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Disulfide-Linked Oligomers of the Major Outer Membrane Protein of Chlamydiae

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The major outer membrane protein of chlamydial elementary bodies was identified in dimer, trimer, and other multimeric forms. These natural multimers were stabilized by disulfide-mediated cross-linking. Such cross-linking of outer membrane proteins may play an important role in the formation and evolution of chlamydial cell wall structure.

The chlamydiae are obligate intracellular pathogens that cause a broad spectrum of diseases in both animals and humans. A unique feature of chlamydiae is their growth cycle, which is characterized by the evolution of the organism from an infectious, metabolically dormant, extracellular form—the elementary body (EB)—to a noninfectious, metabolically active, proliferating, intracellular form—the reticulate body (RB)—which reorganizes back to the EB (21). Many of the pathogenic properties of these organisms are thought to be related to surface structures. The cell envelopes of the EB and the RB forms resemble those of gram-negative bacteria in that both an outer membrane and an inner membrane are present (24). However, in contrast to typical gram-negative bacteria, chlamydiae lack a significant periplasmic space (3), and most attempts to demonstrate peptidoglycan have been unsuccessful. (1, 4, 10). Despite this apparent absence of peptidoglycan, the cell envelope of the chlamydial EB is characteristically rigid and is resistant to mechanical disruption, whereas that of the RB is relatively fragile (2, 3, 10, 12, 23).

Garrett et al. (4) and Caldwell et al. (2) speculated that the rigid structure of the EB cell envelope might be conferred by some undefined envelope components, and Barbour et al. (1) proposed that a protein or lipoprotein layer in the chlamydial cell envelope could be cross-linked through bridges between peptidoglycan-like tetrapeptides on adjacent proteins. However, supportive evidence for these hypotheses has not been described. Manire and Tamura (10, 23) observed that EB cell walls possess cystine and methionine, whereas RB cell walls do not; Hatch et al. (6) reported that envelope proteins of *Chlamydia psittaci* could only be solubilized by sodium dodecyl sulfate (SDS) in the presence of a reducing agent. Taken together, these observations suggest that protein-protein interactions mediated by disulfide bonds could possibly play a role in the structure of EB cell walls. To investigate this possibility, we analyzed purified EBs for the presence of disulfide-linked protein complexes by using one- and two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Partially purified EBs of *C. trachomatis* F/UW-94/Ur, I/UW-12/Ur, and L_2/434/Bu and *C. psittaci* meningopneumonitis (MN)/CAL-10 were obtained after growth in cycloheximide-treated McCoy cell monolayers by differential centrifugation followed by density gradient centrifugation (15). EBs were solubilized by incubation at 100°C for 2 min in one of two buffers: (i) 2.5% (wt/vol) SDS–12.5% (vol/vol) glycerol–0.001% bromophenol blue–62.5 mM Tris (pH 6.8) or (ii) same as (i) but containing either 1.25% (vol/vol) 2-mercaptoethanol or 40 mM dithiothreitol. A duplicate set of sample was alkylated with 100 mM iodoacetamide to prevent reoxidation of sulfhydryl groups. Protein profiles were obtained on linear 4 to 15% polyacrylamide gradient slab gels by using the buffer system of Laemmli (7) (Fig. 1). The major outer membrane protein (MOMP) (2) of these strains did not completely resolve unless the samples were reduced before SDS-PAGE. In addition, the nonreduced samples did not appear to possess unique high-molecular-weight bands that would indicate the presence of disulfide-linked protein complexes. This suggested that (i) nonreduced MOMP existed in a very large complex that did not penetrate the gel, (ii) prior reduction of MOMP was necessary for Coomassie blue staining, or (iii) complexes containing MOMP were present in the gel but were not resolved as discrete bands.

When nonreduced SDS-solubilized EBs were centrifuged at 100,000 × *g* for 1 h, all of the

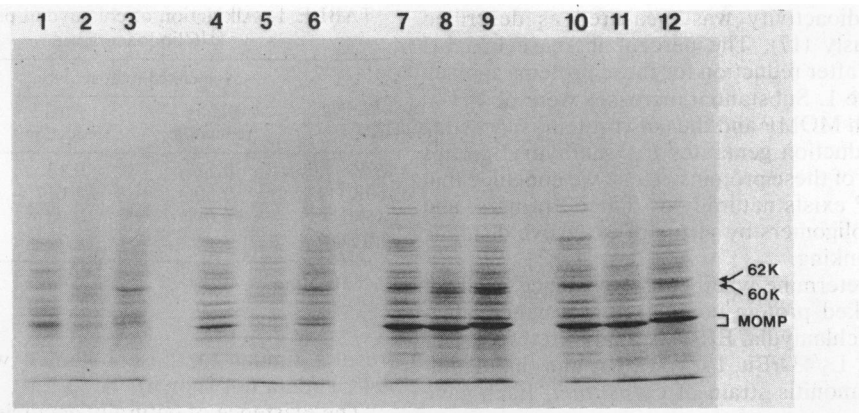

FIG. 1. Coomassie blue-stained 4 to 15% polyacrylamide gradient slab gel of EB proteins of strain L_2/434/Bu (lanes 1, 4, 7 and 10), strain F/UW-94/Ur (lanes 2, 5, 8 and 11), and strain I/UW-12/Ur (lanes 3, 6, 9, and 12). Lanes 1 to 3, Samples not reduced or alkylated; lanes 4 to 6, samples alkylated with iodoacetamide; lanes 7 to 9, samples reduced with dithiothreitol; lanes 10 to 12, samples reduced and alkylated. In each lane 15 μg of protein was resolved. The position of the MOMPs is indicated by a bracket, and the 60K and 62K proteins are indicated by arrows.

MOMP could be demonstrated in the supernatants (data not shown). Therefore, MOMP was not present in an SDS-insoluble, sedimentable complex. To distinguish among the other possibilities, chlamydial EBs were analyzed by two-dimensional SDS-PAGE. For this method, proteins and possible complexes that resolve in the first-dimension gel are subjected to reducing conditions before electrophoresis in the second dimension (16). The application of this technique to the analysis of EB proteins of strain F/UW-94/Ur is shown in Fig. 2. Most of the EB proteins were not affected by reduction and were located along a diagonal line. However, multiple spots which correspond to proteins with the same molecular weight as MOMP were observed below the diagonal line. Extrapolation to a plot of log molecular weight versus migration distance in the first-dimension gel indicated that four of these spots originated from complexes having approximate molecular weights of 42,000 (42K), 82K, 130K, and 165K. These molecular weights correspond to those that would be expected for monomers, dimers, trimers, and tetramers of MOMP. Two other spots of MOMP originated from complexes at the polyacrylamide and agarose gel interfaces of the first dimension. The precise nature of these complexes having very high molecular weights is unknown.

In addition to MOMP, spots corresponding to a 60K protein and a 15K protein were also observed below the diagonal line. The 15K spot appeared to have originated from a dimer of this protein. The 60K spot originated from a high-molecular-weight complex at the origin of the first-dimension gel. The presence of MOMP and 60K components that originate from the same location suggests, but does not prove a covalent association between these proteins.

Because treatment with the reducing agent 2-mercaptoethanol was necessary to convert these complexes into their monomeric constituents, we reasoned that the complexes are stabilized by interchain disulfide bonds. However, 2-mercaptoethanol can potentially alter the migration behavior of membrane proteins by chelation of magnesium ions (11) and by an inherent solvent activity (22). Stabilization by magnesium ions is unlikely for the complexes described here since samples were resolved in the first-dimension gel in the presence of 5 mM EDTA (16). In addition, no differences were observed between samples solubilized in the presence or absence of EDTA. The possible breakdown of the complexes by a solvent activity of 2-mercaptoethanol before resolution in the second dimension was ruled out since identical results were obtained using dithiothreitol, a reducing agent that lacks a similar activity (22).

To obtain evidence that the reducing agents were cleaving disulfide bonds, we measured the uptake of [^{14}C]iodoacetamide by various proteins in SDS-solubilized EBs before and after reduction. For these experiments, two 100-μg samples of EB protein were solubilized by treatment at 100°C for 2 min in 2.5% SDS–0.1 M Tris-hydrochloride (pH 8.0). One of the samples was reduced with 20 mM dithiothreitol. Both samples were cooled to 4°C and alkylated with 50 mM [1-^{14}C]iodoacetamide (2 mCi/mmol) by incubation for 1 h. Free iodoacetamide was removed by passage over a desalting column. The fractions containing EB proteins were fully reduced and then resolved by SDS-PAGE. Bands corresponding to various proteins were excised,

and radioactivity was measured as described previously (17). The percent increase in label uptake after reduction for these proteins is given in Table 1. Substantial increases were observed for both MOMP and the 60K protein, suggesting that reduction generates free sulfhydryl groups in both of these proteins. Thus, we conclude that MOMP exists naturally as dimers, trimers, and larger oligomers by virtue of extensive disulfide cross-linking.

To determine whether the existence of disulfide-linked protein complexes is a general feature of chlamydial EBs, we analyzed three other strains: L_2/434/Bu, I/UW-12/Ur, and the meningopneumonitis strain of *C. psittaci*. Each gave results similar to those obtained with F/UW-94/Ur (data not shown).

TABLE 1. Alkylation of chlamydial proteins with [^{14}C]iodoacetamide

Protein	Associated radioactivity		% Increase
	Before reduction	After reduction	
62K	0.12[a]	0.13	8
60K	0.50	1.61	220
45K	0.08	0.08	0
MOMP	3.44	6.72	95

[a] 10^3 dpm.

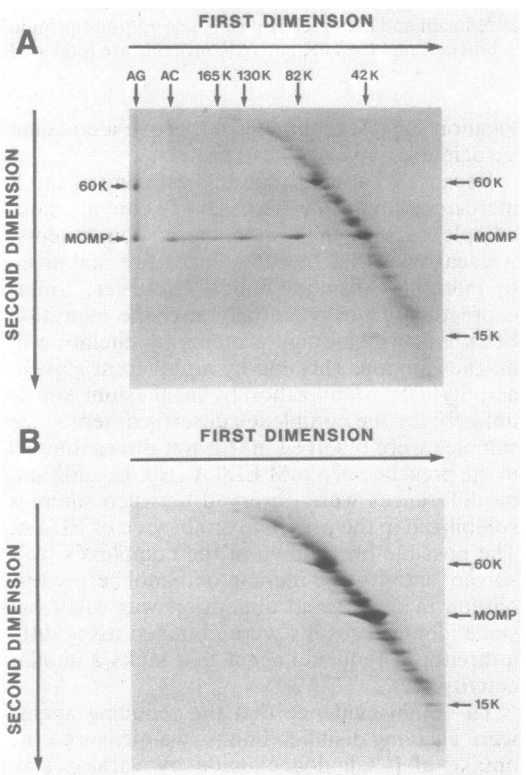

FIG. 2. Two-dimensional SDS-PAGE of EB proteins of strain F/UW-94/Ur. In (A), the sample was alkylated before migration in the first dimension and then reduced before migration in the second dimension; in (B), the sample was reduced and alkylated before migration in the first dimension and then reduced again before migration in the second dimension. The direction of migration during SDS-PAGE is indicated for each dimension. The migration positions of the MOMP complexes in the first dimension are indicated by arrows at the top. The arrows at the sides indicate the position of major proteins in the second dimension. AC and AG, Polyacrylamide and agarose origins, respectively, in the first-dimension gel.

The existence of naturally occurring disulfide-linked protein complexes, such as immunoglobulins, is well known. In addition, the presence of disulfide-linked proteins in bacterial outer membranes has also been described (8, 17). However, the matrix proteins of gram-negative bacteria, which are similar to the MOMP of chlamydiae in terms of relative abundance, molecular weight, and surface exposure, are not covalently linked to each other. Rather, they exist as trimers that are stabilized by noncovalent interactions (25). These trimers function as diffusion channels through the outer membrane (13, 14) and play a secondary structural role through associations with peptidoglycan (5, 9, 20) and other membrane components (18, 19). Therefore, in contrast to gram-negative bacteria, chlamydiae seem to possess a unique mechanism for stabilizing their cell walls which involves extensive disulfide linking of outer membrane proteins. The relationship between this proposed structural determinant and the function and structure of the outer membrane during the chlamydial growth cycle remains to be elaborated. However, recent observations suggest that reduction of EBs in vitro may initiate the differentiation into RBs (T. Hackstadt and H. D. Caldwell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D34, p. 54). In addition, preliminary data suggest that RBs lack the extensive disulfide cross-linking observed in the EBs (W. J. Newhall, unpublished data). Therefore, the formation and cleavage of disulfide bonds in the cell wall may be important aspects of the processes involved in the conversion of one form of chlamydia into another.

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LITERATURE CITED

1. **Barbour, A. G., K.-I. Amano, T. Hackstadt, L. Perry, and H. D. Caldwell.** 1982. *Chlamydia trachomatis* has penicillin-binding proteins but not detectable muramic acid. J. Bacteriol. **151**:420–428.

2. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31**:1161–1176.
3. **Costerton, J. W., L. Poffenroth, J. C. Wilt, and N. Kordova.** 1975. Ultrastructural studies of *Chlamydia psittaci* 6BC *in situ* in yolk sac explants and L cells: a comparison with gram-negative bacteria. Can J. Microbiol. **21**:1433–1447.
4. **Garrett, A. J., M. J. Harrison, and G. P. Manire.** 1974. A search for the bacterial mucopeptide component, muramic acid, in *Chlamydia*. J. Gen. Microbiol. **80**:315–318.
5. **Hancock, R. E. W., R. T. Irvin, J. W. Costerton, and A. M. Carey.** 1981. *Pseudomonas aeruginosa* outer membrane: peptidoglycan-associated proteins. J. Bacteriol. **145**:628–631.
6. **Hatch, T. P., D. W. Vance, Jr., and E. Al-Hossainy.** 1981. Identification of a major envelope protein in *Chlamydia* spp. J. Bacteriol. **146**:426–429.
7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
8. **Lane, B. C., and R. E. Hurlbert.** 1980. Characterization of the cell wall proteins of *Chromatium vinosum*. J. Bacteriol. **141**:1386–1398.
9. **Lugtenberg, B., H. Bronstein, N. Van Selm, and R. Peters.** 1977. Peptidoglycan-associated outer membrane proteins in gram-negative bacteria. Biochim. Biophys. Acta. **465**:571–578.
10. **Manire, G. P., and A. Tamura.** 1967. Preparation and chemical composition of the cell walls of mature infectious dense forms of meningopneumonitis organisms. J. Bacteriol. **94**:1178–1183.
11. **McMichael, J. C., and J. T. Ou.** 1977. Metal ion dependence of a heat-modifiable protein from the outer membrane of *Escherichia coli* upon sodium dodecyl sulfate-gel electrophoresis. J. Bacteriol. **132**:314–320.
12. **Moulder, J. W.** 1966. The relation of the psittacosis group (Chlamydiae) to bacteria and viruses. Annu. Rev. Microbiol. **20**:107–130.
13. **Nakae, T.** 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. **71**:877–884.
14. **Nakae, T.** 1976. Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. J. Biol. Chem. **251**:2176–2178.
15. **Newhall, W. J., V, B. Batteiger, and R. B. Jones.** 1982. Analysis of the human serological response to proteins of *Chlamydia trachomatis*. Infect. Immun. **38**:1181–1189.
16. **Newhall, W. J., V, W. D. Sawyer, and R. A. Haak.** 1980. Cross-linking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **28**:785–791.
17. **Newhall, W. J., V, C. E. Wilde III, W. D. Sawyer, and R. A. Haak.** 1980. High-molecular-weight antigenic protein complex in the outer membrane of *Neisseria gonorrhoeae*. Infect. Immun. **27**:475–482.
18. **Palva, E. T., and L. L. Randall.** 1978. Arrangement of protein I in *Escherichia coli* outer membrane: a cross-linking study. J. Bacteriol. **133**:279–286.
19. **Reithmeier, R. A. F., and P. D. Bragg.** 1977. Cross-linking of the proteins in the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta. **466**:245–256.
20. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. **240**:8019–8029.
21. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Annu. Rev. Microbiol. **34**:285–309.
22. **Shepherd, W. D., and S. Kaplan.** 1978. Effect of heat and 2-mercaptoethanol on intracytoplasmic membrane polypeptides of *Rhodopseudomonas sphaeroides*. J. Bacteriol. **135**:656–667.
23. **Tamura, A., and G. P. Manire.** 1967. Preparation and chemical composition of the cell membrane of developmental reticulate forms of meningopneumonitis organisms. J. Bacteriol. **94**:1184–1188.
24. **Tamara, A., A. Matsumoto, G. P. Manire, and N. Higashi.** 1971. Electron microscopic observations on the structure of the envelopes of mature elementary bodies and developmental reticulate forms of *Chlamydia psittaci*. J. Bacteriol. **105**:355–360.
25. **Tokunaga, M., H. Tokunaga, Y. Okajima, and T. Nakae.** 1979. Characterization of porins from the outer membrane of *Salmonella typhimurium*. 2. Physical properties of the functional oligomeric aggregates. Eur. J. Biochem. **95**:441–448.