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Disulfide-Mediated Interactions of the Chlamydial Major Outer Membrane Protein: Role in the Differentiation of Chlamydiae?

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The effects of exogenous reducing agents on a number of biological properties of purified *Chlamydia trachomatis* LGV-434 and *Chlamydia psittaci* meningopneumonitis elementary bodies (EBs) have been examined in an attempt to identify in vitro correlates of early events in the differentiation of the infectious EB to the replicative cell type, the reticulate body (RB). Treatment of EBs with dithiothreitol elicited a number of changes normally associated with differentiation to the RB. EBs in the presence of 10 mM dithiothreitol displayed enhanced rates of [^{14}C]glutamate oxidation, reduced infectivity, and decreased osmotic stability, and their Machiavello staining properties changed to those characteristic of the RB. A true differentiation of EB to RB did not take place under these conditions, since EBs treated in this manner and examined by transmission electron microscopy did not demonstrate increased size or decreased electron density as do isolated RBs. Additional studies were initiated to identify the macromolecules involved in this process. With polyacrylamide gel electrophoresis and immunoblotting procedures with monoclonal and polyclonal monospecific antibodies, the chlamydial major outer membrane protein was found to be the predominant component that varied under reducing versus nonreducing conditions. Furthermore, the extent of disulfide-mediated cross-linking of the major outer membrane protein varied between the infective and replicative forms of the *C. trachomatis* LGV-434 life cycle. Implications of disulfide interactions in the life cycle of chlamydiae are discussed.

Chlamydia spp. are obligately intracellular procaryotes characterized by a complex life cycle that takes place within phagosomes of eucaryotic cells (4, 31). After endocytosis of the infectious extracellular form, the elementary body (EB), phagosome-lysosome fusion is inhibited by poorly understood mechanisms (14, 15, 23). It is within these vesicles that replication of chlamydiae takes place. Within about 8 h after ingestion, the EBs differentiate into a larger, more pleomorphic, metabolically active cell type known as the reticulate body (RB). The RBs multiply by binary fission within the phagosome (15) until approximately 18 h postinfection, at which time they begin to reorganize and condense back to the EB forms, which are released from the cell to initiate a new infectious cycle.

The cell walls of chlamydiae resemble those of gram-negative bacteria in that they possess both inner and outer membranes, but differ in that they lack demonstrable peptidoglycan (1, 17, 25, 33). There is increasing evidence to suggest that disulfide-mediated interactions of chlamydial outer membrane proteins provide the function of maintaining structural stability in the absence of a peptidoglycan (3, 19, 28). Furthermore, the extent of cross-linking varies between the two stages of the life cycle, the EB cell wall being more cross-linked than that of the RB (19). Recently, porin function was ascribed to the chlamydial major outer membrane protein (MOMP), and this porin function was shown to be regulated by disulfide interactions (3).

Because of the apparent structural and functional importance of disulfide cross-linking of the chlamydial outer membrane, we have examined the effects of reducing agents on some simple biological properties of purified EBs in vitro, and we present preliminary characterization of the molecular interactions that accompany these changes.

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MATERIALS AND METHODS

Organisms. *Chlamydia trachomatis* strain LGV-434 (serotype L2) and *Chlamydia psittaci* meningopneumonitis (Mn) were grown in suspension cultures of mouse L-929 cells, and EBs were purified as previously described (9). RBs were purified as previously described (1), with the following modifications. Suspension cultures of L-929 cells infected 18 to 22 h previously were pelleted at 500 × *g* for 10 min and washed three times with 10 mM sodium phosphate–15 mM NaCl, pH 7.4. The cells were then swollen by suspension in 10 mM Tris–1 mM EDTA–250 mM sucrose, pH 7.4, at 4°C for 1 h, pelleted at 500 × *g* for 10 min, and suspended in cold Hanks balanced salt solution. Cells were disrupted with 10 to 15 strokes in a glass homogenizer with a tight-fitting Teflon pestle and centrifuged at 500 × *g* for 5 min. The supernatants were pooled, and the pellets were disrupted as described above. This process was repeated three times. Disruption of infected cells was monitored by phase microscopy. The pooled supernatants were then layered onto a 30% Renografin (E. R. Squibb & Sons, Inc., Princeton, N.J.) pad and centrifuged at 95,000 × *g* for 30 min. The pellet was suspended in Hanks balanced salt solution and layered over a discontinuous Renografin gradient, and the RB band at the 40 to 44% interface was collected. The RBs were then purified through a 10 to 60% linear Renografin gradient as described previously (9).

Infectivity determinations. Inclusion-forming units were determined as described by Furness et al. (16), except that inclusions were visualized by an indirect immunofluorescence assay employing polyclonal rabbit antisera against the *C. trachomatis* 155-kilodalton (kd) species-specific antigen (10) and fluorescein-conjugated goat anti-rabbit immunoglobulin serum (Cappel Laboratories, Cochranville, Pa.).

Immunological reagents. Preparation and specificity of the immunoglobulin G fraction of hyperimmune rabbit polyclonal antisera against purified sodium dodecyl sulfate (SDS)-denatured *C. trachomatis* L2 MOMP has been described previously (11). The monoclonal antibody (L2-1-45) was prepared as previously described (8) and recognized a type-specific epitope located on the MOMP of the *C. trachomatis* LGV-434 (H. D. Caldwell, manuscript in preparation).

Metabolic studies. Oxidation of [^{14}C]glutamate to CO_2 was determined as described previously (18), except that the EBs were suspended in 50 mM K_2HPO_4–250 mM sucrose at pH 7.5 and the final glutamate concentration was 5 μM (100 mCi/mmol).

PAGE. Polyacrylamide gel electrophoresis (PAGE) was by the method of Laemmli (22) with a acrylamide/bisacrylamide ratio of 30:0.8 and a final gel concentration of 12.5%. In those experiments noted, 2-mercaptoethanol (2-ME) was omitted from the solubilization buffer and replaced with an equal volume of water. Gels were stained with Coomassie brilliant blue R-250 in 25% isopropanol–7% acetic acid and destained in 40% ethanol–5% acetic acid. Silver staining was by the method of Tsai and Frasch (36) as modified by Hitchcock and Brown (21). Fluorography was as described by Bonner and Laskey (6).

Immunoblotting. The immunoblotting procedure of Towbin et al. (35) and Bittner et al. (5) as modified by Batteiger et al. (2) was used. Briefly, transfer to nitrocellulose paper (HAHY; Millipore Corp., Bedford, Mass.) was at 27 V/cm and 1.0 A for 1 h at 17°C in 25 mM sodium phosphate, pH 7.2. Tween 20 at a concentration of 0.05% (vol/vol) in 50 mM $NaPO_4$–150 mM NaCl, pH 7.25, was used as the blocking agent. Incubation with antibodies and ^{125}I-protein A was in 0.05% Tween 20–50 mM $NaPO_4$–150 mM NaCl. After binding of antibodies and protein A, the nitrocellulose sheets were briefly stained with 0.1% amido black 10B in 45% methanol–15% acetic acid (vol/vol) and destained with water to visualize protein bands. This procedure and the transfer of prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.) were used as aids in alignment of autoradiograms with the stained gels.

[^{14}C]iodoacetamide alkylation of EBs and RBs. Purified EBs and RBs (approximately 60 μg of protein of each) were incubated for 2 h at 4°C in 0.6 ml of 10 mM sodium phosphate–15 mM NaCl containing 10 μCi of [1-^{14}C]iodoacetamide (23.6 mCi/mmol; New England Nuclear Corp., Boston, Mass.). After 2 h, 1 μl of 0.1 M iodoacetamide was added to each, and the samples were held for another 1 h at 4°C. Each sample was divided equally and pelleted by centrifugation in a Beckman Microfuge 12 (Beckman Instruments, Inc., Fullerton, Calif.) for 10 min. One half of each sample was solubilized in 50 μl of Laemmli buffer without 2-ME, and the other half was solubilized in Laemmli buffer plus 2-ME. The samples were boiled for 10 min, and 10-μl samples of each were subjected to SDS-PAGE and fluorography.

Electron microscopy. Chlamydiae were fixed for 1 h at room temperature in 0.05 M cacodylate buffer, pH 7.4, containing 2.9% glutaraldehyde. The glutaraldehyde-fixed chlamydiae were pelleted in a microfuge, embedded in 0.5% agarose, and postfixed in 1.5% osmium tetroxide in the same buffer for 45 min at 0°C. The specimens were stained en bloc in 1% uranyl acetate in 0.05 M maleate buffer, pH 5.2, for 45 min, dehydrated in a graded series of ethanol, and embedded in Spurr embedding medium. Silver to gold sections were cut and stained in uranyl acetate and lead citrate and examined with a Hitachi (model HU-11E) electron microscope.

TABLE 1. Effect of reducing agents of the in vitro oxidation of glutamate by *C. trachomatis* LGV-434 and *C. psittaci* Mn EBs

Strain	pmol/h per mg of protein[a]	
	−DTT	+5 mM DTT
C. trachomatis LGV-434	5.8 ± 3.7[b]	27.8 ± 6.9[b]
C. psittaci Mn	220.2 ± 17.8[c]	385.3 ± 61.0[c]

[a] Results are expressed as picomoles of [*U*-^{14}C]glutamate recovered as $^{14}CO_2$. Organisms were suspended at concentrations of 10 to 30 μg of protein per ml of 50 mM K_2HPO_4–250 mM sucrose, pH 7.5, in rubber stopper-sealed flasks (18). The concentration of glutamate was 5 μM (100 mCi/mmol). After 4 h of incubation at 37°C with shaking, the reaction was terminated by the addition of trichloroacetic acid to 5% (vol/vol), and $^{14}CO_2$ was trapped on a hyamine hydroxide-soaked filter suspended from the stopper.

[b] Mean ± standard error of the mean, $n = 4$.

[c] Mean ± standard error of the mean; $n = 3$.

RESULTS

Effects of reducing agents on EB metabolism. Initial experiments tested the effect of reducing agents on the ability of either *C. trachomatis* LGV-434 or *C. psittaci* Mn to oxidize glutamate in an axenic buffer system. Rates of $^{14}CO_2$ evolution from LGV-434 EBs incubated in the presence of 1-[*U*-^{14}C]glutamate were increased up to 7.5-fold by the presence of 5 mM dithiothreitol (DTT) (Table 1). In three separate experiments, glutamate oxidation by *C. psittaci* Mn EBs was greater than that of *C. trachomatis* LGV-434 EBs. Glutamate oxidation by *C. psittaci* EBs was also stimulated by DTT, although the rates of $^{14}CO_2$ evolution were less than twofold greater than control levels in the absence of DTT. Glutamate oxidation by *C. trachomatis* EBs was stimulated over the range of 0.05 to 25 mM DTT. Other monothiol reducing agents such as 2-ME, reducted glutathione, and cysteine, also at 5 mM, were not effective.

Effects of DTT on staining characteristics. Treatment of purified EBs with 5 mM DTT resulted in a change in their Macchiavello staining properties (29) from the red characteristic of EBs to a blue that is typical of RBs. When washed and suspended in buffer without DTT for 12 to 18 h, the EBs regained the red-staining property of EBs. No increase in size of the EBs after DTT treatment was observed.

Osmotic instability in the presence of DTT. *C. trachomatis* LGV-434 EBs were suspended in buffers of decreasing osmolarity in the presence or absence of DTT, and optical density at 540 nm was followed with time. EBs in 250 mM sucrose–10 mM sodium phosphate–5 mM glutamate (SPG) buffer were relatively stable with or without DTT present (Fig. 1). With decreasing osmolarity, however, the presence of DTT resulted in decreased optical densities, indicating osmotic lysis of DTT-treated EBs.

Infectivity. The infectivity of DTT-treated *C. trachomatis* LGV-434 EBs was also examined. In these experiments, EBs were incubated in the presence of DTT, washed in SPG, and then allowed to bind HeLa 229 cell monolayers at 37°C; thus DTT was not present during chlamydial interaction with host cells. Infectivity of EBs, as determined by counting inclusions on HeLa cell monolayers, was reduced, after exposure to 10 mM DTT, to 21.8 ± 9.4% (mean ± standard error of the mean, $n = 4$) of controls in the absence of DTT.

Electron microscopy. Incubation of chlamydial EBs in the presence of reducing agents resulted in the EBs apparently acquiring at least some of the properties associated with the metabolically active, but noninfectious, RB. Electron microscopic examination of ultrathin sections of EBs that had been incubated for up to 24 h in the presence or absence of

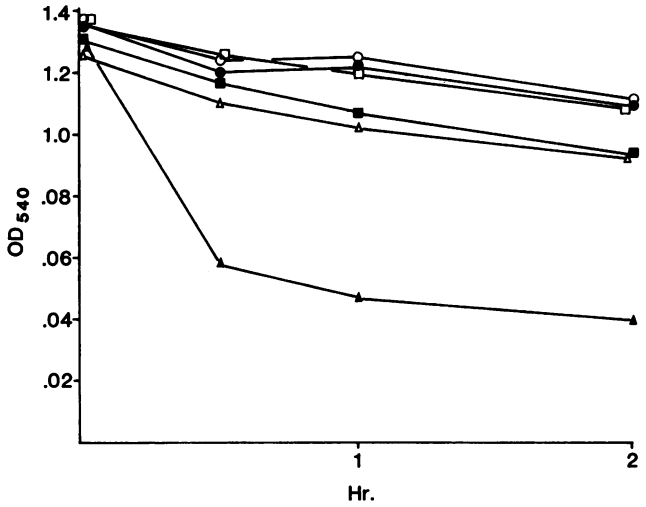

FIG. 1. Effect of DTT on the osmotic stability of purified *C. trachomatis* LGV-434 EBs. The EBs were suspended in SPG, pH 7.2, and diluted in SPG or distilled water in the presence or absence of 10 mM DTT. Symbols: ○, 1× SPG; ●, 1× SPG plus 10 mM DTT; □, 0.5× SPG; ■, 0.5× SPG plus 10 mM DTT; △, 0.125× SPG; ▲, 0.125× SPG plus 10 mM DTT.

DTT revealed no obvious ultrastructural alterations (Fig. 2). Most notably no increase in size of the EBs was observed under these conditions. Thus, the changes in properties described above were not accompanied by an increase in size or loss of electron density as occurs during differentiation in vivo. Electron micrographs of purified EBs and RBs are shown here for comparison.

The above biological data indicated that reduction of EBs in vitro might initiate early stages of chlamydial differentiation; however, it is clear that reduction alone in the absence of host cells was not sufficient for this transformation to be completed. Because of the apparent importance of reduction in triggering chlamydial differentiation, studies were initiated to identify the macromolecules involved in this process.

Effect of reduction on chlamydial polypeptides. *C. psittaci* Mn and *C. trachomatis* LGV-434 EBs were incubated in the absence or presence of 10 mM DTT for 2 h at 37°C. Another sample was incubated in the presence of DTT for 1 h at 37°C and then suspended in SPG without DTT for an additional 18 h at 4°C. The EBs were pelleted by centrifugation, excess buffer was carefully removed, and the cells were solubilized by boiling for 10 min in 2% SDS–10% glycerol in 62.5 mM Tris-hydrochloride, pH 6.8. A portion of each preparation was then analyzed by SDS-PAGE. A silver-stained polyacrylamide gel of *S. psittaci* Mn subjected to the above conditions is pictured in Fig. 3. A number of differences were apparent between EBs incubated in the presence or absence of DTT and those that had been treated with DTT, washed, and suspended in buffer. The most striking difference is that the chlamydial MOMP was not apparent in the gel profiles of EBs that had not been treated with DTT, presumably due to the occurrences of MOMP in multimers and large aggregates that fail to enter even the stacking gel (19, 28; see Fig. 5). Note also in the PAGE profile of those EBs initially incubated in the presence of DTT, washed, and then suspended in buffer without DTT, that monomeric MOMP appears to be present in decreased amounts and migrates with an apparently lower molecular weight. The *C. trachomatis* LGV-434 strain subjected to the same experimental conditions gave virtually identical results.

A silver-stained gel of the *C. trachomatis* LGV-434, similarly incubated in the absence or presence of DTT for 1 h or in the presence of DTT and then washed and incubated for various times in the absence of DTT, is shown in Fig. 4. Between 0 and 2 h after removal of DTT, MOMP resolved into two bands, both of which reacted with the monoclonal antibody against the type-specific determinant of the MOMP (data not shown). By 4 h, the lower of the two bands was most prominent in the stained gel; by 12 h, neither was apparent. In the far right lane in Fig. 4, the EBs were incubated in the presence of the alkylating agent *N*-ethylmaleimide (NEM) for 12 h after the initial 1-h contact period with DTT. By immunoblot analysis (data not shown) of a parallel gel with polyclonal anti-MOMP antibodies, MOMP

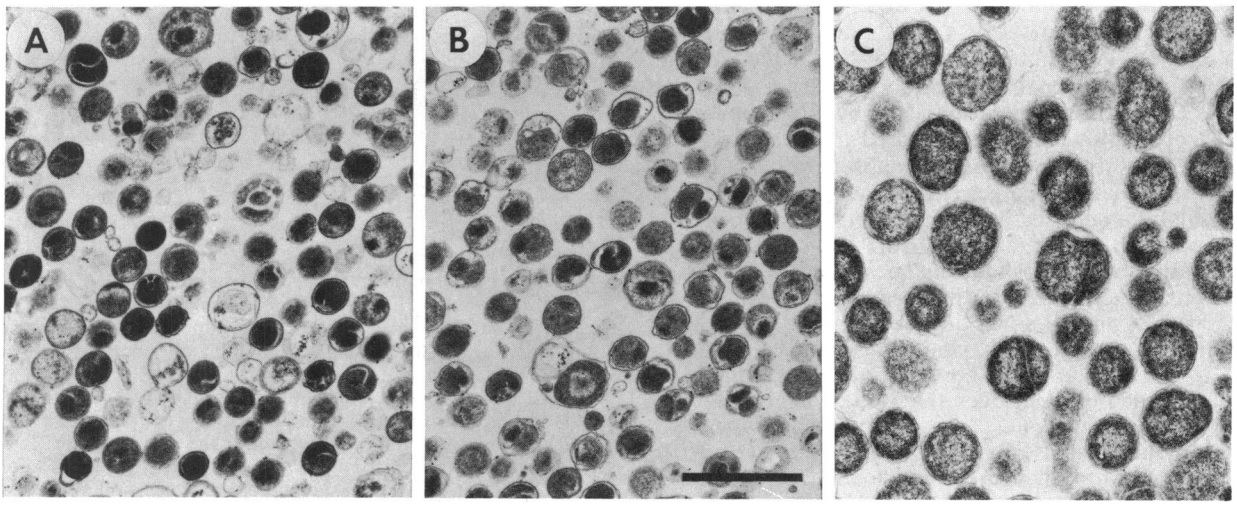

FIG. 2. Electron micrograph of EBs incubated in the absence and presence of 10 mM DTT and comparison with purified RBs. EBs were suspended in SPG plus 25 mM ATP with amino acids and vitamins at the concentration of Eagle minimum essential medium in the absence of DTT (A) or the presence of 10 mM DTT (B). Purified RBs (C) are shown for comparison. The bar indicates 1 μm. The magnification of all three panels is identical. It should be noted that intermediate forms of the RB-to-EB transition occur and that these intermediate forms may copurify with EBs. The EB preparation used here may thus contain intermediate forms whose susceptibility to DTT is unknown.

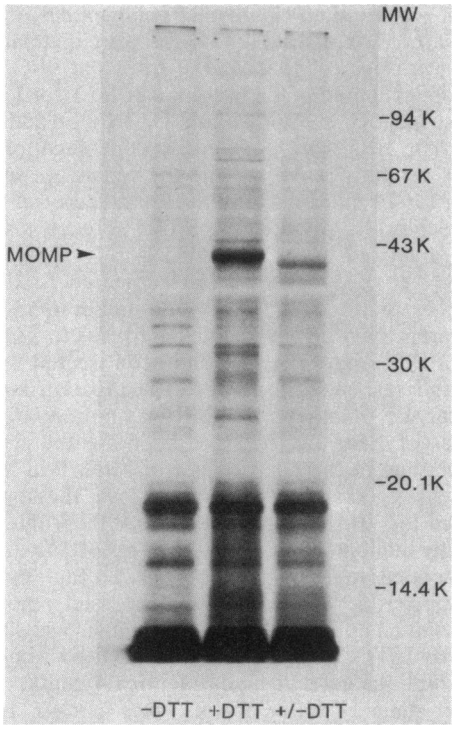

FIG. 3. Silver-stained SDS-PAGE profile of *C. psittaci* Mn EBs incubated in the absence of DTT, in 10 mM DTT, or in 10 mM DTT for 1 h at 37°C and then washed and incubated an additional 18 h at 4°C in the absence of DTT. Organisms were solubilized for electrophoresis in Laemmli digestion buffer without 2-ME.

was, with time, apparent in aggregates migrating above the position of monomer MOMP. Antigenic fragments of MOMP, migrating below the position of monomer MOMP, were not detected.

Comparison of EBs and RBs. The effects of reducing agents on the various biological properties of EBs and the correlation of these effects with the migration of MOMP in polyacrylamide gels suggested that differences in disulfide-mediated associations of MOMP may be involved in the differentiation of EBs to RBs. To examine this possibility, purified *C. trachomatis* LGV-434 EBs and RBs were subjected to SDS-PAGE, and MOMP was detected by immunoblotting with monoclonal antibody. Intact purified EBs and RBs (Fig. 2) were either alkylated by incubation in the presence of 5 mM NEM or not alkylated and then solubilized for electrophoresis in the presence or absence of 2-ME. Both a Coomassie blue-stained gel and an autoradiograph of the immunoblot are depicted in Fig. 5. In those samples solubilized in the presence of 2-ME, MOMP from either EBs or RBs migrated to the position of the 39.5-kd monomer. In those samples solubilized in the absence of 2-ME, MOMP from both EBs and RBs was detected in what appear to be monomers, dimers, trimers, and larger oligomeric aggregates. In EB preparations, however, the large oligomeric aggregates failed to enter even the 5% acrylamide stacking gel. These large aggregates were present in both the alkylated and nonalkylated EBs, but were not detected in RB preparations. Note that the immunoreactive species of MOMP migrating just below monomer MOMP is not apparent in either the stained gel or the immunoblot of NEM-alkylated organisms.

The EBs or RBs were either not alkylated or alkylated with NEM before solubilization to block reactivity of free sulfhydryls. In those preparations solubilized in the absence of 2-ME without prior alkylation, approximately equal amounts of monomeric MOMP were apparent on the Coomassie brilliant blue-stained gel of both EBs and RBs. However, in this case, without prior alkylation, most of the monomeric MOMP from either EBs or RBs migrated to the position (about 36 kd) of the lower of the two MOMP bands described above. If alkylated before solubilization, MOMP migrated to the position of the 39.5-kd monomer form, with apparently more monomeric MOMP observed from RBs solubilized in the absence of 2-ME than from EBs solubilized under the same conditions. It appears, therefore, that alkylation of chlamydiae may be necessary to prevent disulfide exchange or cross-linking of cysteine-containing polypeptides during solubilization. We feel that the polypeptide profiles of EBs and RBs alkylated before solubilization are therefore more reflective of their natural conditions. It must be kept in mind, however, that both EBs and RBs had been put through an extensive purification process during which some oxidation or cross-linking might occur; thus the SDS-PAGE profiles may not accurately reflect the extent of outer membrane cross-linking in vivo.

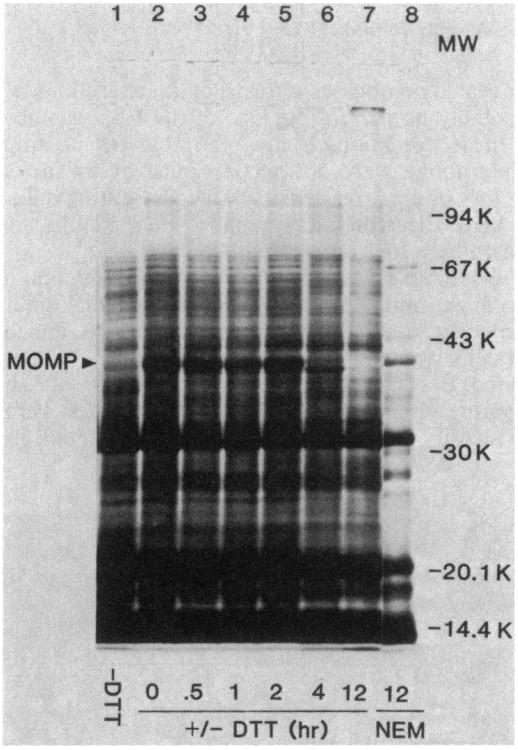

FIG. 4. Silver-stained SDS-PAGE profile of *C. trachomatis* LGV-434 EBs demonstrating reassociation of MOMP with time after removal of DTT. A parallel Western blot indicated the formation of higher-molecular-weight species, apparently dimers, trimers, and larger aggregates. The band immediately under MOMP reacts with antibodies to MOMP. This lower-molecular-weight (36,000) MOMP polypeptide is believed to be MOMP, but with internal disulfide bridges resulting in a slightly lower apparent molecular weight. Lane 1 is a control incubated in the absence of DTT. Lanes 2 through 9 were incubated in the presence of 10 mM DTT for 1 h, and the organisms were washed before resuspension in the absence of DTT and incubation at 37°C for the indicated times. Lane 9 was similarly treated, except NEM was added after washing to alkylate-free sulfhydryl groups. Blocking free sulfhydryl groups with NEM apparently interferes with reorganization of the MOMP. All were solubilized for electrophoresis in Laemmli buffer without 2-ME.

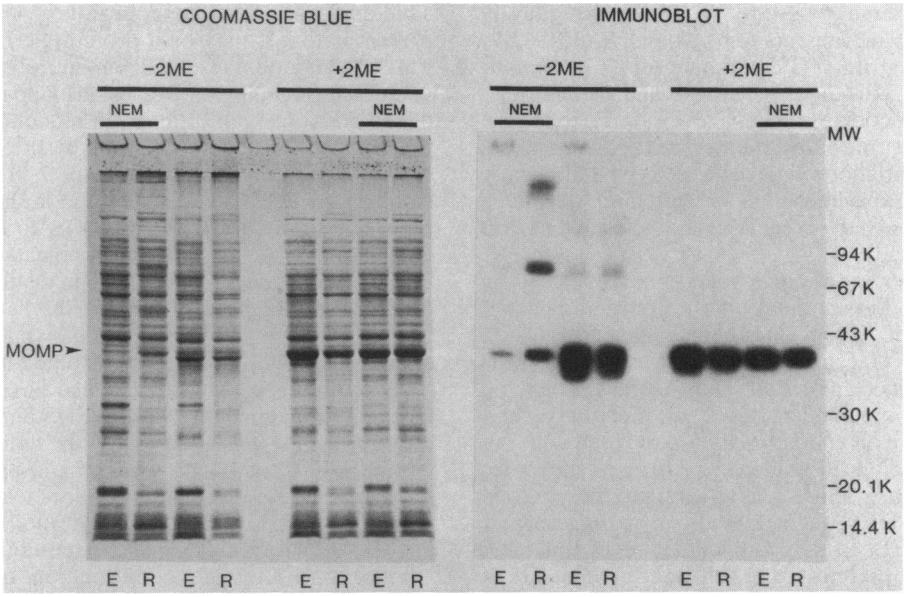

FIG. 5. Comparison of the SDS-PAGE profiles *C. trachomatis* LGV-434 EBs (E) with reticulate bodies (R). The chlamydiae were solubilized for electrophoresis either in the presence or absence of 2-ME. In the outside lanes of each gel, the organisms were first alkylated with NEM before solubilization to block reformation of disulfide bonds. Parallel gels were run and stained either with Coomassie blue or immunoblotted with monoclonal antibody against MOMP.

Reactivity of exposed sulfhydryls. The differences in disulfide-mediated interactions of MOMP observed between EBs and RBs suggested the probability of a greater number of reduced sulfhydryls on the RB surface. To test this hypothesis, purified EBs and RBs were alkylated with [^{14}C]iodoacetamide and subjected to SDS-PAGE and fluorography to identify those polypeptides containing free sulfhydryl groups (Fig. 6). EBs were poorly labeled by iodoacetamide. MOMP was the predominant protein labeled on EBs, and four other polypeptides were weakly reactive. Only one polypeptide, migrating just below the 14.4-kd marker, was labeled equivalently on both EBs and RBs. In general, many more RB polypeptides were reactive with [^{14}C]iodoacetamide. MOMP was intensely reactive. A large number of RB polypeptides were labeled; however, this may be due in part to the increased permeability of the RB outer membrane, allowing greater penetration of the label and reaction with polypeptides and sulfhydryls not exposed on the surface. Therefore, the increased number of polypeptides labeled by [^{14}C]iodoacetamide and the apparently increased labeling of MOMP on RBs may not necessarily be due to an increased number of sulfhydryls, but may be due simply to differences in accessibility of these groups. Such an effect has been previously described for bacterial spores during germination (32).

DISCUSSION

Tamura and Manire (33) were the first to suggest, based on amino acid compositions of EB and RB outer membranes, that disulfide bonding of membrane proteins might be involved in the structural stability of these bacteria, which lack demonstrable peptidoglycan (1, 17, 25, 33). Chlamydial EBs extracted with the detergent Sarkosyl retain the characteristic rigid spherical morphology of the EBs (9), leading to the suggestion that protein interactions might contribute to the structural stability of chlamydiae. The findings that detergents plus reducing agents, but not detergents alone, solubilized EB outer membrane proteins has been taken as evidence that the structural stability of EBs is conferred by disulfide bonding of outer membrane proteins (3, 19, 20, 28). The increased osmotic fragility of EBs in the presence of reducing agents demonstrated here further supports a role for disulfide bonding in maintaining EB structure.

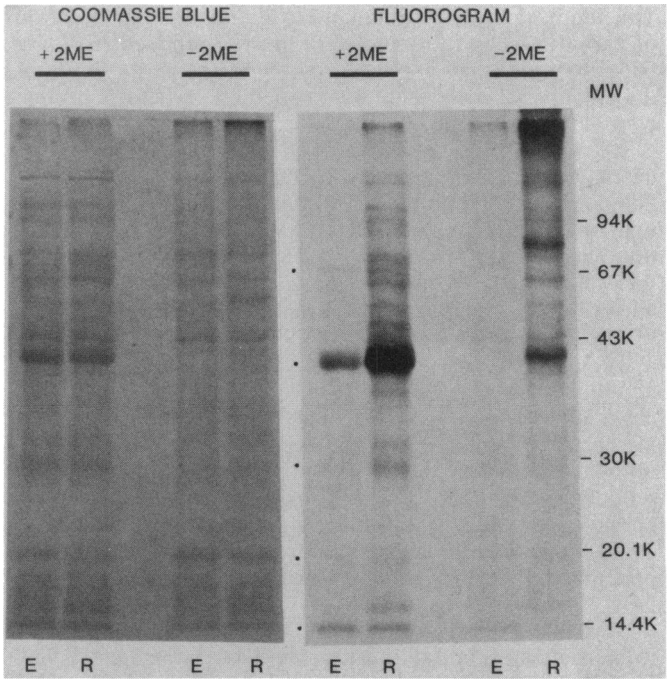

FIG. 6. Polypeptides of *C. trachomatis* LGV-434 EBs (E) and RBs (R) containing free sulfhydryl groups reactive with [1-^{14}C]iodoacetamide. The chlamydiae were solubilized for electrophoresis either in the presence or absence of 2-ME. The left panel depicts the Coomassie brilliant blue-stained gel before fluorography. A fluorogram of that gel is depicted in the right panel. Dots to the left of the fluorogram indicate the positions of weakly reactive polypeptides on EBs.

The disulfide-mediated cross-linking of the structurally rigid EB outer membrane appears to be extensive (3, 19, 28), but, as shown for *C. psittaci* (19) and here for *C. trachomatis*, it is less extensive in the more fragile and pleomorphic RB. Therefore, reduction or cleavage (or both) of these disulfide interactions of outer membrane proteins likely accompanies differentiation of the EB to the metabolically active RB. In the experiments described here, we have examined some effects of exogenous reducing agents on purified chlamydial EBs.

A number of properties of chlamydial EBs are altered by reducing conditions. Among the properties observed changing after exposure of EBs to DTT are enhanced rates of glutamate oxidation, Macchiavello staining properties characteristic of RBs, reduced infectivity, and decreased osmotic stability. Increased metabolism, lack of infectivity, and osmotic instability are all characteristics associated with the RB (31). No morphological changes were apparent under these same conditions. Thus, it is clear that the changes induced in EBs by DTT did not result in a triggering of differentiation from EBs to RBs as occurs in vivo. Reducing conditions alone are, therefore, not sufficient to stimulate in vitro a complete EB-to-RB transition.

Sarov and Becker (30) observed the increased rates of incorporation of nucleotides into the RNA of EBs in vitro in the presence of 2-ME. More recently, Bavoil et al. (3) have ascribed porin functions to MOMP. They demonstrated that MOMP displayed enhanced pore-forming activity when first reduced with DTT and then alkylated with iodoacetamide to prevent reoxidation. Our results were similar to those of Sarov and Becker (30); we observed somewhat increased rates of oxidation of radiolabeled glutamate by intact EBs when reducing agents were included in the assay mixture. This increased rate of glutamate oxidation and the retention of carbol fuchsin by unreduced, but not reduced, EBs are consistent with and explained by increased permeability of the chlamydial outer membrane when the structural protein components are in a reduced state.

We found that exposure of EBs to DTT resulted in decreased infectivity, suggesting that certain oxidized disulfide bonds may be required for infectivity. The design of the experiment was such that the EBs were washed after reduction and that DTT was not present during interaction with the HeLa cells. We were unable to test the effect of alkylation to prevent reoxidation of these reduced sulfhydryls since iodoacetamide or NEM abolished infectivity of reduced or unreduced EBs (Hackstadt and Caldwell, unpublished observations). It is apparent from Fig. 4 that reoxidation of reduced disulfide bonds among the outer membrane proteins of the EBs occurs once reducing conditions are removed. Although reoxidation occurs, the EBs reduced and then reoxidized do not regain infectivity. This is perhaps not surprising, since unnatural mixed disulfides could be formed upon reoxidation, or some essential component may be lost from the reduced and permeabilized EBs.

During the reoxidation of MOMP after removal of EBs from reducing conditions, a protein migrating just below MOMP on polyacrylamide gels, with an apparent molecular mass of approximately 36 kd, becomes apparent. This protein reacts with antibodies to MOMP, is not apparent when the EBs are alkylated before solubilization, and becomes the predominant species of MOMP during reoxidation of reduced EBs, but is not seen after longer periods of reoxidation. This protein appears analogous to the 36-kd protein previously observed to copurify with MOMP (9). One possible explanation for the appearance of this MOMP-related protein is that it may be a proteolytic fragment of MOMP. This explanation does not seem entirely satisfying, since no additional fragments of MOMP are apparent even after 12 h of reoxidation when little or no monomeric MOMP is seen (Fig. 4) and shifts its position to the gel upward if first electrophoresed in the absence of 2-ME and then re-electrophoresed in a second dimension in the presence of 2-ME (data not shown). The data lead us to favor an alternative explanation that this protein represents an aberrant migration of MOMP due to an intrachain disulfide bond leading to an unfavorable conformation. If this is indeed the case, it suggests that, in addition to the known intermolecular interactions of MOMP, different disulfide-mediated conformations of monomer MOMP are also possible. An artifactual basis of this phenomenon in SDS-denatured MOMP on polyacrylamide gels is certainly one consideration. Another is that in this highly cross-linked and predominant protein, inter- and intramolecular disulfide exchange may be commonplace and function to regulate not only porin activity, but also structural rigidity and membrane fluidity in general.

Since this alternate conformation of MOMP was not present when EBs or RBs were first alkylated before solubilization, it suggests that at least one free sulfhydryl of MOMP is available for alkylation. However, it appears that not all MOMP monomers as they exist on the EB surface are equivalently and as extensively cross-linked. A number of structural features of the EB and RB surface have been described (12, 24, 26, 27). Hexagonally arrayed lattice-like structures have been described on the inner surface of the EB outer membrane (12, 27). Chang et al. recently demonstrated that this structure from RBs was more soluble in detergent than was the structure from EBs (12). Since MOMP appears to be linked in both homooligomers and heterooligomers (20, 28), the intermolecular interactions of MOMP and the role of these complexes in maintaining a variety of supramolecular structures (12, 24, 26, 27) will be of interest.

The extent of cross-linking of the EB outer membrane requires that reductive cleavage of disulfide bonds precede differentiation to the larger and pleomorphic RB. The source of reducing power for this cleavage remains unknown. Host-supplied reducing compounds may play a role in the initial reduction of bonds involved in maintaining the rigid EB structure. Both porin activity (3) and outer membrane fluidity would be predicted to increase, thus enhancing permeability and allowing transport of required nutrients that would lead to increased chlamydial metabolism and generation of endogenous reducing power. As the infectious cycle progresses, a depletion of metabolic substrates would occur, favoring oxidation of reduced sulfhydryls among RB outer membrane proteins. Blebbing of the RB outer membrane has been described by Tanami and Yamada (34). If the process of cross-linking outer membrane proteins via disulfide bonds stimulated blebbing, a mechanism for the reduction of RB mass may be provided. This cross-linking might then promote a rigid outer membrane structure, leading to the condensation process involved in the reorganization of the RB to the infectious EB.

Although purely speculative, an interesting alternative mechanism for cleavage of the critical outer membrane disulfides on the EB surface would be exchange and mixed disulfide formation with host cell proteins. Such a mechanism might explain the highly efficient internalization properties characteristic of chlamydiae (7). Precedence for this type of interaction may be found in the interaction of insulin

with its cell surface receptor (13) and the proposed role of disulfides in the internalization of diphtheria toxin (37). Such a covalent interaction of an obligate intracellular parasite, like chlamydiae, with eucaryotic cell surface receptors would be unique and could potentially influence internalization and inhibition of phagosome-lysosome fusion. We are presently attempting to explore these possibilities.

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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. **Batteiger, B., W. J. Newhall V, and R. B. Jones.** 1983. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
3. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44**:479–485.
4. **Becker, Y.** 1978. The chlamydiae: molecular biology of procaryotic obligate parasites of eucaryotes. Microbiol. Rev. **42**: 274–306.
5. **Bittner, M., P. Kupferer, and C. F. Morris.** 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose of nitrocellulose sheets. Anal. Biochem. **102**:459–471.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
7. **Byrne, G. I., and J. W. Moulder.** 1978. Parasite-specified phagocytosis of *Chlamydia psittaci* and *Chlamydia trachomatis* by L and HeLa cells. Infect. Immun. **19**:598–606.
8. **Caldwell, H. D., and P. J. Hitchcock.** 1984. Monoclonal antibody against a genus-specific antigen of *Chlamydia* species: location of the epitope on chlamydial lipopolysaccharide. Infect. Immun. **44**:306–314.
9. **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.
10. **Caldwell, H. D., and C. C. Kuo.** 1977. Purification of a *Chlamydia trachomatis* specific antigen by immunoadsorption with monospecific antibody. J. Immunol. **118**:437–441.
11. **Caldwell, H. D., and J. Schachter.** 1982. Antigenic analysis of the major outer membrane protein of *Chlamydia* spp. Infect. Immun. **35**:1024–1031.
12. **Chang, J. J., K. Leonard, T. Arad, T. Pitt, Y. N. Zhang, and L. H. Zhang.** 1982. Structural studies of the outer envelope of *Chlamydia trachomatis* by electron microscopy. J. Mol. Biol. **161**:579–590.
13. **Clark, S., and L. C. Harrison.** 1982. Insulin binding leads to the formation of covalent (-S-S-) hormone receptor complexes. J. Biol. Chem. **257**:12239–12244.
14. **Eissenberg, L. G., P. B. Wyrich, C. H. Davis, and J. W. Rumpp.** 1983. *Chlamydia psittaci* elementary body envelopes: ingestion and inhibition of phagosome lysosome fusion. Infect. Immun. **40**:741–751.
15. **Friis, R.** 1972. Interaction of L-cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110**:706–721.
16. **Furness, G., D. M. Graham, and P. Reeve.** 1960. The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. J. Gen. Microbiol. **23**:613–619.
17. **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.
18. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. U.S.A. **78**:3240–3244.
19. **Hatch, T. P., I. Allen, and J. H. Pearce.** 1984. Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. J. Bacteriol. **157**:13–20.
20. **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.
21. **Hitchcock, P. J., and T. M. Brown.** 1983. Morpholoical heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Lawn, A. M., W. A. Blyth, and J. Taverne.** 1973. Interaction of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy. J. Hyg. **71**:515–528.
24. **Louis, C., G. Nicolas, F. Eb, J.-F. Lefebvre, and J. Orfila.** 1980. Modifications of the envelope of *Chlamydia psittaci* during its developmental cycle: freeze-fracture study of complementary replicas. J. Bacteriol. **141**:868–875.
25. **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.
26. **Matsumoto, A.** 1973. Fine structures of cell envelopes of *Chlamydia* organisms as revealed by freeze-etching and negative staining techniques. J. Bacteriol. **116**:1355–1363.
27. **Matsumoto, A., and G. P. Manire.** 1970. Electron microscopic observations on the fine structure of cell walls of *Chlamydia psittaci*. J. Bacteriol. **104**:1332–1337.
28. **Newhall, W. J. V., and R. B. Jones.** 1983. Disulfide linked oligomers of the major outer membrane of chlamydiae. J. Bacteriol. **154**:998–1001.
29. **Rake, G., and H. P. Jones.** 1942. Studies on lymphogranuloma venereum. I. Development of the agent in the yolk sac of the chicken embryo. J. Exp. Med. **75**:323–338.
30. **Sarov, I., and Y. Becker.** 1971. Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity in purified trachoma elementary bodies: effect of sodium chloride on ribonucleic acid transcription. J. Bacteriol. **107**:593–598.
31. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Annu. Rev. Microbiol. **34**:285–309.
32. **Setlow, P., and A. Kornberg.** 1969. Biochemical studies of bacterial sporulation and germination. XVII. Sulfhydryl and disulfide levels in dormancy and germination. J. Bacteriol. **100**:1155–1160.
33. **Tamura, A., and G. P. Manire.** 1967. Preparation and chemical composition of the cell membranes of developmental reticulate forms of meningopneumonitis organisms. J. Bacteriol. **94**: 1184–1188.
34. **Tanami, Y., and Y. Yamada.** 1973. Miniature cell formation in *Chlamydia psittaci*. J. Bacteriol. **114**:408–412.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
36. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
37. **Wright, H. T., A. W. Marston, and D. J. Goldstein.** 1984. A functional role for cysteine disulfides in the transmembrane transport of diphtheria toxin. J. Biol. Chem. **259**:1649–1654.