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Single channel analysis of recombinant major outer membrane protein porins from *Chlamydia psittaci* and *Chlamydia pneumoniae*

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Abstract We recently demonstrated that the major outer membrane protein of *Chlamydia psittaci*, the primary vaccine candidate for combating chlamydial infections, functions as a porin-like ion channel. In this study, we have cloned, expressed and functionally reconstituted recombinant major outer membrane proteins from *C. psittaci* and *Chlamydia pneumoniae* and analysed them at the single channel level. Both form porin-like ion channels that are functionally similar to those formed by native *C. psittaci* major outer membrane protein. Also, like the native channels, recombinant *C. psittaci* channels are modified by a native major outer membrane protein-specific monoclonal antibody. This is the first time that native function has been demonstrated for recombinant chlamydial major outer membrane proteins. Future bilayer reconstitution will provide a strategy for detailed structure/function studies of this new subclass of bacterial porins and the work also has important implications for successful protein refolding and the development of improved subunit vaccines.

Key words: *Chlamydia*; Major outer membrane protein; Planar lipid bilayer; Porin; Recombinant protein

1. Introduction

Chlamydia species are obligate, intracellular pathogens distinguishable from other gram negative bacteria by a distinctive biphasic developmental cycle in which an extracellular infectious form, the elementary body (EB), alternates with an intracellular metabolically active form, the reticulate body (RB). These organisms are responsible for a broad range of diseases in humans and animals. *Chlamydia trachomatis* is the leading cause of human sexually transmitted bacterial disease resulting in infertility and in some developing countries it is also the largest single cause of preventable blindness (trachoma). *Chlamydia pneumoniae*, known to cause pneumonia and bronchitis [1], has also been implicated as a causal factor in asthma [2], atherosclerosis [3] and, more recently, Alzheimer's disease [4]. The two animal pathogens, *Chlamydia psittaci* and *Chlamydia pecorum*, are major causes of ruminant abortion and infertility and infection with the ovine enzootic abortion (OEA) subtype of *C. psittaci* can cause abortion in women [5].

MOMP, a 40 kDa major outer membrane protein, common to all four species of *Chlamydia*, is central to many aspects of chlamydial biology. It is also a target for several neutralising antibodies, making it the primary focus for vaccine development. We recently reported the purification, structural analysis and functional reconstitution of native OEA *C. psittaci* MOMP [6]. Circular dichroism (CD) indicated that MOMP has a β-sheet-rich, porin-like secondary structure and the reconstitution of MOMP into planar lipid bilayers gave rise to porin-like ion channels [6]. This work extended the liposome swelling studies of Bavoil et al. [7] and added fresh insights into MOMP structure/function relationships. For example, our observations suggested that MOMP associates as a trimer within the outer membrane, similar to the trimeric arrangement of other bacterial porins. In addition, we proposed a possible physiological function for MOMP, following demonstration of the porin's ability to transport nucleoside triphosphates, which may be required to initiate the metabolic activity of the intracellular RB following its conversion from the EB in the early stages of the organism's complex intracellular developmental cycle.

In order to enable detailed studies of MOMP structure/function relationships, we have cloned, expressed and functionally reconstituted recombinant MOMPs from both OEA *C. psittaci* and *C. pneumoniae*. To our knowledge, this is the first time that native function has been demonstrated for recombinant chlamydial MOMPs and the refolding of recombinant chlamydial porins into functional, native conformations has important implications for future structure/function studies and vaccine development.

2. Materials and methods

2.1. Materials

The Expand Long Template PCR System was obtained from Boehringer, Mannheim. Expression vector pET-22b(+) and host strain BL21 (DE3) were from Novagen, while host strain XL-1 blue was from Stratagene. *n*-octyl-β-D-glucoside was purchased from Calbiochem and bilayer lipid was obtained from Avanti polar lipids. The monoclonal antibody (mAb) 4/11 [8] recognises a linear epitope of OEA *C. psittaci* MOMP and the neutralising mAb A11 [9], which recognises a conformational epitope of native MOMP, was kindly provided by A. Andersen. The *C. pneumoniae* native MOMP-specific mAb was purchased from Dako.

2.2. Preparation of chlamydial genomic DNA

The OEA *C. psittaci* S26/3 isolate was grown in McCoy cells [10] and elementary bodies were purified from infected cells by density gradient centrifugation. Genomic DNA was extracted as described previously [10]. *C. pneumoniae* IOL-207 genomic DNA was a gift from the late J. Treharne.

2.3. Generation of MOMP expression constructs

The *MOMP* genes were amplified from *C. psittaci* and *C. pneumoniae* genomic DNA using the expand long template PCR system according to the manufacturers' instructions. The primer pairs used were 5′-ATCGATGGC**CATATG**TGGGAAGGTG-3′ plus 5′-GGGC**GAATTC**TTATGCGAATGGAT-3′ and 5′-TGATGGT**CATAT-**

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GTGGGAAGGTGCTGCAGG-3′ plus 5′-AGCGGCCGCTCA-GAATCGAACT-3′ for *C. psittaci* (GenBank accession number X51859) and *C. pneumoniae* (M69230), respectively. The PCR reaction conditions were as follows: 1 min denaturation at 94°C for 1 cycle, 45 sec denaturation at 94°C, 1 min annealing at 50°C and 2 min extension at 68°C for 30 cycles and a final 8 min extension at 68°C for 1 cycle. The resulting 1.21 kb (*C. psittaci*) and 1.36 kb (*C. pneumoniae*) PCR fragments were cloned in frame into the expression vector pET-22b(+) via engineered *Nde*I/*Eco*RI (natural *Eco*RI site) and *Nde*I/*Not*I restriction enzyme sites, respectively, in the primers (these sites are indicated in bold in the primer sequences) by using procedures described previously [11]. Initial cloning was performed in *recA* mutant strain XL-1 Blue, which lacks the gene for T7 RNA polymerase, to allow examination of the construct sequences. The *C. psittaci* construct encodes a truncated version of MOMP starting at the first natural methionine (useful for creating a *Nde*I site for cloning into the pET vector) and therefore lacks the first 16 amino acids of the mature protein [12]. *C. pneumoniae* MOMP was engineered in a similar way to introduce a methionine residue (via the insertion of a *Nde*I site) at the corresponding position.

2.4. The production of recombinant and native MOMPs

The native *C. psittaci* MOMP was obtained as previously described [6]. To obtain recombinant proteins, the pET constructs described above were transformed into expression host BL21 (DE3) and protein expression was induced as described previously [13]. The expressed products formed inclusion bodies which were purified by a procedure involving sonication and differential centrifugation to remove outer membranes and other bacterial components, as described in Novagen's pET system manual. The proteins were analysed by SDS-PAGE and immunoblotting, as previously described [6]. Very small amounts of recombinant MOMPs were solubilised from inclusion bodies using 2% (w/v) *n*-octyl-β-D-glucoside containing 1 mM dithiothreitol (OG/DTT) prior to bilayer incorporation. In addition, *Escherichia coli* outer membrane vesicles were prepared from the non-induced bacteria by a procedure involving sonication and differential centrifugation (see above) and these were also solubilised in OG/DTT prior to incorporation.

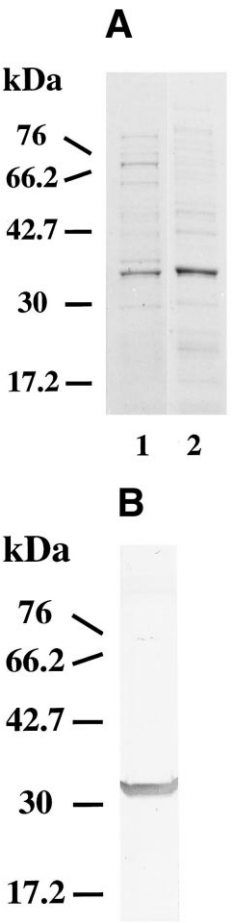

Fig. 1. SDS-PAGE and immunoblot analyses of purified *C. psittaci* and *C. pneumoniae* recombinant MOMPs. (A) SDS-PAGE analysis of MOMP from (1) *C. psittaci* and (2) *C. pneumoniae* using 12.5% (w/v) gels with Coomassie staining. (B) *C. psittaci* MOMP immunoblotted and probed with mAb 4/11. Molecular masses are as indicated.

2.5. Planar bilayer reconstitution

Recombinant MOMPs were incorporated into 0.3 mm diameter planar lipid bilayers cast from decane suspensions of diphytanoylphosphatidylcholine using standard reconstitution and recording methods, as previously described for native *C. psittaci* MOMP [6]. Control experiments were carried out using *E. coli* BL21(DE3) outer membrane proteins (Omps) which were also solubilised in OG/DTT and reconstituted in exactly the same way as inclusion body proteins. The bilayers were bathed in 50–500 mM KCl buffered with 10 mM Tris-HCl, pH 7.4, and the solutions were changed by perfusion (10 volumes) or by the addition of aliquots of 3 M KCl, as required. The *cis* chamber was voltage-clamped (using a biologic RK-300 patch clamp amplifier) at a range of potentials relative to the *trans* chamber, which was grounded, and positive currents flowing from *cis* to *trans* were recorded as 'upwards' deflections. The data were low-pass filtered (100–300 Hz, −3 dB point, Bessel-type response) and analysed using pClamp 6 (Axon instruments). Single channel slope conductances were measured from current/voltage plots and relative anion versus cation permeabilities (P_A/P_C) were calculated from reversal potentials using the following equation:

$$P_A/P_C = \{n - \exp(E/K)\}/\{[n.\exp(E/K)] - 1\}$$

where n is the *cis*:*trans* concentration ratio (corrected for activities using standard tables), E is the reversal potential (in mV) and K is 26 mV at room temperature. Measurements of P_o, the probability of a single channel being open, were made using pClamp 6 as described and discussed previously [14]. Conductance, permeability and P_o data were analysed by non-paired *t*-testing. The *C. psittaci* oligomeric MOMP-specific neutralising monoclonal antibody A11 (final dilution of 1/500) was added to both the *cis* and *trans* chambers.

3. Results

3.1. SDS-PAGE and immunoblot analysis of recombinant MOMPs

Standard inclusion body preparations containing recombinant *C. psittaci* and *C. pneumoniae* MOMPs routinely yielded 10–50 mg/l of protein. SDS-PAGE analyses of the recombinant proteins are shown in Fig. 1A. Both proteins migrated with an apparent molecular mass of 37 kDa when solubilised in SDS sample buffer and denatured by boiling. Recombinant *C. psittaci* MOMP was recognised by MOMP-specific mAb 4/11 following immunoblotting (Fig. 1B). As expected, the denatured protein did not react when it was probed with *C. psittaci* native MOMP-specific mAb A11 (results not shown). The commercially available native *C. pneumoniae* MOMP-specific mAb also failed to recognise denatured recombinant *C. pneumoniae* MOMP (results not shown).

3.2. Reconstitution of C. psittaci and C. pneumoniae recombinant MOMPs

C. psittaci and *C. pneumoniae* OG/DTT-solubilised MOMPs (~1 ng/ml) were introduced into the *cis* bilayer chamber in the presence of a 500 mM KCl *cis* versus 50 mM KCl *trans* gradient and became incorporated into the bilayer within 5–10 min to give rise to ion channel-like unit conductances. The addition of similar volumes of OG/DTT alone had no effect. Channel incorporation appeared to be autocatalytic, as previously described for native *C. psittaci*

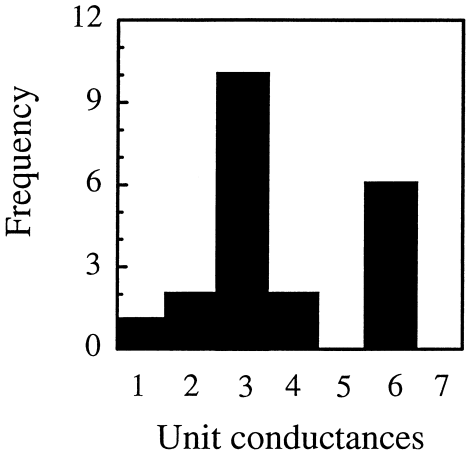

Fig. 2. Incorporation of unit conductances into planar bilayers. The number of recombinant *C. psittaci* MOMP channels incorporated in 21 independent experiments.

MOMP [6] and so after the first channels appeared the *cis* chamber was perfused with fresh 50 mM KCl in an attempt to limit the channel content. We counted the number of discrete unit conductances in 21 independent experiments with recombinant *C. psittaci* MOMP and as observed with the native protein [6], channels tended to appear in groups of three (Fig. 2).

3.3. Single channel recordings of recombinant MOMPs

Fig. 3 shows representative ion channel recordings resulting from recombinant *C. psittaci* and *C. pneumoniae* MOMPs and native *E. coli* Omps. The bilayers were voltage-clamped at −100 mV or −150 mV (*cis* minus *trans*) as indicated and exposed to 300 mM KCl containing 10 mM Tris-HCl, pH 7.4. As noted earlier, the apparent 'trimeric' behaviour of both recombinants was similar to that previously observed for native *C. psittaci* MOMP porin [6]. The unit amplitudes and gating behaviour of the recombinant *C. pneumoniae* MOMP channels were subtly different from those of *C. psittaci* MOMP. For example, this porin required a relatively high 'threshold potential' of about −150 mV to bring about a significant channel closure (although this was still insufficient to close the *E. coli* Omp channels). After comparing them to *E. coli* Omps (Section 3.4), the chlamydial channels were themselves compared in more detail (Section 3.5).

3.4. Comparison with E. coli Omps

To investigate the possibility that the recombinant *C. psittaci* and *C. pneumoniae* MOMPs prepared from inclusion bodies had been contaminated by novel *E. coli* porins, outer membrane proteins were prepared from non-induced bacteria, solubilised in OG/DTT and incorporated into bilayers. The *E. coli* porins displayed very different single channel properties to those of the chlamydial porins (Fig. 3C and Table 1). The native *E. coli* Omps (probably OmpF or OmpC, see Table 1 legend) had a substantially lower conductance than the chlamydial MOMPs and remained open even at relatively high membrane potentials. In addition, the *E. coli* channels were more selective for cations than either of the recombinant MOMPs (Table 1). These Omp-like channels were never seen in any reconstitution of MOMP inclusion body proteins and MOMP-like channels could not be reconstituted from non-induced bacteria.

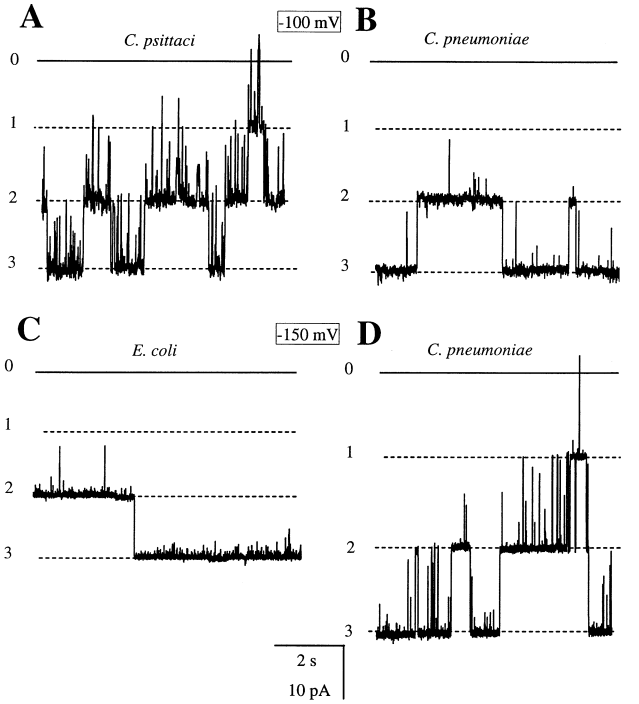

Fig. 3. Comparison of recombinant *C. psittaci* and *C. pneumoniae* MOMPs and *E. coli* Omps. Representative recordings of planar bilayers containing the indicated proteins, in the presence of 300 mM KCl, 10 mM Tris-HCl (pH 7.4) in both chambers, at a holding potential (*cis* minus *trans*) of either −100 mV (A, B) or −150 mV (C, D). The dotted lines indicate unit current levels marked 1–3 (openings are downwards at negative potentials) and the solid line represents the baseline level (0 openings), corresponding to closure of all the 'unit' conductances. Filtered at 100 Hz.

Table 1
Single channel properties of native and recombinant chlamydial porins and native *E. coli* Omp

	[KCl] (mM) (*cis*/*trans*)	*C. psittaci* MOMP		*C. pneumoniae* MOMP	*E. coli* OMP
		Native	Recombinant	Recombinant	Native
g (pS)	50/50	120 ± 18(4)	130 ± 8(3)^{NS}	150 ± 25(4)*	82 ± 8(3)*
	150/150	210 ± 25(4)	230 ± 25(3)^{NS}	260 ± 21(4)*	nd
	300/300	320 ± 32(4)	340 ± 10(3)^{NS}	410 ± 47(4)*	nd
P_{Cl}/P_K	250/50	2.0 ± 0.8(4)	0.38 ± 0.2(3)**	0.49 ± 0.07(4)**	0.28 ± 0.02(3)***
Po	−70 mV	0.71 ± 0.11(5)	0.69 ± 0.06(4)^{NS}	nd	nd
	+70 mV	0.52 ± 0.05(5)	0.48 ± 0.06(4)^{NS}	nd	nd

Conductances (g), relative anion versus cation selectivities (P_{Cl}/P_K) and Po values at ± 70 mV are shown as mean ± S.D. (for *n* experiments). The differences from native *C. psittaci* are not significant (NS), or significant at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). nd = not determined. The native *C. psittaci* conductances are from [6] and the properties of the *E. coli* Omp are typical of Omp F or Omp C [17].

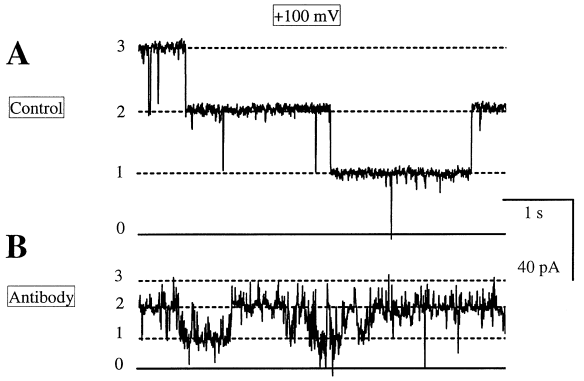

Fig. 4. The effect of native MOMP-specific mAb A11 on recombinant *C. psittaci* channels. Bilayer bathed in 300 mM KCl, 10 mM Tris-HCl (pH 7.4). A, control: B, mAb A11 (in the presence of 1 mg/ml BSA) added to both the *cis* and *trans* chambers to a final dilution of 1:500. Other labelling is as in Fig. 3. Filtered at 100Hz.

3.5. Ionic selectivity, conductance and gating behaviour

Whereas the conductances of native [6] and recombinant *C. psittaci* MOMPs were the same over a range of KCl activities, those of recombinant *C. pneumoniae* MOMP were significantly higher, as summarised in Table 1. There was, however, a significant difference in relative anion versus cation selectivity between native and recombinant *C. psittaci* MOMPs. Although both were only poorly selective between anions and cations, the recombinant channels were weakly cation selective, while the native protein was weakly anion selective. Finally, a comparison of the *P*o values for native and recombinant *C. psittaci* porins at membrane potentials of −70 mV and +70 mV revealed no significant differences.

3.6. Effect of oligomer-specific antibody A11 on recombinant *C. psittaci* MOMP channels

The recombinant *C. psittaci* porin exhibited the same asymmetric response to holding potentials of opposite polarity as previously reported for the native protein [6]. Note the increase in 'flickery' short-lived closures for recombinant *C. psittaci* MOMP at negative (Fig. 3A) as compared to positive (Fig. 4A) holding potentials. Addition of a *C. psittaci* native MOMP-specific neutralising mAb, A11, affected both the gating and the unit amplitude of the recombinant channel. Fig. 4 shows a recording of the channel behaviour before (A) and 2 min after (B) the addition of 0.25% (v/v) mAb A11 to both the *cis* and *trans* chambers. Following antibody addition, the unit conductance amplitudes were significantly reduced and there were also more 'flickery' closures than in the control trace. These effects were very similar to those observed following the addition of mAb A11 to the native MOMP channel [6]. The addition of the commercially available *C. pneumoniae* native MOMP-specific mAb had no discernible effect on the behaviour or properties of the *C. pneumoniae* MOMP channels.

4. Discussion

These results represent the first successful expression and functional reconstitution of recombinant MOMPs from *Chlamydia* species. We have shown that the recombinant *C. psittaci* protein is functionally similar to the native protein [6], in that it exhibits a similar tendency to appear in groups of 3 channels and has similar conductance and gating behaviour. Recombinant *C. pneumoniae* MOMP has broadly similar single channel properties to those of *C. psittaci* MOMP.

Secondary structure information and confirmation of the ion channel function of chlamydial MOMPs [6] places these proteins firmly in the porin superfamily, where they represent a new porin subclass. The channel gating characteristics, conductances and selectivities are also clearly different from those of the *E. coli* Omp-like channels purified from the non-induced bacteria. In addition, the behaviour of recombinant *C. psittaci* MOMP channels is modified by the addition of native MOMP-specific neutralising mAb A11, in a manner very similar to its effect on the native protein [6]. Not only do these results confirm that the channel activity results from reconstituted MOMP and not contaminating *E. coli* Omps, they also provide additional evidence that recombinant MOMP is refolding to a native conformation. Although the conformational epitope recognised by mAb A11 is unknown [9], it is likely to be a surface-exposed sequence in the outer channel vestibule. This is consistent with the idea that bound antibody may partially obstruct the channel (thus reducing the single channel conductance) and the bound protein might also intermittently occlude the channel, giving rise to poorly-resolved 'flickery' closures (Fig. 4B).

Addition of a commercially supplied mAb said to be *C. pneumoniae* MOMP-specific had no effect on our recombinant *C. pneumoniae* channels. This result is unsurprising. In contrast to the MOMPs of the other chlamydial species, the MOMP of *C. pneumoniae* appears to be less immunogenic and antigenically complex [15]. Indeed, surface exposure of this MOMP has not been confirmed, leading some researchers to hypothesise that it lies beneath a layer formed by other chlamydial antigens [16]. In view of these results, there may be some doubt over the specificity of this antibody which was in fact raised against *C. pneumoniae* EBs.

The crystal structures of several porins have been determined. The proteins form β-barrels containing 16 anti-parallel β-strands [17] with short periplasmic loops and longer external loops, one of which is an 'eyelet' pore-confined loop that greatly limits single channel conductance. Without this partial obstruction, the single channel conductance would be at least an order of magnitude higher [18], yet MOMP conductances are very similar to those of the so called 'general diffusion' porins [17]. We thus speculate that chlamydial porins have a similar structure. The small difference in ion selectivity between recombinant and native *C. psittaci* MOMPs may be due to the 16 amino acid truncation of the recombinant protein. If MOMP is structurally similar to other porins, as we suggest, this truncation could result in the loss of two anti-parallel β-strands. This might in turn result in the β-barrels having a slightly reduced diameter and more importantly, the orientation of residues in the channel vestibule and eyelet region might be slightly different, accounting for the small change in selectivity.

Finally, there is no evidence to suggest that all the expressed recombinant protein is refolding to a native conformation. Indeed, the correct folding of these proteins may be closely related to the actual process of bilayer insertion. It is even conceivable that some or all of the reconstituted channels arise from a small population of recombinant proteins that had originally been incorporated into *E. coli* outer membranes. These may subsequently have contaminated our inclu-

sion body preparations, although in that case it is difficult to explain the absence of co-incorporated *E. coli* Omps. In future work we will attempt to improve and optimise the yield of refolded protein, but it is clear that bilayer reconstitution offers an important route to detailed structure/function studies on recombinant chlamydial MOMPs and this work also has broad implications for the production of improved immunogenic MOMP proteins as future subunit vaccines.

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