°C , and SDS/PAGE showed that these samples had slowly reverted to unfolded monomers (results not shown). CD of the dimer in phospholipids was not possible, as this caused the formation of trimers and, interestingly, monomers (results not shown). This hints that the dimer to trimer transition may result from a dimer-dimer interaction in which one monomer is eventually excluded.

Both dimers and trimers show clear co-operative unfolding transitions upon heating which confirms that the dimer is very close to native state and is not a molten globule [37]. However, while the refolded trimer matches the high-temperature stability of the native trimer (results not shown), the dimer is less stable by approx. 20°C . This was confirmed by DSC which also revealed that the calorimetric enthalpy of the unfolding transition was reduced > 10 -fold (Figure 3C). Thermal denaturation of dimers and trimers was found to be irreversible [15] and so can be used only as a qualitative guide to stability. Trimeric OmpF is resistant to degradation by proteases, whereas the unfolded forms are degraded rapidly [17]. The dimer resisted treatment with proteinase K for 1 h, whereas the monomeric forms observed by SDS/PAGE were degraded completely (see the Supplementary Data at <http://www.BiochemJ.org/bj392/bj3920375add.htm>).

The OmpF ion channels have been studied extensively and show voltage-dependent gating in artificial BLMs (bilayer lipid membranes) [32,38–40]. This gating manifests as an increased probability of closing above a critical applied transmembrane voltage (V_{app}), but whether it has a physiological role is unclear. In 1 M NaCl and at a V_{app} above 140 mV, native OmpF trimer channels close in three equal (830 pS) steps [32,39]. These three ion channels correspond to the three independent channels observed in the high-resolution X-ray structure (Figure 1) [7]. This has allowed calculation of conductance values in agreement with the hypothesis that one monomer forms one channel [41]. Furthermore, mutagenesis of residues in the channel lumen leads

Figure 3 Solution behaviour of dimers and trimers

(A) Size-exclusion chromatography of trimers (solid line) and dimer (broken line) in 0.1 % (w/v) DM, 150 mM NaCl and 25 mM Tris/HCl. Measured elution volumes of LamB (trimer molecular mass of 160 kDa) and OmpA transmembrane domain (monomer molecular mass of 28 kDa) are shown by arrows. (B) Far-UV CD spectra of OmpF. Trimers [solid line, refolded initially in 0.5 % (w/v) DG/0.2 % (w/v) DM, 50 mM Tris/HCl, 1 mM DTT and 0.1 mM EDTA, and then buffer-exchanged] and dimers (broken line) in 1 % (w/v) DM/1 % (v/v) OG, 50 mM Tris/HCl, 1 mM DTT and 0.1 mM EDTA. The dotted line is native OmpF from [36], in 0.1 M NaCl, 20 mM sodium phosphate, 0.2 mM EDTA, 1 % (v/v) octyl-PGE, 0.2 mM DTT and 3 mM sodium azide. (C) Thermal stability of OmpF dimers and trimers. Left y-axis: proportion of unfolded dimer (●) and trimer (○) at each temperature measured by far-UV CD at 208 nm (dimer) and 218 nm (trimer). Right y-axis: DSC measurements of partial specific heat capacity (C_p) against temperature, for dimer (broken line) and trimer (solid line).

to expected increases in conduction [42]. After closing of the channels, removal of the V_{app} causes slow re-opening of channels, and reversal of V_{app} causes a more rapid re-opening. Furthermore, substates of closure often occur which are smaller than the defined channel size. Thus the single-channel analysis of OmpF reveals the behaviour of individual monomers within the trimer.

The dimer form was inserted into BLMs by adding small amounts of detergent-solubilized protein to the *cis* side of the bilayer. Thus the conditions of measurement change in two important respects: first, detergent is replaced by membrane phospholipids, and, secondly, the protein is at a very low concentration in a two-dimensional matrix. Thus the dimers in the BLM, at the concentrations needed for single-channel measurements (one to ten proteins in an area of 0.01 nm^2), are extremely

Figure 4 Single-molecule BLM studies

(A) Closures of single-dimer channels (upper trace) at $V_{\text{app}} = 180$ mV (lower trace). A single dimer was present which first closed its small channel (1). A second dimer inserted which again first closed its small channel which flickers shut before fully closing [(2) and inset]; the second native size closure could result from either dimer (3). Later, a small channel opens and closes (4). A brief period with $V_{\text{app}} = 0$ mV allows a single dimer to re-open which then closes in two steps, this time with the native sized channel first (5). (B) A single dimer is closed by $V_{\text{app}} = 180$ mV and opened by $V_{\text{app}} = 0$ mV. First, the small channel closes, then the large channel closes, but to a noisy state before re-opening, closing to a quiet state and then via a small channel to fully closed. It then re-opens with a large step which remains the only level open, even at $V_{\text{app}} = 0$ mV. Finally, this large step changes to a small conductance state, indicating interchange between states.

dilute. This means that they will probably lose their detergent molecules rapidly and they are extremely unlikely to fold further by intermolecular interaction. Hence, although the progression from dimeric to trimeric ion channels in the BLM would confirm the intermediate role of the dimer, the experiment cannot be performed at high protein concentrations. Insertion of dimeric OmpF was observed at a constant V_{app} by sudden defined increases in membrane current. When the voltage was > 150 mV, these channels closed in two steps. Thus it was always observed that dimeric preparations caused channels to insert in units of two while trimeric samples caused channels to insert in threes. These single-molecule data confirm that a dimeric form remains intact in the BLM, but we cannot measure its stability and secondary structure as for the detergent form. Surprisingly, the two channels within the dimer were of two different sizes (Figure 4A). The larger channel corresponds to the size (840 pS) seen in native trimers, but the smaller one is only 624 pS. When experiments were carried out with single dimers in the membrane, every one showed the two unequal steps. Importantly, the order of closing was nearly random, implying that each state was pre-formed in the membrane before closure and was not the result of the larger or smaller form having closed previously. Furthermore, there are rare examples (Figure 4B) where channels swap sizes when in the membrane. This can be observed when one channel has already closed and the remaining one adopts the alternative size. The low frequency of these events and the time resolution of the data acquisition mean that the possibility of the simultaneous closure of a large channel and opening of a small one can be discounted. Thus the two monomer forms appear to be stably different and in

Figure 5 Single-channel events from refolded dimer (solid bars) and trimer (open bars)

n_T and n_D indicate the number of channel events from dimer and trimer samples respectively recorded in each 45-pS-wide bin from 315 to 1100 pS. Gaussian fits of dimer data show two distinct populations not seen in the trimer sample.

slow equilibrium. Analysis of a large number of channels shows the expected bimodal distribution for dimers and unimodal for trimers (Figure 5). Slightly more large events are seen, and this may indicate a slight preference for these to close first.

The macroscopic stability analysis (CD, DSC, etc.) led to the assumption that the dimer creates a distinct less intricate subunit interface by the association of two partially folded forms. However, the single-molecule analysis shows that the dimer is composed of two unequal monomers: one like that found in native trimers, $M_{(T)}$, and one characteristic of the dimer form, $M_{(D)}$. The $M_{(D)}$ resembles channel substates seen sometimes in native OmpF and hence may not be far from native itself. Since OmpF forms an odd-numbered oligomer, the monomer interfaces are essentially asymmetric, and it appears that this is also a feature of the dimer. Thus one monomer may correctly latch on to the neighbour via loop 2 to create the dimer, but which of the two provides the non-native channel we cannot say (Figure 6). The smaller conductance form disappears upon trimer formation, and refolding of this subunit may provide some of the folding free energy that causes trimer formation. Dimers of the related protein, OmpC, have been isolated from *E. coli* strains which express mutant OmpF [43]. Why mutation of one Omp should cause this change in another is not clear, but provides more evidence that dimers may be important *in vivo*. These OmpC dimers show many of the features of the OmpF dimers, e.g. lower stability and smaller average channel size, but individual channels were not measured, and we can only guess that they are also asymmetric. Asymmetric dimer structures have been observed using X-ray crystallography [44] (where they may be induced by crystal contacts and flexible structures) and can be induced by ligand binding [45], but this is the first indication of such a structure on a protein-folding pathway. That this dimer is really on the folding pathway is probably in need of further proof, but, coupled with data from previous work [17], the current evidence is: the progression from dimer to trimer shown in Figure 2(A); the slow appearance of trimer from dimer under the 'dimer-folding conditions'; and the similar behaviour of the published *in vivo* dimer intermediate [16]. Garel [31] suggested that, since monomers in C3 symmetrical

Figure 6 Folding pathway for the OmpF trimer as revealed by macroscopic and single-channel methods

The dimer consists of a native state and a partially folded monomer. The model shows the dimer binding via loop 2 of one monomer with the remaining loop 2 unfolded. The eventual formation of trimers may be either from recruitment of monomers or by recombination with other dimers.

structures form asymmetric relationships, folding could proceed from asymmetric dimers which have already adopted their final positions [46] (Figure 6). The single-molecule data here are the first to show this route for any protein. How the two molecules adopt their respective roles is not clear, but the slow exchange between the two sites is indicative of the relative stability of this arrangement.

Recently, an OmpF dimer has been extensively characterized by Watanabe and Inoko [47] using X-ray and light scattering, and CD. The CD spectrum of the dimer shows more native structure than that shown in the present study.

In conclusion, the efficient folding of OmpF trimers is possible *in vitro* using detergents. The trimers which lack bound LPS resemble the naturally folded species, and the dimer provides clear evidence for an unexpected folding pathway involving an asymmetric dimer.

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