

Functional refolding of the *Campylobacter jejuni* MOMP (major outer membrane protein) porin by GroEL from the same species

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Functional and structural studies of outer membrane proteins from Gram-negative bacteria are frequently carried out using refolded proteins. Recombinant proteins are produced in *Escherichia coli* as inclusion bodies and then tediously refolded by dilution in buffered detergent solutions. In the present work, we obtained the refolding of MOMP (major outer membrane protein) from *Campylobacter* assisted by the molecular chaperone GroEL. Refolded MOMP recovered its native pore-forming activity when reconstituted in planar lipid bilayers. Both proteins were purified from the *Campylobacter jejuni* strain 85H. The purity of GroEL was assessed by silver staining and MS. Its native ultrastructure

was observed by the use of transmission electron microscopy. Denaturation of MOMP was performed in urea at 65 °C followed by dialysis against 100 mM acetic acid, and was assessed by CD analysis. MOMP refolding reached a maximum efficiency in the presence of GroEL (at a MOMP/GroEL molar ratio of 9:1) and ATP. Under these conditions, 95 % of denatured MOMP was refolded after a 15 min incubation. This approach represents an alternative method in studies of membrane protein refolding.

Key words: β -barrel, channel, heat-modifiable, outer membrane protein, porin, refolding.

INTRODUCTION

Although several structures of bacterial OMPs (outer membrane proteins) are now available, a large number of these proteins are still structurally and functionally poorly characterized. Membrane protein studies are frequently compromised by the difficulties encountered during the purification process. In the large majority of cases, heterologous membrane proteins are produced in *Escherichia coli* in the form of cytoplasmic inclusion bodies in order to avoid the toxic integration of protein into the membrane. Inclusion bodies must be solubilized, and then the protein must be refolded correctly to allow further structural and functional studies [1]. Although refolding of membrane proteins has been successfully achieved in the past, the number of potential unfolded candidates is increasing rapidly. Therefore it appears to be of major interest to develop new approaches for the *in vitro* folding of membrane proteins.

MOMP (major outer membrane protein) of *Campylobacter* has been studied extensively in our laboratory. This porin forms hydrophilic channels through the outer membrane. Two-dimensional crystallographic analysis showed that MOMP is structurally related to the family of trimeric bacterial porins [2]. However, MOMP trimers present a peculiar sensitivity to detergents, and folded monomers are recovered following treatment of purified trimers with 2 % (w/v) SDS. MOMP folded monomer showed an apparent molecular mass of 35 kDa on SDS/PAGE. CD analysis also demonstrated that the folded monomer mainly comprised β -sheet secondary structure, in agreement with the so-called β -barrel structure of porins [3]. MOMP folded monomers are able to form channels in artificial lipid bilayers with the same conductance properties as monomers embedded into trimers, which suggests that the folded monomer is the functional unit of the MOMP porin [4]. The native monomer could be heat-denatured at temperatures above 50 °C, yielding a pseudo

α -helical conformation, a characteristic of SDS-denatured β -barrels [5]. The denatured monomer presents an apparent molecular mass of 45 kDa by SDS/PAGE [3].

According to these data, the refolding of MOMP can be monitored easily by the shift of its apparent molecular mass on SDS/PAGE (from 45 to 35 kDa). Similar shifts in apparent molecular mass have been described previously for the *E. coli* porin PhoE [6–8]. Thus we could analyse the kinetics of MOMP refolding and determine the parameters which may influence this folding.

Chaperones promote the correct folding of nascent polypeptides, and prevent the aggregation of hydrophobic surfaces [9]. GroEL [HSP60 (heat-shock protein 60)], which consists of a 'double-doughnut' complex of 14 identical subunits of approx. 60 kDa, is essential for growth and viability under many environmental conditions [10]. *In vivo*, GroEL is involved in the folding of approx. 10 % of newly synthesized polypeptides [11]. Several studies have demonstrated the catalyst activity of GroEL to fold soluble proteins such as lysozyme [12] and malate dehydrogenase [13]. The size cut-off for GroEL substrates is consistent with the capacity of the catalytic chaperone cage, which allows substrates below 55 kDa [14]. GroEL from *Campylobacter jejuni* has been purified previously as a recombinant protein from *E. coli* [15] or from *C. jejuni* [16]. In the latter case, transmission electron microscopy demonstrated that GroEL oligomers present a typical double-doughnut shape [16]. Since OMPs are frequently produced as inclusion bodies and subsequently refolded *in vitro* [1], and since GroEL could be re-used several times as a folding agent [12], we reasoned that it could be used for the *in vitro* folding of OMPs.

In the present study, we show that native GroEL purified from *C. jejuni* is able to mediate the folding of *in vitro* fully denatured MOMP. After reconstitution in artificial membranes, the refolded MOMP exhibited its native pore-forming activity.

Abbreviations used: p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; HSP, heat shock protein; MALDI-MS, matrix-assisted laser-desorption ionization-time-of-flight MS; MOMP, major outer membrane protein; nMOMP, uMOMP and rMOMP, native, unfolded and refolded MOMP respectively; octyl-POE, n-octyl-polyoxyethylene; OMP, outer membrane protein; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK').

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Campylobacter jejuni 85H [17] was grown on Columbia Agar (Biomérieux, Marcy l'Etoile, France) at 42 °C in a microaerophilic environment.

Purification of MOMP from *C. jejuni* 85H

Purification of MOMP was achieved according to Dé and co-workers [4]. Briefly, OMPs were isolated from *C. jejuni* 85H and resuspended in octyl-POE (n-octyl-polyoxyethylene; Bachem AG, Bubendorf, Switzerland) at a final concentration of 1% (w/v). Porins in octyl-POE micelles were recovered by ultracentrifugation (100 000 g, 1 h, 4 °C). After ion-exchange chromatography followed by chromatofocusing on MonoQ and MonoP columns (Amersham Biosciences), purified MOMP was dialysed against 20 mM sodium phosphate (pH 7.6) containing 0.3% octyl-POE.

Purification of native GroEL from *C. jejuni*

C. jejuni 85H was grown at 42 °C on Columbia Agar for 2 days. Cells from 40 plates were harvested and resuspended in pre-heated Yeast-Tryptone and stressed for 1 h at 46 °C. These conditions had been shown to be efficient for overexpression of HSPs. Cells were then harvested by centrifugation (10 000 g, 30 min), resuspended in a lysis buffer containing 50 mM Tris/HCl (pH 8), 1 mM EDTA, 0.2 mM Tos-Lys-CH₂Cl (tosyl-lysylchloromethane; 'TLCK') and 0.3 mg/ml lysozyme and frozen at -20 °C. Cells were sonicated for 10 × 2 min, with a resting time of 2 min between each period in order to avoid over-heating and protein denaturation. Cell debris was removed by centrifugation (1000 g, 30 min). The supernatant volume was measured and a saline buffer containing 50 mM Tris/HCl (pH 7.5), 2 M NaCl, 2 M KCl, 20 mM MgCl₂ and 0.2 mM Tos-Lys-CH₂Cl was added to obtain final concentrations of 500 mM NaCl, 500 mM KCl, and 5 mM MgCl₂.

The sample was loaded on to a pre-equilibrated ATP-agarose column (Sigma) with a flow rate 0.1 ml/min, as described previously for periodontopathogenic bacteria [18]. After a wash, proteins were eluted with the same buffer containing 5 mM ATP. Fractions containing GroEL were pooled and concentrated (Centriprep; Millipore, Bedford, MA, U.S.A.). A final gel filtration (Superose 6; Amersham Biosciences) in 50 mM Tris/HCl, pH 7.5, 75 mM NaCl, 20 mM KCl and 5 mM MgCl₂ gave pure native GroEL from *C. jejuni*.

Transmission electron microscopy

Specimens of purified GroEL (30 µg/ml) from *C. jejuni* were prepared by staining for 1 min in aqueous uranyl acetate (1%, w/v; pH 5.5) on glow discharged carbon grids. Grids were blotted dry and examined in a Zeiss EM 109 electron microscope at an accelerating voltage of 80 kV.

MS analysis

MALDI (matrix-assisted laser-desorption ionization-time-of-flight)-MS was performed on a reflectron time-of-flight mass spectrometer equipped with delayed extraction (Voyager DE-RP; Perceptive Biosystems Inc.). A 0.7 µl sample was directly mixed on the support with an equal volume of sinapinic matrix (saturated solution of sinapinic acid in 40% acetonitrile/60% water/0.1% in trifluoroacetic acid).

Refolding of MOMP from *C. jejuni* using GroEL

A solution of 0.5 mg/ml MOMP from *C. jejuni* was diluted in 100 mM Tris/HCl, pH 6.7, containing 8 M urea and 150 mM dithiothreitol. MOMP was unfolded by a 5 min incubation at 65 °C and a rapid cooling on ice, and then was dialysed extensively against 100 mM acetic acid at 4 °C. Dialysed MOMP was diluted 4-fold in refolding buffer containing 100 mM Tris/HCl, pH 6.7, 10 mM ATP, 0.3% octyl-POE, 5 mM MgCl₂, 5 mM KCl and 20 mM NaCl (final concentrations). The mixture was then incubated for various periods of time at 25 °C. GroEL monomers and uMOMP (unfolded MOMP) were present at molar concentrations of 0.31 µM and 2.78 µM respectively; thus the GroEL/MOMP ratio reached 1:9 (mol/mol).

CD spectroscopy

CD spectra were recorded for solutions of proteins (0.5 mg/ml) using a CD6 spectropolarimeter (Jobin-Yvon, Longjumeau, France) at room temperature. Ellipticity values are expressed as mean residue molar ellipticity in units of degrees · cm⁻² · dmol⁻¹. Each spectrum was acquired with 0.02 cm path-length cells, between 190 and 250 nm, in steps of 0.5 nm and with an integration time of 2 s. Experimental data were corrected by subtracting the values of a blank obtained under the same conditions in the absence of protein. Both experimental and blank values were the means from five recordings.

Gel filtration

Gel filtration chromatography was performed on an Akta Explorer 10 liquid chromatography system equipped with a Superose 6 HR 10/30 column (Amersham Biosciences). The column was equilibrated with 2 column volumes of buffer [50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.3% octyl-POE and 0.1% (w/v) SDS for the nMOMP (native MOMP) monomer; or 100 mM acetic acid and 100 mM NaCl for uMOMP]. uMOMP was obtained after denaturation and acetic acid dialysis (see above). The nMOMP monomers were obtained after treatment of nMOMP with 1% (w/v) SDS as described previously [3]. The protein sample (1 ml) was applied and eluted in equilibration buffer. The absorption of the eluate was followed at 280 nm.

SDS/PAGE analysis

The method of Laemmli [19] was used to perform SDS/PAGE analysis (10% slab gels; Mini-Protean II; Bio-Rad Laboratories). Samples were resuspended in a buffer containing a decreased SDS concentration (0.05%, w/v). Taking into account the peculiar properties of MOMP, which maintained its native secondary structure in up to 2% (w/v) SDS solution at temperatures of up to 50 °C with an apparent molecular mass of 35 kDa in SDS/PAGE analysis, samples were not heated before loading except when specified. The migration was performed at 160 V for 1 h and proteins were stained with Coomassie Brilliant Blue (0.1%, w/v).

ATPase activity of GroEL protein

Determination of the ATPase activity of GroEL was performed according to Mendoza et al. [20]. Briefly, GroEL (4 µg) was incubated in 50 mM Tris/HCl, 10 mM MgCl₂ and 10 mM KCl, pH 7.5, in the presence of 1 mM ATP and 0.3 mM SDS. Control experiments were performed in the absence of ATP, SDS or both. After a 4 h incubation at 50 °C, an aliquot was mixed with 360-3 diagnostic reagent (Sigma), which forms a complex with P_i after

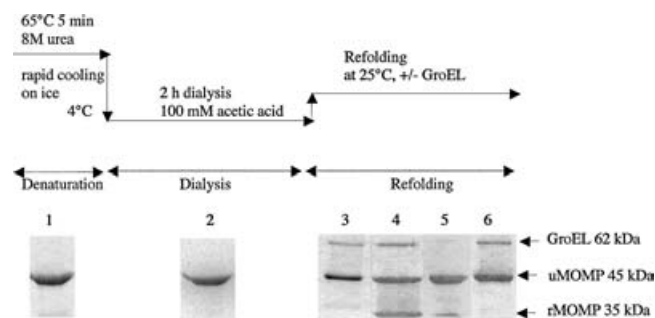


Figure 1 Steps in the procedure used to assay MOMP refolding

SDS/PAGE analysis of MOMP denatured after incubation for 5 min at 65 °C in 8 M urea and rapid cooling on ice (lane 1); MOMP was then dialysed (lane 2), and finally the soluble part was refolded in a refolding buffer containing ATP for 1 h (lane 3) or 4 h (lane 4) in the presence of GroEL, or for 4 h in the absence of GroEL (lane 5), or for 4 h in the presence of p[NH]ppA instead of ATP (lane 6). Arrows indicate GroEL, uMOMP and rMOMP.

ATP hydrolysis. The absorbance was monitored at 340 nm. A standard curve obtained with sodium phosphate buffer was used to determine the amount of P_i released.

Reconstitution in planar lipid bilayers

The method has been described previously by Dé and co-workers [4]. Briefly, solvent-free planar lipid bilayers were formed by the Montal and Muller technique [21], as modified by Saint and co-workers [22]. Current fluctuations were recorded using a BLM 120 amplifier (Biologic, Claix, France) and stored on a digital tape recorder (DTR 1202; Biologic). Currents and amplitude histograms were obtained from the stored signals using Satori software from Intracell (Royston, Herts., U.K.).

Asolectin IV-S from soybean (Sigma, St Quentin Fallavier, France) was used as lipid. GroEL or MOMP that was either unfolded or had been refolded for 1 h was added to the measurement compartments containing 4 ml of electrolyte solution (1 M NaCl, 10 mM Hepes, pH 7.4) at concentrations ranging from 0.25 to 0.5 ng/ml.

RESULTS

Characterization of uMOMP

The ability to achieve a soluble and stable uMOMP conformation was a prerequisite step for studying the refolding efficiency of the GroEL chaperone. nMOMP was purified according to Dé and co-workers [4]. After several assays, we selected the following denaturing conditions for nMOMP, which allowed us to avoid aggregation and spontaneous refolding of MOMP. nMOMP was denatured at 65 °C for 5 min in 8 M urea (Figure 1, lane 1) and further dialysed against 100 mM acetic acid for 2 h at 4 °C (Figure 1, lane 2). The obtained uMOMP showed an apparent molecular mass of 45 kDa in SDS/PAGE analysis (Figure 1, lanes 1 and 2), compared with an apparent molecular mass of 35 kDa observed for nMOMP [3].

In order to confirm that this apparent molecular mass of 45 kDa reflects a drastic disruption of secondary structure, CD experiments were carried out to analyse the secondary structures of uMOMP and nMOMP. The nMOMP CD spectrum was characteristic of the anti-parallel β -sheet secondary structure of porins, as observed previously by our group [3] (Figure 2, solid line). In contrast, uMOMP showed a loss of secondary structure (Figure 2, dotted line). One possible explanation for this loss of signal was that uMOMP had become aggregated after acetic acid

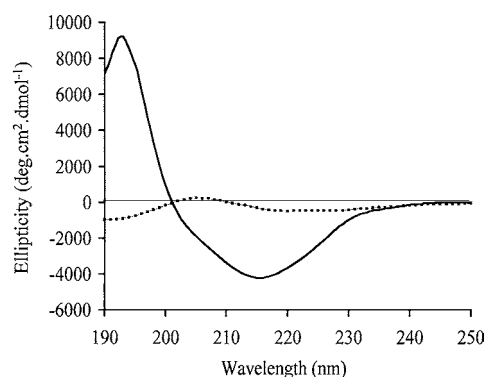


Figure 2 CD spectra in acetic acid of nMOMP (solid line) and uMOMP (dotted line) of *C. jejuni*

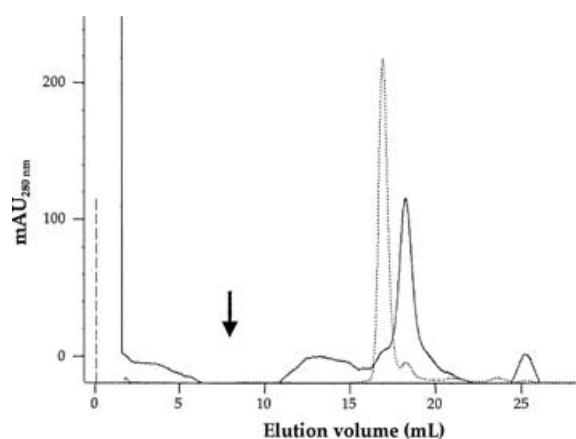


Figure 3 Gel filtration experiment comparing uMOMP (solid line) and nMOMP monomers (dotted line)

The arrow indicates the elution volume of Blue Dextran used as a calibration control. AU, absorbance units.

dialysis. To address this, uMOMP was analysed by size exclusion chromatography. The elution profile of uMOMP was compared with those of Blue Dextran (see Figure 3 legend) and of MOMP monomers obtained after treatment of nMOMP with 1% (w/v) SDS [3]. A major peak was observed which corresponded to treated nMOMP, as analysed by SDS/PAGE (Figure 3, solid line). The elution volume of uMOMP was slightly different from that of the monomers obtained from the native protein (Figure 3, dotted line), which demonstrates a peculiar conformation of the unfolded protein. However, as compared with the elution volume of Blue Dextran indicated by an arrow in Figure 3, no large aggregates were observed under the conditions used.

Characterization of purified GroEL from *C. jejuni*

The homogeneity of the GroEL preparation was assessed by silver-stained SDS/PAGE (results not shown). MS analysis detected a unique molecular species, with a calculated MALDI-MS molecular mass of 57924 Da, which is in good agreement with the theoretical mass of 57971 Da for GroEL from the *C. jejuni* NCTC 11168 strain (http://www.sanger.ac.uk/Projects/C_jejuni/) [24]. GroEL was in its native oligomeric state, as confirmed by transmission electron microscopy, with which the typical sevenfold rotation symmetry of the tetradecamer [(GroEL)₁₄] was observed on top-on view and side-on view (results not shown).

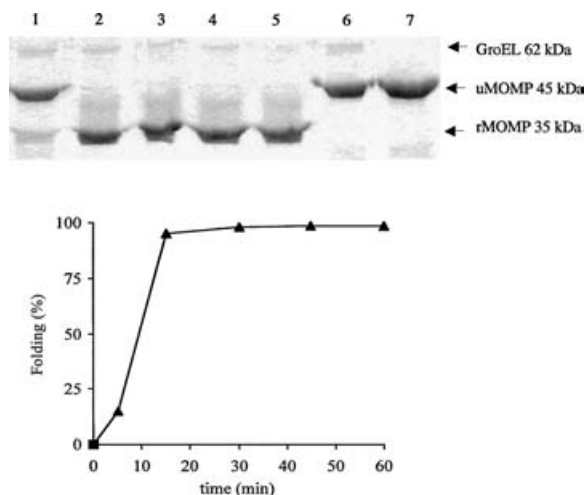


Figure 4 Kinetics of the refolding of denatured MOMP by GroEL from *C. jejuni*

Lanes 1–5, MOMP refolding in buffer containing GroEL and 0.3 mM SDS after 5 min (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4) and 60 min (lane 5); lane 6, MOMP refolding in buffer containing heat-denatured GroEL (65 °C, 5 min) and 0.3 mM SDS after 60 min; lane 7, refolding in the absence of GroEL after 60 min. Arrows indicate GroEL, uMOMP and rMOMP.

These results also showed that we obtained purified GroEL devoid of GroES contamination.

Refolding of uMOMP by GroEL

The folding activity of native GroEL was assayed using uMOMP. Native GroEL and uMOMP were incubated in refolding buffer at 25 °C according to Guise and Chaudhuri [12]. Under these conditions, we observed a shift in the apparent molecular mass of MOMP from 45 to 35 kDa (Figure 1, lane 4). The latter band corresponded to the folded form of MOMP, as described above. We named this refolded form of MOMP rMOMP. The refolding efficiency was weak after 1 h, since the majority of MOMP was still unfolded (Figure 1, lane 3). However, after a 4 h incubation in the presence of GroEL in the refolding buffer, MOMP was in large part refolded (> 50%) (Figure 1, lane 4). Moreover, as a control, MOMP refolding was assayed in the absence of GroEL, and we observed only very weak (< 5%) spontaneous refolding after a 4 h incubation (Figure 1, lane 5). These results suggest that GroEL may favour slow refolding of uMOMP under these conditions.

GroEL activity is well known to depend on ATPase activity. No refolding of uMOMP could be observed after 4 h in the presence of p[NH]ppA (adenosine 5'-[β,γ -imido]triphosphate), a non-hydrolysable ATP analogue (Figure 1, lane 6). With p[NH]ppA in the reaction mixture, no refolding was observed even in the presence of SDS (results not shown). This suggests that ATP is required for assisted MOMP refolding. Moreover, Li and co-workers [25] showed that the ATPase activity of GroEL increased with increasing SDS concentration up to 0.3 mM. Thus the refolding of uMOMP may depend on a low SDS concentration. This low SDS concentration could be used in our assay, since we showed previously that SDS treatment (up to 2%, w/v) of purified rMOMP at 25 °C dissociated the trimers into monomers, which (i) maintained their native β -sheet secondary structure [3,4], (ii) had a molecular mass lower than that of denatured monomers [3], and (iii) preserved their pore-forming functional activity [4]. Moreover, tetradecameric GroEL protein is not dissociated at SDS concentrations lower than 0.8 mM [25]. Thus, to investigate the effect of SDS on refolding efficiency, experiments were carried out with SDS concentrations ranging from 0 to 3 mM (incubation at 25 °C). After 1 h in a refolding mixture containing 0.3 mM SDS, 99% of rMOMP was recovered; however, only a small proportion of MOMP was refolded with the higher SDS concentrations tested (results not shown). According to these observations, subsequent refolding assays were performed at 25 °C in the presence of 0.3 mM SDS.

MOMP refolding kinetics

The kinetics of refolding were investigated in refolding buffer containing 10 mM ATP, 0.3 mM SDS and GroEL. After a 5 min incubation in this buffer, rMOMP represented approx. 15% of total MOMP (Figure 4, lane 1). This percentage increased rapidly to 95% after a 15 min incubation (Figure 4, lane 2) to reach a maximal level of 99% after 60 min (Figure 4, lanes 3–5). These results showed that MOMP refolding is a fast (less than 15 min) and efficient (up to 99%) process under *in vitro* conditions. As controls, refolding experiments were performed either with heat-denatured GroEL (Figure 4, lane 6) or without GroEL (Figure 4, lane 7). In both cases, no heat-modifiable rMOMP was detectable.

Channel-forming activity of rMOMP

The pore-forming activity of rMOMP was studied using reconstitution in planar lipid bilayers [4]. rMOMP caused well-isolated single events, leading to measurement of the porin single-channel conductance. Figure 5(A) presents the current variation obtained

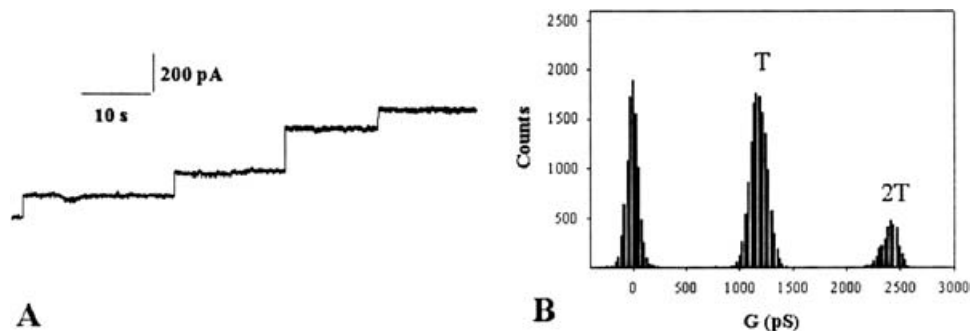


Figure 5 Single-channel activity of MOMP refolded by GroEL from *C. jejuni*

(A) Single-channel activity in 1 M NaCl induced by MOMP refolded by GroEL for 1 h. The recording demonstrates the incorporation of trimers at an applied potential of +90 mV. The associated histogram (B) shows two single-channel conductance levels of 1180 ± 80 pS (T) and 2410 ± 75 pS (2T).

after the incorporation of rMOMP in a preformed bilayer. Two main conductances were observed, corresponding to a trimer (1180 ± 80 pS; T) and two trimers (2410 ± 75 pS; 2T) (Figure 5B) as described previously by Dé and co-workers [4]. Under the same conditions, GroEL and uMOMP did not present any significant pore-forming activity (results not shown). The results showed that, with respect to the electrophysiological parameters associated with pore-forming activity, a fully active MOMP was obtained after the GroEL-dependent refolding process.

DISCUSSION

Protein folding is an essential step to ensure functional assembly. Moreover, the folding of β -barrel OMPs promotes trimer assembly and integration of the channel into the outer membrane. These steps have been studied extensively both *in vivo* and *in vitro*. Lipopolysaccharide and phospholipids are key elements promoting assembly of the *E. coli* OmpF porin *in vivo* [26] and the *E. coli* PhoE porin *in vitro* [27]. However, refolding *in vitro* involves dilution of denatured protein into buffered solutions containing large amounts of detergents [1]. Moreover, the kinetics of spontaneous folding obtained in these models are not relevant to physiological conditions. In the work presented here, we demonstrate for the first time that native GroEL purified from *C. jejuni* refolds a fully uMOMP prepared from the same strain, and that the refolding of MOMP promotes the recovery of its pore-forming activity.

To evaluate the interactions of MOMP and GroEL in an *in vitro* model, we first studied MOMP denaturation. We had demonstrated previously that 2% SDS and heating to over 50 °C could dissociate MOMP into monomers [3], leading to a switch from β -sheets to pseudo- α -helical secondary structure, as also observed for *E. coli* OmpF [28]. In the present study, treatment at 65 °C in 8 M urea was used instead of SDS treatment. The structure of soluble uMOMP dialysed against acetic acid was analysed by CD spectroscopy, which indicated a typical disordered structure. The uMOMP obtained was soluble without any detergent addition. Outer membrane β -barrel proteins do not show any stretch of hydrophobic amino acid residues, suggesting that a disordered structure may be soluble in water without any detergent addition. Moreover, dialysis against 100 mM acetic acid (pH 2.5) may prevent the aggregation of MOMP, the pI of which reaches 4.56 [29]. We hypothesize that uMOMP may exhibit a disordered structure which could be recognized by chaperones, including GroEL, exerting a binding pressure towards the fully unfolded proteins [30–32]. It has been shown recently by Conlan and Bayley [33] that the OmpG porin from *E. coli* could be obtained in a soluble form after denaturation and that this soluble form behaved as a stable unfolded structure when analysed by size exclusion chromatography. We have applied the same procedure to uMOMP. Under these conditions, the elution volume of uMOMP was approx. 18 ml, which is far from the void volume, in accordance with the results obtained by Conlan and Bayley [33]. Moreover, the elution profile of uMOMP was compared on the same column with that of nMOMP. In this latter case the elution volume of nMOMP was approx. 17 ml. Taken together, these results show that the soluble uMOMP exhibited a peculiar conformation, in agreement with the results obtained by Conlan and Bayley [33] with the OmpG porin.

In a second step, we evaluated the refolding activity of GroEL on uMOMP. GroEL is a chaperone that plays an important role in the folding of newly synthesized proteins, and in stress tolerance by preventing protein misfolding and aggregation. The conditions of MOMP refolding were very difficult to finalize. MOMP was refolded efficiently in the presence of GroEL at a

molar ratio of monomeric GroEL/MOMP of 1:9. This efficiency was improved when 0.3 mM SDS was added to the refolding mixture containing GroEL. Since refolding efficiency depended on the addition of 0.3 mM SDS in the folding buffer, and since GroEL ATPase activity has been shown by others to also increase in these conditions [25], it was tempting to hypothesize that the refolding efficiency of uMOMP depends on GroEL ATPase activity. However, although under our conditions GroEL ATPase activity was approx. 0.012 μ mol/min per mg of GroEL, which is in agreement with previous reports [20,34], this activity was not significantly modified by SDS. This suggests another role for SDS. Other detergents such as Triton X-100 are known to play an important role in the *in vitro* assembly of the PhoE protein, and De Cock and co-workers [35] suggested that the detergent could act *in vitro* as a molecular chaperone. Such a molecule may stabilize PhoE in an assembly state. This was not the case with SDS in our experiment, since uMOMP remained unfolded after a 4 h incubation in the absence of GroEL (Figure 4, lane 7).

Interestingly, the refolding reaction was almost complete after a 15 min incubation of uMOMP in the refolding mixture, suggesting a fast and efficient response under these conditions. The key point of our study showed that treatment with GroEL is able to generate rMOMP from uMOMP, and that the rMOMP obtained exhibits pore-forming activity in lipid planar bilayers. In contrast with uMOMP, this rMOMP is able to insert in artificial membranes and generate channels, clearly demonstrating its correct folding. Moreover, these channels presented conductance values similar to those observed previously with native trimers, suggesting that rMOMP could be inserted into membranes as trimeric forms [4]. Since outer membrane lipid components have been shown to be involved in OmpF assembly [26], incorporation of rMOMP into lipid planar bilayers may facilitate its trimerization, as observed by Van Gelder and co-workers for PhoE [7].

In the present study, the role of the co-chaperone GroES was not analysed. The purification of GroEL was carried out by affinity chromatography on ATP-agarose followed by exclusion chromatography. As confirmed by MS, the final preparation was devoid of GroES. We showed that MOMP refolding required both GroEL and ATP. The non-hydrolysable ATP analogue p[NH]ppA was not able to promote refolding when added instead of ATP. This suggests that the release of MOMP from GroEL requires ATP hydrolysis. A study by Kubo and co-workers [36] showed that the refolding of enolase (a protein of approx. 47 kDa) by GroEL did not require GroES provided that the nucleotide was ATP. GroES was only required when other nucleotides were used. In a more recent study, Mizobata and co-workers [37] showed that a GroEL mutant that is unable to bind GroES was still capable of improving the refolding yield of lactate dehydrogenase. MOMP is a β -barrel protein; such proteins show efficient spontaneous refolding and, when folded, are very resistant to denaturation. Taken together, these data strongly suggest that the role of GroEL in MOMP refolding may be different from its role in the refolding of small, monomeric and water-soluble proteins. In this particular case, GroES appeared not to be necessary to release MOMP from GroEL provided that ATP was added, similarly to enolase refolding.

To conclude, analysis of the interactions between MOMP and GroEL should highlight a possible role for GroEL in structural studies on membrane proteins.

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REFERENCES

- 1 Buchanan, S. K. (1999) Beta-barrel proteins from bacterial outer membranes: structure, function and refolding. *Curr. Opin. Struct. Biol.* **9**, 455–461
- 2 Zhuang, J., Engel, A., Pagès, J.-M. and Bolla, J.-M. (1997) The *Campylobacter jejuni* porin trimers pack into different lattice types when reconstituted in the presence of lipid. *Eur. J. Biochem.* **244**, 575–579
- 3 Bolla, J.-M., Loret, E., Zalewski, M. and Pagès, J.-M. (1995) Conformational analysis of the *Campylobacter jejuni* porin. *J. Bacteriol.* **177**, 4266–4271
- 4 Dé, E., Jullien, M., Labesse, G., Pagès, J.-M., Molle, G. and Bolla, J.-M. (2000) MOMP (major outer membrane protein) of *Campylobacter jejuni*; a versatile pore-forming protein. *FEBS Lett.* **469**, 93–97
- 5 Eisele, J.-L. and Rosenbuch, J. P. (1990) *In vitro* folding and oligomerization of a membrane protein. Transition of bacterial porin from random coil to native conformation. *J. Biol. Chem.* **265**, 10217–10220
- 6 De Cock, H., Hendricks, R., de Vrije, T. and Tommassen, J. (1990) Assembly of an *In vitro* synthesized *Escherichia coli* outer membrane porin into its stable trimeric configuration. *J. Biol. Chem.* **265**, 4646–4651
- 7 Van Gelder, P., De Cock, H. and Tommassen, J. (1994) Detergent-induced folding of the outer-membrane protein PhoE, a pore protein induced by phosphate limitation. *Eur. J. Biochem.* **226**, 783–787
- 8 Van Gelder, P. and Tommassen, J. (1996) Demonstration of a folded monomeric form of porin PhoE of *Escherichia coli* *in vivo*. *J. Bacteriol.* **178**, 5320–5322
- 9 Guagliardi, A., Cerchia, L., Bartolucci, S. and Rossi, M. (1994) The chaperonin from the archaeon *Sulfolobus solfataricus* promotes correct refolding and prevents thermal denaturation *In vitro*. *Protein Sci.* **3**, 1436–1443
- 10 Fayet, O., Ziegelhoffer, T. and Georgopoulos, C. (1989) The groES and groEL heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**, 1379–1385
- 11 Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F. and Hartl, U. (1999) Identification of *in vivo* substrates of the chaperonin GroEL. *Nature (London)* **402**, 147–154
- 12 Guise, A. D. and Chaudhuri, J. B. (1998) Recovery and reuse of the molecular chaperone GroEL for *In vitro* protein refolding. *Biotechnol. Prog.* **14**, 343–346
- 13 Tieman, B. C., Jonhston, M. F. and Fisher, M. T. (2001) A comparison of the GroE chaperonin requirements for sequentially and structurally homologous malate dehydrogenase. *J. Biol. Chem.* **276**, 44541–44550
- 14 Xu, Z., Horwich, A. L. and Sigler, P. B. (1997) The crystal structure of the asymmetric GroEL-GroES-(ADP)₇ chaperonin complex. *Nature (London)* **388**, 741–749
- 15 Thies, F. L., Weishaupt, A., Karch, H., Hartung, H.-P. and Giergerich, G. (1999) Cloning, sequencing and molecular analysis of the *Campylobacter jejuni* groESL bicistronic operon. *Microbiology* **149**, 89–98
- 16 Takata, T., Wai, S. N., Takade, A., Sawae, Y., Ono, J. and Amako, K. (1995) The purification of a GroEL-like stress protein from aerobically adapted *Campylobacter jejuni*. *Microbiol. Immunol.* **39**, 639–645
- 17 Kelle, K., Pagès, J.-M. and Bolla, J.-M. (1998) A putative adhesin gene cloned from *Campylobacter jejuni*. *Res. Microbiol.* **149**, 723–733
- 18 Hinode, D., Grenier, D. and Mayrand, D. (1996) A general procedure for the isolation of heat-shock proteins from peridontopathogenic bacteria. *J. Microbiol. Methods* **25**, 349–355
- 19 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 20 Mendoza, J. A., Warren, T. and Dulin, P. (1996) The ATPase activity of chaperonin GroEL is highly stimulated at elevated temperatures. *Biochem. Biophys. Res. Commun.* **229**, 271–274
- 21 Montal, M. and Muller, P. (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3561–3566
- 22 Saint, N., Lou, K. L., Widmer, C., Luckey, M., Schirmer, T. and Rosenbuch, J. P. (1996) Structural and functional characterization of OmpF porin mutants selected for larger pore size. II. Functional characterization. *J. Biol. Chem.* **271**, 20676–20680
- 23 Reference deleted
- 24 Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holtroyd, S. et al. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences *Nature (London)* **403**, 665–668
- 25 Li, S., Wang, L.-T. and Zhou, H.-M. (1999) SDS-induced conformational changes and inactivation of the bacterial chaperonin GroEL. *J. Protein Chem.* **18**, 653–657
- 26 Bolla, J.-M., Ladzinski, C. and Pagès, J.-M. (1988) The assembly of the major outer membrane protein OmpF of *Escherichia coli* depends on lipid synthesis. *EMBO J.* **7**, 3595–3599
- 27 De Cock, H., Pasveer, M., Tommassen, J. and Bouveret, E. (2001) Identification of phospholipids as new components that assist in the *In vitro* trimerization of a bacterial pore protein. *Eur. J. Biochem.* **268**, 865–875
- 28 Rosenbusch, J. P. (1974) Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. *J. Biol. Chem.* **249**, 8019–8029
- 29 Bacon, D. J., Johnson, W. M. and Rodgers, F. G. (1999) Identification and characterisation of a cytotoxic porin-lipopolysaccharide complex from *Campylobacter jejuni*. *J. Med. Microbiol.* **48**, 1–10
- 30 Khan, R. H. and Khan, F. (2002) Models for protein folding and nature's choice of protein as catalyst. *Biokhimiya (Moscow)* **67**, 520–524
- 31 Flynn, G. C., Beckers, C. J., Baase, W. A. and Dahlquist, F. W. (1993) Individual subunits of bacterial luciferase are molten globules and interact with molecular chaperones. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10826–10830
- 32 Buckle, A. M., Zahn, R. and Fersht, A. R. (1997) A structural model for GroEL-polypeptide recognition. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3571–3575
- 33 Conlan, S. and Bayley, H. (2003) Folding of a monomeric porin, OmpG, in detergent solution. *Biochemistry* **12**, 9453–9465
- 34 Sagane, Y., Hasegawa, K., Mutoh, S., Kouguchi, H., Suzuki, T., Sunagawa, H., Nakagawa, T., Kamaguchi, A., Okasaki, S., Nakayama, K. et al. (2003) Molecular characterization of GroES and GroEL homologues from *Clostridium botulinum*. *J. Protein Chem.* **22**, 99–108
- 35 De Cock, H., van Blockland, S. and Tommassen, J. (1996) *In vitro* insertion and assembly of outer membrane protein PhoE of *Escherichia coli* K-12 into the outer membrane. Role of Triton X-100. *J. Biol. Chem.* **271**, 12885–12890
- 36 Kubo, T., Mizobata, T. and Kawata, Y. (1993) Refolding of yeast enolase in the presence of the chaperonin GroE. The nucleotide specificity of GroE and the role of GroES. *J. Biol. Chem.* **268**, 19346–19351
- 37 Mizobata, T., Kawagoe, M., Hongo, K., Nagai, J. and Kawata, Y. (2000) Refolding of target proteins from a "rigid" mutant chaperonin demonstrates a minimal mechanism of chaperonin binding and release. *J. Biol. Chem.* **275**, 25600–25607

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