Characterization of the Disulfide Bonds and Free Cysteine Residues of the *Chlamydia trachomatis* Mouse Pneumonitis Major Outer Membrane Protein†

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ABSTRACT: Members of the genus *Chlamydia* lack a peptidoglycan layer. As a substitute for peptidoglycan, it has been proposed that several cysteine rich proteins, including the major outer membrane protein (MOMP), form disulfide bonds to provide rigidity to the cell wall. Alignment of the amino acids sequences of the MOMP from various serovars of *Chlamydia* showed that they have from 7 to 10 cysteine residues and seven of them are highly conserved. Which of these are free cysteine residues and which are involved in disulfide bonds is unknown. The complexity of the outer membrane of *Chlamydia* precludes at this point the characterization of the structure of the cysteines directly in the bacteria. Therefore, mass spectrometric analysis of a purified and refolded MOMP was used in this study. Characterization of the structure of this preparation of the MOMP is critical because it has been shown, in an animal model, to be a very effective vaccine against respiratory and genital infections. Here, we demonstrated that in this MOMP preparation four cysteines are involved in disulfide bonds, with intramolecular pairs formed between Cys^{48} and Cys^{55} and between Cys^{201} and Cys^{203}. A stepwise alkylation, reduction, alkylation process using two different alkylating reagents was required to establish the Cys^{48}−Cys^{55} disulfide pair. The other residues in MOMP, Cys^{51}, Cys^{136}, Cys^{226}, and Cys^{351}, are free cysteines and could potentially form disulfide-linked complexes with other MOMP or other membrane proteins.

The genus *Chlamydia* contains four species, the human pathogens *Chlamydia trachomatis* and *Chlamydia pneumoniae* and the animal pathogens *Chlamydia pecorum* and *Chlamydia psittaci* (*1*). *C. trachomatis* infection is the most common sexually transmitted bacterial disease in the world and affects more than 4 million people annually in the United States (*2*−*4*). Furthermore, *C. trachomatis* is the etiological agent of trachoma, the most common cause of preventable blindness throughout the world (*5*). On the basis of serological methods, 18 serovars (A−K, Ba, Da, Ia, L1, L2, L3, and L2a) of human *C. trachomatis* have been identified (*6*). In addition, the *C. trachomatis* mouse pneumonitis (MoPn)[1] serovar was isolated from mice inoculated with respiratory tract tissues from humans with respiratory infections (*7*).

Chlamydia are obligate intracellular bacteria that have a unique developmental cycle characterized by a small (0.3 μm), infectious elementary body (EB) that is metabolically inactive and osmotically stable, and a large (1 μm) parasitic reticulate body (RB) that replicates and is osmotically fragile (*8*). The developmental cycle starts when the EB enters the host cell, converts into the RB, and uses products from the host cell as a metabolic source (*8*, *9*). The MOMP has a molecular mass of 40 kDa, and in the EB, this protein accounts for 60% of the mass of the outer membrane and appears to have a porin function (*8*−*12*). MOMP contains four variable domains (VD1−4) that are antigenic and define the classification of the *C. trachomatis* serovars (*13*, *14*). Because of its antigenic property, MOMP has been a major target for the development of a vaccine (*14*−*18*). A proposed secondary structure for MOMP includes 16 transmembrane segments with large loops facing outward and short periplasmic connecting segments (*19*). Furthermore, as is the case for similar proteins in other bacteria, MOMP most likely forms trimers in the membrane (*20*).

Approximately 80% sequence identity is found when the amino acid (AA) sequences of the MOMP from *C. trachomatis* serovars, including MoPn (Swiss-Prot entry P75024), are aligned using the Blast-ClustalW program. Eight of the conserved amino acids are cysteine residues, found at positions 48, 51, 55, 136, 201, 203, 226, and 351 of the MOMP of MoPn (the B serovar lacks Cys^{226}) (*21*). Several proteins, including MOMP and the cysteine-rich proteins (CRPs), 9 kDa OmcA and 60 kDa OmcB, are thought to form a cross-linked protein complex via disulfide bonds in the EB (*8*, *10*, *20*, *22*). This protein complex has been proposed to be the substitute for peptidoglycan, a component that provides rigidity to the wall of Gram-negative bacteria that so far has not been observed in *Chlamydia* (*8*, *23*, *24*).

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[1] Abbreviations: MoPn, mouse pneumonitis; EB, elementary body; RB, reticulate body; MOMP, major outer membrane protein; AA, amino acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; IAM, iodoacetamide; LC−ESI-MS/MS, liquid chromatography−electrospray ionization-tandem mass spectrometry.

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However, information about disulfide linkages and free cysteine residues is lacking in *Chlamydia*. Furthermore, there are some conflicting results. For example, while some authors have shown that the MOMP can be extracted from the EB without reducing agents, others have indicated that reducing agents are required (*10*, *12*). These differences may be ascribed to variations among the *Chlamydia* isolates or to the methodological approaches used to analyze these organisms.

Pal et al. (*17*, *18*) have developed a procedure for the isolation and refolding of MOMP, and have reported on its immunological properties. These authors demonstrated that purified, native MOMP that had been refolded was effective at protecting mice from *C. trachomatis* infection and infertility. In this study, we have used a combination of chemical modifications and mass spectrometric analysis (*25*, *26*) to identify the disulfide linkage pattern and free cysteine residues in this purified, native *C. trachomatis* MoPn MOMP. We found that four of the eight highly conserved cysteine residues in the refolded MOMP are engaged in disulfide bonds, with pairs formed between Cys^{48} and Cys^{55} and between Cys^{201} and Cys^{203}. The other residues in MOMP, Cys^{51}, Cys^{136}, Cys^{226}, and Cys^{351}, are free cysteines.

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise described, were obtained from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Santa Clara, CA).

Preparation of the C. trachomatis MoPn MOMP. The extraction and purification of the *C. trachomatis* MoPn MOMP were performed as previously described (*17*). In brief, *C. trachomatis* MoPn grown in tissue culture was pelleted by centrifugation, resuspended in PBS (pH 7.4), and treated with 25 μg/mL DNAse for 2 h at 4 °C. The preparation was centrifuged, and the pellet was extracted twice with 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Anatrace, Maumee, OH) in 0.2 M phosphate buffer (pH 5.5) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Calbiochem, San Diego, CA), 1 mM EDTA, and 100 mM dithiothreitol (DTT) (Roche Diagnostic Corp., Indianapolis, IN). The pellet was again extracted with 2% Anzergent 3-14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) (Anatrace) in the buffer described above. To further purify the MOMP, the supernatant was applied to a 30 cm × 2 cm hydroxyapatite (Bio-Gel HTP Gel; Bio-Rad Laboratories, Hercules, CA) column equilibrated with 0.1% Z3-14 in 0.02 M phosphate buffer (pH 5.5) and eluted with a linear gradient from 0.02 to 0.5 M in the same buffer. The fractions showing an increase in absorbance at 280 nm were analyzed by SDS−polyacrylamide gel electrophoresis (PAGE), and the MOMP was identified by Western blot analysis using the monoclonal antibody MoPn40 to the *C. trachomatis* MoPn MOMP. Fractions containing a single band were pooled, and the MOMP was refolded by dialysis in 0.1 M phosphate buffer (pH 7.8), containing 3 mM reduced glutathione, 0.3 mM oxidized glutathione, 1 mM EDTA, and 0.05% Z3-14. The MOMP was concentrated using a Centricon-10 filter (Millipore Corp., Bedford, MA) and dialyzed, before characterization, against 20 mM phosphate buffer (pH 7.4) and 0.15 M NaCl containing 0.05% Z3-14.

Preparation and Characterization of Monoclonal Antibodies to the C. trachomatis MoPn MOMP. Monoclonal antibodies (mAb) were prepared and characterized as previously described (*27*). Briefly, 4−6-week-old female BALB/c (H-2^d) mice (Simonsen Laboratories, Gilroy, CA) were immunized with *C. trachomatis* MoPn EB and their spleen cells fused with the S194/5XX0.Bu.1 mouse myeloma cell line. Hybridoma cells were screened by indirect immunofluorescence using *C. trachomatis* MoPn EB fixed with acetone. Positive clones were subsequently tested by Western blot analysis, and the epitope of positive clones binding to the MOMP was determined using a commercially available kit (Chiron Mimotopes Peptide System, San Diego, CA).

To test the reactivity of the mAb to the purified MOMP preparation, an enzyme-linked immunosorbent assay (ELISA) was performed in a 96-well microtiter plate (Corning, Palo Alto, CA). Nonfixed live *C. trachomatis* MoPn EB, at a concentration of 10 μg of protein/mL, were resuspended in PBS (10 mM, pH 7.4), and 100 μL of the suspension was added per well. The *C. trachomatis* MoPn MOMP, purified as described above, was added in 100 μL aliquots per well to the plates at a concentration of 5 μg/mL. The plates were incubated overnight at 4 °C. The mAb (mAb 13-2 and mAb 18b to MOMP and mAb 02 to the 60 kDa cysteine rich protein) were added at a concentration of 5 μg/mL and incubated for 2 h at 37 °C. The antigen−antibody complexes were detected using horseradish peroxidase-conjugated goat anti-mouse pan Ig antibody. For color development, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and H_2O_2 were used as the substrate. The reaction was followed at 405 nm using an ELISA reader (Bio-Rad Laboratories).

Alkylation of Cys Residues. Fifty microliters of MOMP (1 μg/μL) was alkylated with *N*-ethylmaleimide (NEM, 50 mM) in the dark at room temperature for 24 h. The NEM-labeled MOMP sample was transferred to a Microcon YM-30 (Millipore) unit and filtered (8000*g*) three times with 200 μL of 50 mM ammonium bicarbonate buffer (AB buffer) (pH 7.8) to remove unreacted NEM and detergent.

Digestion with Trypsin and Endoproteinase Glu-C. Following ultrafiltration, the MOMP sample was digested with trypsin (Promega, Madison, WI) in 80 μL of AB buffer overnight at 37 °C. The ratio of proteolytic enzyme versus MOMP was 1/40 (w/w). Half of the tryptic digest was further incubated overnight at 37 °C with endoproteinase Glu-C (Roche Applied Science, Indianapolis, IN).

Identification of Free Cys Residues and Disulfide Bonded-Peptides by LC−ESI-MS/MS. Liquid chromatography−electrospray ionization-tandem mass spectrometry (LC−ESI-MS/MS) analysis was performed using an LCQ Classical ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) with a modified electrospray ionization source as previously described (*25*). Briefly, the LC−ESI-MS/MS data acquisition was set up to collect ion signals from the eluted peptides using an automatic, data-dependent scan procedure in which a cyclic series of three different scan modes (full scan, zoom scan, and MS/MS scan) were performed. HPLC analysis was conducted using a MicroLC system (Micro-Tech Scientific, Vista, CA) with a capillary C18 column (100 mm × 75 μm; Nucleosil, 5 μm particle size) at a flow rate of 0.3 μL/min. The enzymatically digested peptides were separated using mobile phases A and B, with a three-step linear gradient from 5 to 10% B over the first 10 min, followed by a 10 to 35%

[1]MKKLLKSVLAFAVLGSASSLHALPVGNPAEPSLMIDGILWEGFGGDPC[48]DPC[51]TTWC[55]DAISL

[61]RLGYYGDFVFDRVLKTDVNKQFEMGAAPTGDADLTTAPTPASRENPAYGKHMQDAEMFTN

[121]AAYMALNIWDRFDVFC[136]TLGATSGYLKGNSAAFNLVGLFGRDETAVAADDIPNVSLSQAVV

[181]ELYTDTAFAWSVGARAALWEC[201]GC[203]ATLGASFQYAQSKPKVEELNVLC[226]NAAEFTINKPKGYV

[241]GQEFPLNIKAGTVSATDTKDASIDYHEWQASLALSYRLNMFTPYIGVKWSRASFDADTIR

[301]IAQPKLETSILKMTTWNPTISGSGIDVDTKITDTLQIVSLQLNKMKSRKSC[351]GLAIGTTIV

[361]DADKYAVTVETRLIDERAAHVNAQFRF

FIGURE 1: Overview of the complete amino acid (AA) sequence of the MOMP, including the cysteine residues and the corresponding disulfide bonds determined in this study (denoted with arrows). Underlined residues make up the predicted membrane-spanning regions (*19*).

B gradient over the next 40 min, and from 35 to 50% B over the final 10 min; mobile phase A is 0.1% HCOOH in water, and mobile phase B is 0.1% HCOOH in acetonitrile.

RESULTS

Structure of MOMP. The objective of this study was to examine the free cysteine residues and disulfide bonds of a refolded mature MOMP, containing the AA sequence from AA 23 to 397 (the leading peptide of 22 AA is cleaved off by the bacteria). Figure 1 shows an overview of the complete AA sequence of MOMP, including the cysteine residues, and the corresponding disulfide bonds determined in this study.

Binding of Monoclonal Antibodies (mAb) to the C. trachomatis MoPn. Monoclonal antibodies raised to *C. trachomatis* MoPn EB were tested using several immunoassays. The initial screening was performed using immunofluorescence microscopy with acetone-fixed *C. trachomatis* MoPn EB as the antigen. The three mAb tested gave positive signals (data not shown). By Western blot analysis, mAb 13-2 was found to bind to the MOMP while mAb 18b did not react. Using synthetic peptides we mapped mAb 13-2 to the MOMP VD-4 peptide: TTWNPTIS. The mAb 02 recognized the 60 kDa cysteine rich protein (data not shown).

An ELISA was performed with nonfixed live EB and the same *C. trachomatis* MoPn MOMP preparation used here to determine the disulfide bonds. As shown in Figure 2, mAb 13-2 and mAb 18b bound to the purified MOMP and also recognized the *C. trachomatis* MoPn EB. Tissue culture media, utilized to grow the hybridomas, used as a negative control, did not bind. mAb 02 did not bind to the MOMP or to the live EB, indicating that the epitope in the 60 kDa cysteine rich protein is not surface-exposed.

Characterization of Free Cysteine Residues and Disulfide Bonds of MOMP by Mass Spectrometry. Because MOMP contains closely spaced cysteine residues, it was necessary to utilize an alkylation procedure that minimized the possibility of thiol−disulfide exchange reactions (*25*, *26*). The procedure that was employed involves four steps: (1) alkylated free Cys residues with an excess of NEM (50 mM), (2) proteolytic digestion of the protein with trypsin and endoproteinase Glu-C to obtain peptides that contain NEM-labeled Cys and disulfide bonds, (3) capillary LC−ESI-MS/MS analysis of the chromatographically separated peptides, and (4) identification of the free Cys residues and disulfide-linked peptides using protein database searching programs Sequest and Mascot.

Using LC−ESI-MS/MS analysis in combination with protein database searching, we confirmed more than 95%

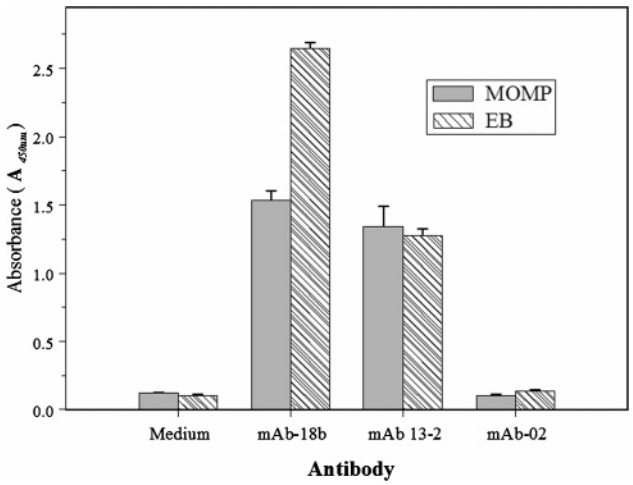

FIGURE 2: ELISA using purified MOMP and live *C. trachomatis* MoPn EB as antigens. Monoclonal antibodies 13-2 and 18b to the *C. trachomatis* MoPn MOMP and mAb 02 to the 60 kDa CRP were reacted with the purified MOMP and EB. Tissue culture media were used as a negative control. The error bars were calculated from the standard deviation of four measurements.

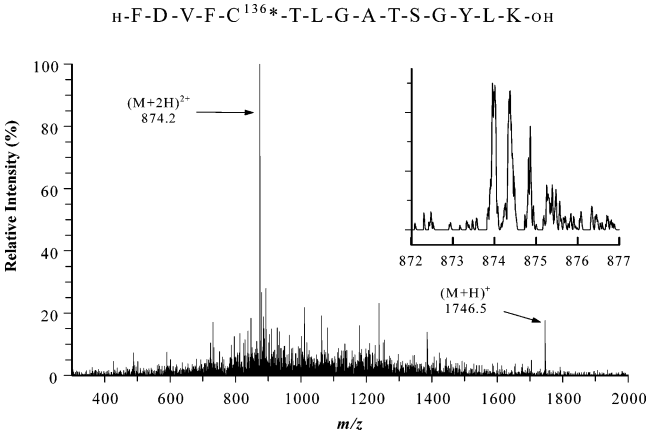

FIGURE 3: Full scan mass spectrum and corresponding inset zoom scan of AA 132−146. This figure shows that Cys[136] is alkylated with NEM with a doubly charged ion at *m*/*z* 874.2. The asterisk denotes the NEM-labeled Cys[136].

of the amino acid sequence predicted for the mature form (AA 23−397) of the MOMP. Typically, the Sequest cross-correlation scores (XCorr) of the MS/MS spectra for the detected MOMP peptides ranged from 6.14 to 1.60, and the Mascot ions scores for the tryptic peptides of the MOMP ranged from 137 to 26. To exclude the false-positive identification, all MS/MS spectra were manually inspected to confirm the sequence of the detected peptides. Among the eight, highly conserved Cys residues in the MOMP, we detected three tryptic peptides (AA 132−146, 219−237, and 350−364), each containing a Cys residue (i.e., Cys[136], Cys[226], and Cys[351], respectively) that was alkylated by NEM. The peptide (AA 132−146) that contains Cys[136] was detected as a doubly charged ion at *m*/*z* 873.9 (see the ion peak in the inset of Figure 3), matching the calculated $(M + H)^+$ ion of 1746.8 Da for an alkylated Cys-containing peptide. MS/MS analysis of the precursor ion at *m*/*z* 873.9 (Figure 4) confirmed the peptide sequence as shown by dominant N- and C-terminal fragments (b_{2-4}, b_7, b_9, b_{13}, b_{14}, and y_{5-12}). The fragmentation pattern demonstrated that Cys[136] was modified by NEM since a mass difference of 228 Da was observed between fragments y_{11} (*m*/*z* 1238.4) and y_{10} (*m*/*z*

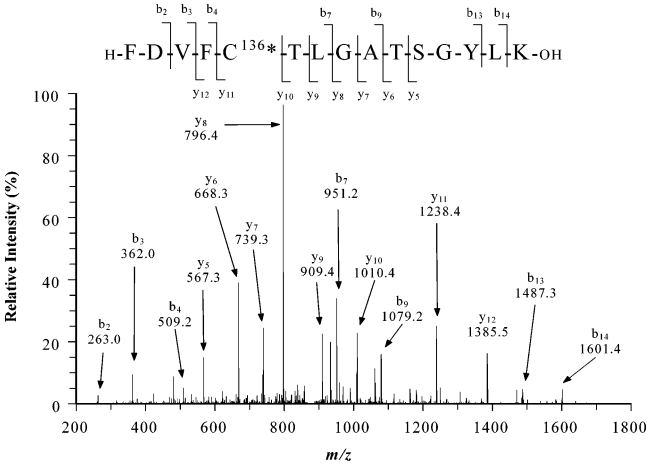

FIGURE 4: MS/MS spectrum of AA 132−146. The corresponding MS/MS spectrum for the ion at *m*/*z* 874.2 confirms that the dominant fragments, y_n ($n = 5-12$), arise from the NEM-labeled Cys[136]-containing peptide in which the mass difference between y_{11} and y_{10} equals a molecular mass of 103 Da (Cys) + 125 Da (NEM).

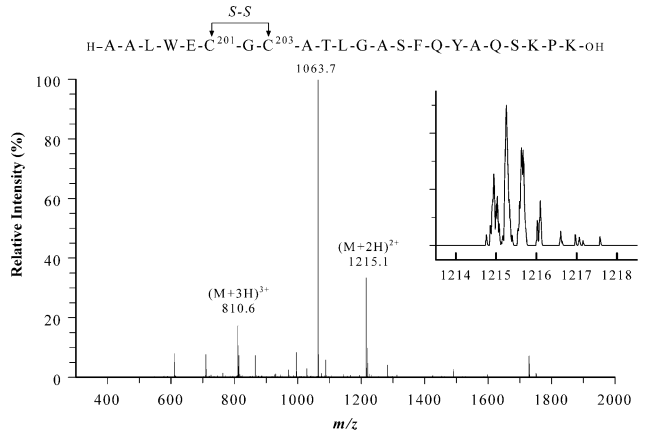

FIGURE 5: Full scan mass spectrum and corresponding inset zoom scan of the disulfide-bonded peptide (AA 196−218). Full scan mass spectrum and corresponding inset zoom scan of the disulfide-bonded peptide (AA 196−218) between Cys[201] and Cys[203] for a doubly charged ion at *m*/*z* 1215.1.

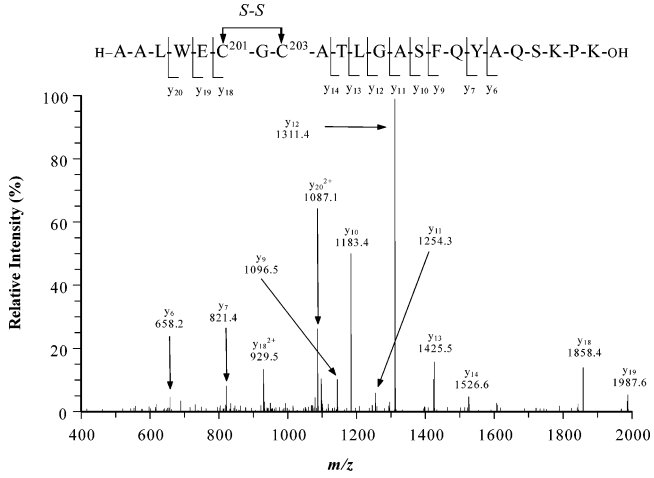

FIGURE 6: MS/MS spectrum for the ion at *m*/*z* 1215.1. The corresponding MS/MS spectrum for the ion at *m*/*z* 1215.1 confirms that the dominant fragments, y_n ($n = 6, 7, 9-14$, and $18-20$), arise from the disulfide-bonded peptide between Cys[201] and Cys[203].

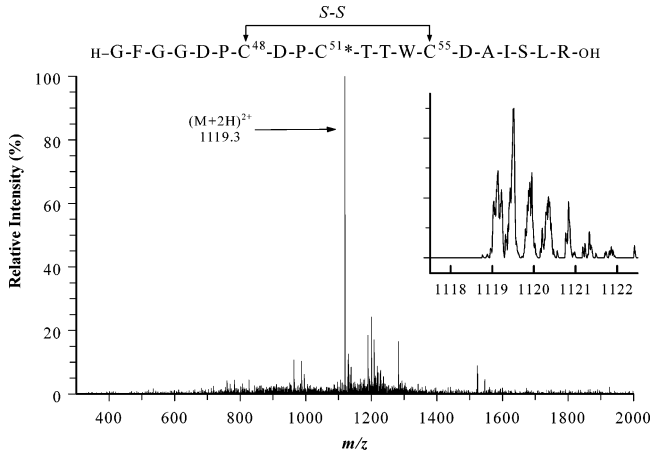

FIGURE 7: Full scan mass spectrum and corresponding inset zoom scan of the disulfide-bonded peptide (AA 42−61). Full scan mass spectrum and corresponding inset zoom scan of the disulfide-bonded peptide (AA 42−61) between Cys[48] and Cys[55] for a doubly charged ion at *m*/*z* 1119.3 from MOMP that was digested with trypsin and endoproteinase Glu-C. The asterisk denotes the NEM-labeled Cys[51].

1010.4), equal to the mass of Cys (103 Da) plus the mass of NEM (125 Da). Similarly, we detected doubly charged ions at *m*/*z* 1129.0 and 794.9 for peptides that contain Cys[226] and Cys[351], respectively (data not shown). MS/MS analysis also confirmed that Cys[226] and Cys[351] were alkylated by NEM. These results demonstrate that Cys[136], Cys[226], and Cys[351] exist as free Cys residues in the MOMP.

Analysis of the tryptic digest from the MOMP also demonstrated that Cys[201] and Cys[203] form a disulfide bond. Figure 5 shows the MS spectrum for this peptide with prominent ions at *m*/*z* 810.6 and 1215.1 for a triply and doubly charged ion, respectively. These ions correspond to an intramolecular, disulfide-bonded (Cys[201]−Cys[203]), peptide (AA 196−218). The detected mass from the zoom spectrum (shown in the inset of Figure 5) matches the calculated mass of the disulfide-bonded peptide (2428 Da). If the peptide contained free Cys residues, a mass of 2430 Da for AA 196−218 would be observed. MS/MS analysis of the doubly charged ion at *m*/*z* 1215.1 (Figure 6) showed dominant ions for C-terminal fragment ions (y_6, y_7, y_{9-14}, and y_{18-20}), confirming the peptide sequence with a disulfide bond formed between Cys[201] and Cys[203].

The tryptic peptide (AA 23−61) that contains Cys[48], Cys[51], and Cys[55] was detected at *m*/*z* 1424.5 as a triply charged ion, matching the mass expected for the peptide (4269 Da) containing a NEM-labeled Cys and a disulfide (data not shown). MS/MS analysis of this ion produced few fragments, which were insufficient to confirm the sequence of the peptide. MS/MS analysis of a large peptide (>4K Da) often results in a poor fragmentation. To overcome this problem, we digested the tryptic peptides of the MOMP with endoproteinase Glu-C to produce a smaller peptide. Figure 7 shows the mass spectrum and the corresponding zoom scan (inset) of the doubly charged ion $(M + 2H)^{2+}$ at *m*/*z* 1119 for AA 42−61. This peptide was generated from the tryptic peptide (AA 23−61) that was cleaved at the C-terminus of Glu[41]. The detected mass of the peptide (AA 42−61) matches that calculated (2235.9 Da) for the peptide which contained one alkylated Cys residue with NEM and a disulfide bond. MS/MS analysis of the precursor ion at *m*/*z* 1119, shown in Figure 8, revealed a dominant fragment ion, y_{15}^{2+}, which

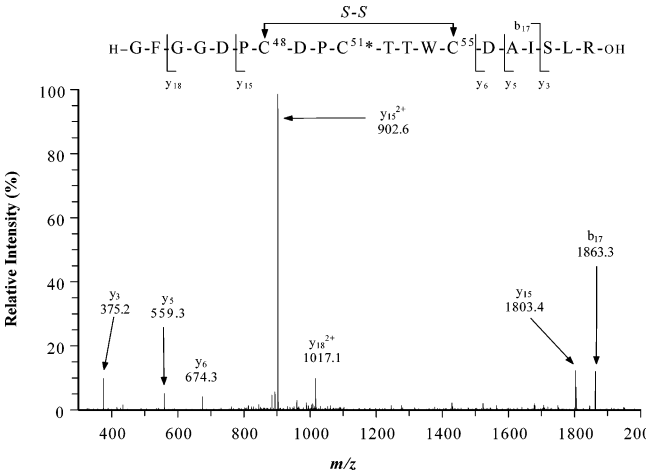

FIGURE 8: Corresponding MS/MS spectrum for the ion at *m*/*z* 1119.3. The corresponding MS/MS spectrum for the ion at *m*/*z* 1119.3 shows that the dominant fragments, y_n (n = 3, 5, and 6), y_{15}^{2+}, y_{18}^{2+}, and b_{17}, arise from the disulfide-bonded peptide between Cys[48] and Cys[55].

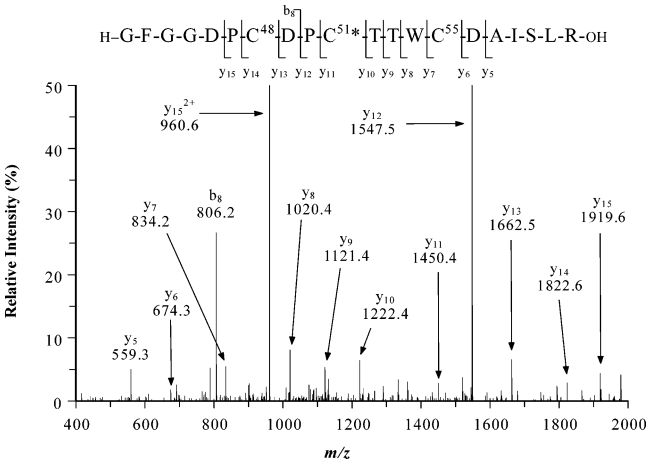

FIGURE 9: MS/MS spectrum from AA 42−61. MS/MS spectrum of the peptide of AA 42−61 that was further reduced and alkylated with DTT and then with IAM. The fragments, y_n (n = 5−15), confirm that Cys[48] and Cys[55] form a disulfide bond and Cys[51] is a free Cys since Cys[48] and Cys[55] were labeled with IAM while Cys[51] (denoted with an asterisk) was labeled with NEM.

was generated from a cleavage at the N-terminus of Pro[47]. If the disulfide bond involved Cys[48] and Cys[51], additional fragments which contain AA 52−55 would be expected (*25*).

To obtain additional evidence to support the proposed disulfide between Cys[48] and Cys[55], the disulfide bond in the peptide was reduced with DTT and the resulting free Cys residues were alkylated with iodoacetamide (IAM). Eliminating the disulfide linkage should result in fragments in the spectrum that can be used to determine which Cys residues were originally involved in a disulfide bond. The resulting peptide was detected as a dominant peak at *m*/*z* 1177 for a doubly charged ion in a full scan mass spectrum (data not shown). The observed peptide mass of 2342 Da confirms that one Cys residue was alkylated with NEM, whereas the other two Cys residues were alkylated with IAM. MS/MS analysis of the precursor ion at *m*/*z* 1177 (Figure 9) showed dominant fragment ions, y_{12} and y_{15}, which were generated from a cleavage at the N-terminus of Pro[50] and Pro[47] residues, respectively. Moreover, there are noticeable fragments denoted as y_{5-11} and y_{13-15} that unambiguously demonstrate that Cys residues are involved in the disulfide linkage. The mass difference of 228 Da was detected between y_{10} (*m*/*z* 1222.4) and y_{11} (*m*/*z* 1450.4), demonstrating that Cys[51] was alkylated by NEM, and thus was a free Cys residue in the parent peptide. A mass difference of 160 Da was found between y_{14} (*m*/*z* 1822.6) and y_{13} (*m*/*z* 1662.5), and between y_7 (*m*/*z* 834.2) and y_6 (*m*/*z* 674.3) in the spectrum, demonstrating that Cys[48] and Cys[55] were involved in a disulfide bond before they were reduced by DTT and alkylated by IAM. In conclusion, our results demonstrate that among eight highly conserved Cys residues of MOMP, Cys[51], Cys[136], Cys[226], and Cys[351] are free Cys residues and the other four form two disulfide bonds between Cys[48] and Cys[55] and between Cys[201] and Cys[203]. No evidence of phosphorylation and N-linked glycosylation was observed.

DISCUSSION

In Gram-negative bacteria, a layer of peptidoglycan provides rigidity to the cell wall. Attempts to identify peptidoglycan in members of the genus *Chlamydia* have so far been unsuccessful (*23*, *24*, *28*, *29*). Interestingly, these organisms have penicillin-binding proteins, and furthermore, DNA sequencing of the *C. trachomatis* genome showed that these bacteria have a complete set of genes for the synthesis of peptidoglycan (*30*, *31*). The lack of a peptidoglycan layer has prompted several investigators to postulate alternative structures that can provide rigidity to the cell wall of this bacterium while at the same time allowing for the expansion of the membrane when the EB transform to RB (*8*, *20*, *22*, *28*).

In the envelope of *Chlamydia*, in addition to the MOMP, two other cysteine rich proteins (CRPs) have been identified (*32*, *33*). The OmcB has approximately 500 AA, of which almost 8% are cysteines, while for OmcA around 20%, of the approximately 70 AA, are cysteines (*8*, *32*, *33*). In the case of *C. trachomatis*, the MOMP is the primary vaccine candidate (*14*, *16*). Thus, understanding the structure of this protein can be critical for the formulation of an efficacious vaccine. Optimally, the characterization of the structure of the MOMP should be performed directly in RB and EB. Methodological limitations preclude at this point performing this type of analysis. Here, as a surrogate, we have characterized a purified preparation of the MOMP that has been shown to be biologically relevant when used as a vaccine because of its ability to protect against a genital and a respiratory chlamydial challenge (*17*, *18*).

In EB, the MOMP is thought to have interpeptide cross-links to itself and maybe to form a disulfide cross-linked complex with other CRPs (*8*, *20*, *22*, *34*). In contrast, in RB, the MOMP is detected mainly as a monomer, indicating that some intermolecular disulfides have been reduced (*8*, *20*, *22*). These data suggest that over the developmental cycle of *Chlamydia* several conformations of the disulfide bonds and cysteines are likely to occur. These findings, however, are based on evaluations of gel electrophoresis experiments, and there are no data on the direct characterization of intra- or interpeptide bonds of the envelope proteins. Here we have, for the first time, evaluated the distribution of free and disulfide-linked Cys residues in a native preparation of the MOMP, and determined that four Cys residues, Cys[51], Cys[136], Cys[226], and Cys[351], are found in the free form and the other

form two intramolecular disulfide bonds between Cys^{48} and Cys^{55} and between Cys^{201} and Cys^{203}. These data agree with the results obtained by Newhall and Jones (*34*) that determined that approximately 50% of the cysteine residues in the MOMP of EB are oxidized.

Rodriguez-Marañon et al. (*19*) applied several algorithms and an artificial neural network in analyzing the topology of the MOMP and proposed a 16-strand β-barrel porin-type configuration, a structure motif found for a family of proteins that occur in the outer membrane of Gram-negative bacteria (*35*). This model locates all Cys residues except Cys^{226}, proposed to be located in a β-sheet, in the external loops on the outer surface of the membrane. Interestingly, Cys^{226} is not conserved in the B serovar, suggesting that, at least in some of the serovars of *Chlamydia*, a maximum of three intramolecular disulfide bonds may be required to maintain a functional MOMP. Incorporating our current findings into this proposed model for the MOMP suggests that the disulfide bonds between Cys^{48} and Cys^{55} and between Cys^{201} and Cys^{203} of MOMP monomer create two short looplike structures in L1 and L4. The disulfide bonds formed in L1 and L4 could result in the folding back of L1 and/or L4 toward the outer membrane surface that may regulate the entrance of various molecules through the MOMP channel. This may explain the finding that the MOMP, only in the reduced form, functions as a porin (*10*).

Disulfide bonds formed between cysteine residues are important in the folding process and contribute to the native three-dimensional structures of proteins. Disulfide bonds in some proteins are also found to participate in catalyzing other disulfide bond formation via the reduced and oxidized state of cysteine (*36*, *37*). The thioredoxin superfamily of proteins contains a Cys-Xxx-Xxx-Cys (C-X-X-C) active site motif that catalyzes formation of disulfide bonds in various substrates. The *Escherichia coli* periplasmic protein DsbA contains the C-X-X-C motif that catalyzes disulfide bond formation in a group of proteins localized in the bacterial cell envelope (*37*). There is evidence that thiol−disulfide exchange interactions occur between the protein disulfide isomerase (PDI) on the surface of infected host cells and the EB of *C. trachomatis* serovar E and are involved in entry of EB into the cell (*38*). The MOMP contains a C-X-X-C (Cys^{48}-Asp-Pro-Cys^{51}) sequence that is conserved in *Chlamydia*. Interestingly, the C-X-X-C motif is also found in the other two CRPs, 9K Da OmcA and 60K Da OmcB (*32*, *33*). Together with a recent study, which shows that two PDI genes of *C. trachomatis* are expressed differentially during the developmental cycle of *Chlamydia* (*39*), these data suggest that the MOMP, with other CRPs and PDI, may be involved in assisting in disulfide bond formation in the outer membrane.

In conclusion, we have shown that, in a purified preparation of the *C. trachomatis* MoPn MOMP extracted directly from the organism in the presence of DTT and subsequently refolded with glutathione, there are two intrapeptide disulfide bonds and four free cysteines. We realize that this particular conformation may not exist in vivo. Most likely, however, this is one of the several structures that the MOMP adopts during the developmental cycle of *Chlamydia*. With the advent of new methodologies, the feasibility of determining the conformation of the cysteines directly in this bacterium may become feasible. In the meantime, characterization of purified preparations of chlamydial proteins may help us understand the structure of this organism so that we can implement more effective therapeutic and preventive measures.

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