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Role of Disulfide Bonding in Outer Membrane Structure and Permeability in *Chlamydia trachomatis*

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The outer membrane of *Chlamydia trachomatis* can be efficiently solubilized by a variety of mild detergents in the presence of the reducing agent dithiothreitol. This allows purification of the chlamydial major outer membrane protein at high yield in very gentle conditions by using its differential solubility in Sarkosyl and octylglucoside in the presence of dithiothreitol. The major outer membrane protein of the L2 serovar is an acidic protein with a pI of ca. 5. It contains three cysteine residues that allow it to form a disulfide-linked proteinaceous network responsible for the characteristic rigid outer membrane of the elementary body. By the use of an in vitro reconstitution assay developed by Nikaido and his co-workers, it was shown that the outer membrane contains pores with an "exclusion limit" between molecular weights 850 and 2,250. In addition, the "opening-closing" of the pores was shown to be controlled through a simple reduction-oxidation mechanism. A model that outlines the role of disulfide bonding in the physiology of chlamydial development is presented.

Chlamydiae are obligate intracellular parasites that obtain nutrients and energy from an infected host (3, 27). They undergo a unique developmental growth cycle. The infectious particles (elementary bodies; EBs) are somewhat similar to spores of other bacteria: they are relatively small, apparently metabolically inactive, and resistant to a variety of physical insults, e.g., sonication, variations of osmotic pressure, etc. Infection is initiated when the host cell phagocytoses at least one EB particle. In its new environment, the EB differentiates into a metabolically active form, the reticulate body (RB). RBs are larger and sensitive to environmental variations. They are the actively multiplying form of chlamydiae. They eventually condense themselves to form infectious EBs that are released into the surrounding environment upon lysis of the host cell.

At all stages of development chlamydial cells appear to be surrounded by a double membrane system, a characteristic feature of gram-negative bacteria. The chlamydial cell envelope differs, however, by the apparent absence of a peptidoglycan layer in the space between the two membranes (2). This implies that chlamydiae must have some other structural component to fill the role that peptidoglycan plays in typical gram-negative bacteria (maintaining shape and resistance to variations of osmotic pressure). Largely on the basis of indirect evidence, it has been suggested that these functions are filled by a disulfide-linked proteinaceous network in the outer membrane (2, 29). Although consistent with the needs of the EB, this model probably does not apply in the case of the RB. As opposed to EBs, RBs have a fragile outer membrane of poorly defined shape and are probably subject to much less physical stress inside the phagosome.

Outer membranes of gram-negative bacteria also function as permeability barriers. In most cases, the major outer membrane protein (MOMP [by weight]) has been characterized as a pore-forming protein or porin (21; L. Shaltiel Zalman, Ph.D. thesis, University of California, Berkeley, 1982). With conspicuous regularity, porins of gram-negative bacteria and mitochondria are acidic proteins of 30,000 to 40,000 daltons that are present in the outer membrane as oligomeric structures. MOMP has been shown to be one of the major antigens of the chlamydial surface; it has a molecular weight of 39,500 (range, 38,000 to 42,000) (4, 6).

In this study, we investigated the structure and function of the EB outer membrane, particularly with regard to (i) its ability to rigidify itself through protein covalent cross-linking mediated by disulfide bonding, and (ii) its ability to form water-filled pores allowing passive diffusion of hydrophilic solutes. Finally, we discuss a model that takes into account these features together with physiological requirements specific to the chlamydial developmental cycle.

MATERIALS AND METHODS

Organism and growth conditions. *Chlamydia trachomatis* L2/434/Bu was used throughout this study. It was grown in suspension cultures of L-929 cells (5).

Purification of chlamydiae. Chlamydiae were purified by the procedure of Caldwell et al. (4), except for the following modifications: (i) centrifugation through the 35% Renografin cushion was omitted, (ii) supernatants from the 500 × *g* centrifugation were centrifuged at 30,000 × *g* for 30 min at 4°C, and (iii) pellets were suspended in 25 ml of Hanks balanced salt solution containing RNase and DNase, incubated for 1 h at 37°C, and applied to Renografin gradients.

Purification of MOMP. MOMP was purified from EBs (20 mg of protein) by a three-step extraction: (i) extraction of EBs with 1% Sarkosyl, (ii) extraction of the Sarkosyl-insoluble fraction (chlamydial outer membrane complexes; COMCs) with 1% Sarkosyl–10 mM dithiothreitol (DTT), and (iii) extraction of the Sarkosyl–DTT-insoluble fraction with 1% octylglucoside–10 mM DTT. All steps were carried out in 10 mM sodium phosphate buffer (pH 7.4). Extraction mixtures (5, 7.5, and 7.5 ml, respectively) were each incubated for 30 min at 37°C with several short (under 30 s) sonications in a Bransonic bath sonicator and centrifuged at 40,000 rpm in a Beckman 75 Ti rotor for 1 h at 20°C. Each pellet was suspended in buffer (1.5 ml) before the next step.

Liposome swelling assay. The liposome swelling assay was done by the method of Luckey and Nikaido (16) with the following modifications: (i) a mixture of acetone-extracted egg yolk phosphatidylcholine (type IX-E; Sigma Chemical

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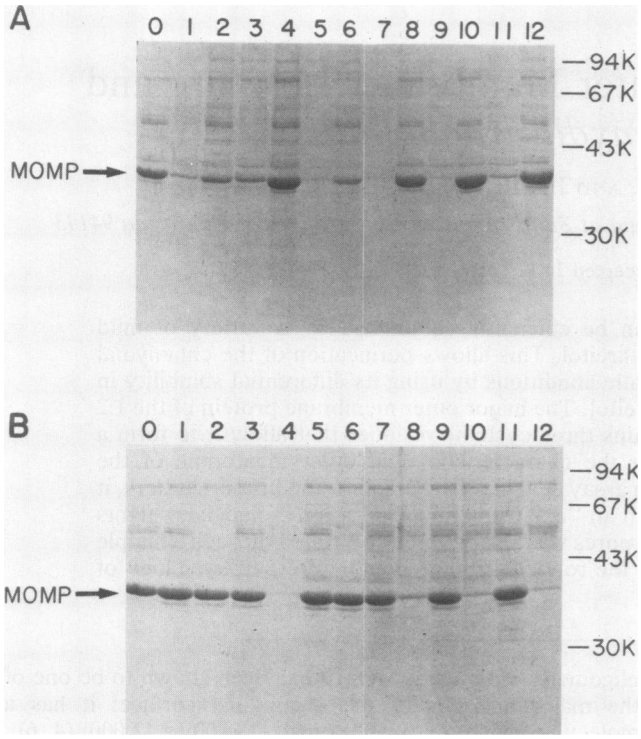

FIG. 1. Solubilization of COMCs with various detergents. EBs (20 mg of protein) were extracted in 10 mM sodium phosphate buffer (pH 7.4) containing 1% Sarkosyl (final volume, 5 ml). The conditions of extraction throughout this experiment were as described in the text. The Sarkosyl-insoluble material (COMCs) was resuspended in 1.8 ml of 50 mM sodium phosphate buffer (pH 7.4). A portion of this (0.1 ml) was used for each extraction (0.5 ml final volume). Detergent was added (2% final concentration) in the presence (even numbers) or the absence (odd numbers) of 10 mM DTT. The detergents used for the various lanes were Sarkosyl (1 and 2), SDS (3 and 4), deoxycholate (5 and 6), Triton X-100 (7 and 8), octylglucoside (9 and 10), and Zwittergent (11 and 12). Lane 0 (panels A and B), 3 μl of COMCs; lanes 1 through 12 (panel A), 15 μl of each extract; lanes 1 through 12 (panel B), 15 μl of each residue after suspension in 0.5 ml of 2% SDS–10 mM DTT. The positions of MOMP and molecular weight standards are indicated.

Co.) and dicetylphosphate (Sigma) was used (23); (ii) dextran T-40 (Pharmacia Fine Chemicals) was used at a final concentration of 15%; and (iii) the optical density was recorded at 540 nm with a Gilford 250 spectrophotometer.

Protein samples used in the swelling assay were COMCs suspended in 50 mM sodium phosphate buffer (pH 7.4) or COMCs treated sequentially with DTT and iodoacetamide as follows. COMCs (200 μg) were suspended in 62.5 mM Tris-hydrochloride (pH 7.4) containing 25 mM DTT (0.4 ml final volume) and incubated for 15 min at 20°C. The suspension was then incubated for another 15 min at 20°C after addition of 0.1 ml iodoacetamide (0.1 M final concentration). The DTT–iodoacetamide-treated COMCs were then pelleted at 10,000 rpm in an SS34 rotor for 10 min at 20°C, and suspended in an appropriate amount of 50 mM sodium phosphate buffer (pH 7.4). This material was used in the swelling assay. Buffer blanks were assayed in parallel.

Analytical methods. Protein content was determined by the method of Lowry et al. (15), with bovine serum albumin as standard. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was run as described by Laemmli (13). Two-dimensional gel electrophoresis was done as described by O'Farrell (24), with the modification of Ames and Nikaido (1) for membrane proteins. Ampholines (pH 3.5 to 10) (LKB Instruments Inc.) were used. Amino acid analysis was performed on a Beckman 120C amino acid analyzer. Cysteine and methionine contents were determined after performic acid oxidation of the protein.

RESULTS

Solubilization of the chlamydial outer membrane in the presence of a reducing agent. Caldwell and colleagues first demonstrated that the chlamydial outer membrane can be differentially extracted by Sarkosyl treatment (4). We found, based on the similarity of the SDS-polyacrylamide gel electrophoresis patterns, that the same result can be obtained by using the nonionic detergent octylglucoside (data not shown). The COMCs obtained in this way resemble emptied ghosts of the EB and can be solubilized by SDS after 1 h at 37°C (4). We sought a milder detergent to replace SDS for solubilization of the COMCs and further purification of MOMP. This was done by taking advantage of the known sensitivity of MOMP to reducing agents (20). Figure 1 shows that (i) regardless of the detergent used, MOMP solubilization was always enhanced, sometimes markedly, when DTT was present in the extraction mixture; (ii) MOMP could be readily and fully solubilized by mild nonionic (Triton X-100, octylglucoside) or dipolar ionic (Zwittergent) detergents in the presence of DTT; and (iii) MOMP was poorly extracted by mild anionic (deoxycholate, Sarkosyl) detergents in the presence of DTT. In addition, MOMP was also readily solubilized when a cationic detergent (Cetavlon) was used with DTT (data not shown). Except for SDS, a strong anionic detergent, no detergent extracted MOMP efficiently in the absence of a reducing agent. The yield of the extraction with SDS was also greatly improved in the presence of DTT (Fig. 1).

When the DTT concentration present during the extraction was reduced, the COMCs remained in the insoluble fraction; only cytoplasmic and solubilized membrane proteins were released in the extract (Fig. 2, lanes I to Q). This

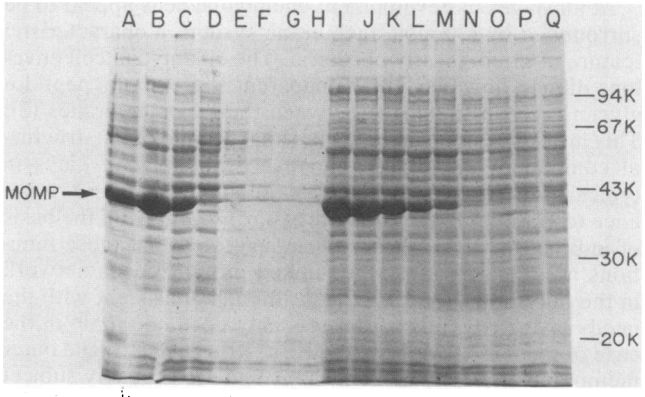

FIG. 2. Influence of detergent and reducing agent concentrations on the solubilization of chlamydial outer membrane proteins. EBs (0.4 μg of protein) were extracted in 50 mM sodium phosphate buffer (pH 7.4) containing octylglucoside and DTT at various concentrations. The conditions of extraction were as described in the text (extraction final volume, 0.1 ml). The octylglucoside concentrations (percent) were 2 (B), 1 (C), 0.4 (D), 0.2 (E), 0.1 (F), 0.02 (G), none (H), 2 (I to Q). The DTT concentrations (millimolar) were 10 (B to I), 5 (J), 2.5 (K), 1 (L), 0.5 (M), 0.1 (N), 0.05 (O), 0.01 (P), none (Q). Lane A represents 40 μg of the starting material. A portion of each extract (10 μl) was applied to the gel. The positions of MOMPs and molecular weight standards are indicated.

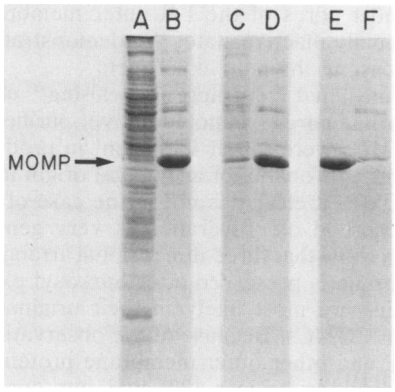

FIG. 3. Purification of MOMP in octylglucoside-DTT. MOMP was purified as described in the text. Samples for SDS-polyacrylamide gel electrophoresis were (A) 10 μl of Sarkosyl-soluble fraction; (B) 2 μl of Sarkosyl-insoluble fraction; (C) 10 μl of Sarkosyl–DTT-soluble fraction; (D) 3 μl of Sarkosyl–DTT-insoluble fraction; (E) 10 μl of octylglucoside–DTT-soluble fraction; (F) 7 μl of octylglucoside–DTT-insoluble fraction. The position of MOMP is indicated.

result suggests that detergent alone renders the outer membrane permeable to large macromolecules, presumably by extensively solubilizing the phospholipid bilayer areas of the membrane, without disrupting the proteinaceous skeleton of the membrane, i.e., the COMC. Reducing agent alone did not allow the release of soluble proteins from the EB, showing that the integrity of the membrane and its structural role were maintained after reduction (Fig. 2, lanes B to H). It is noteworthy, however, that a small amount of known COMC-associated proteins (including MOMP) appeared in the soluble fraction after DTT treatment in the absence of detergent (Fig. 2, lane H). This indicates that (i) these proteins were readily released from the EB upon DTT action without the preliminary disruption of the membrane by a detergent, thus confirming their presence on the outer surface of the EB, and (ii) in their native state in the EB outer membrane, these proteins were probably cross-linked by disulfide bridges, most likely in the form of large hetero-oligomers.

Purification and characterization of MOMP. The purification procedure was modified from that of Caldwell et al. (4). Solubilization and chromatography in SDS were bypassed with the intent of conserving the native conformation of the protein by the use of a mild nonionic detergent, octylglucoside. Advantage was taken of the differential solubilization of MOMP in Sarkosyl-DTT and octylglucoside-DTT. Sarkosyl-generated COMCs were solubilized in Sarkosyl-DTT, a treatment that extracted most contaminating proteins but very little of MOMP (Fig. 3). MOMP could then be solubilized in octylglucoside-DTT with a yield consistently greater than 90% (Fig. 3). MOMP purity was >90% as judged from the densitometric scanning of the Coomassie blue-stained gels. An even higher degree of purity could be achieved by chromatography on hydroxyapatite as described by Caldwell et al. (4), but by using octylglucoside instead of SDS in the elution buffer. Two-dimensional gel electrophoresis of purified EBs and of the purified protein showed that MOMP was one of the most acidic polypeptides of the EB, with a pI of approximately 5 (Fig. 4 and 5).

The amino acid analysis of the purified protein (Table 1) reveals that (i) MOMP contains several cysteine residues that can account for its ability to cross-link through disulfide bridges (20), and (ii) MOMP is very similar in amino acid content to MOMPs from other organisms, such as *Escherichia coli* porin and *Neisseria gonorrhoeae* protein pI, two proteins known to exist in the form of oligomers producing water-filled pores across the membrane (9, 19). On the other hand, the amino acid composition of MOMP is different (namely, less hydrophobic) from that of a typical integral membrane protein, the *lacY* gene product from *E. coli*.

This leads to the possibility that chlamydial MOMP also functions as a porin. This was investigated next.

Pores in the outer membrane of *C. trachomatis*. Pore-forming activity was tested by using the swelling assay developed by Nikaido and his co-workers (16, 22). Outer membrane vesicles can be reconstituted by mixing pore-forming outer membrane proteins with phosphatidylcholine and dicetylphosphate. When such vesicles are diluted in a sugar solution, the flow of sugar and accompanying water to the vesicle interior causes them to swell until osmotic equilibrium is reached. This change in vesicle size can be followed against time as a decrease in turbidity of the vesicle suspension. Figure 6 shows the results typically obtained when *C. trachomatis* COMCs were used in the reconstituted system. Small sugars penetrated the vesicles at various initial rates between that of buffer alone and that of a slowly penetrating tetrasaccharide, stachyose. In addition, the rates were faster with small sugars than with large ones, indicating a size dependence of diffusion across the chlamydial outer membrane, as has been observed in other gram negative bacteria.

The swelling assay normally allows the determination of the radius of the pore, if one assumes that it is a hollow cylinder of constant radius along its length and applies the Renkin equation (25) to the rates of diffusion obtained with sugars of different sizes. The COMCs allowed diffusion of sucrose (molecular weight, 342) inside the liposomes at a rate of 10 to 20% that of arabinose (molecular weight, 150). This corresponds to a pore radius of 0.65 to 0.90 nm, which in turn corresponds to an "exclusion limit" range of 850 to 2,250 daltons. A more accurate determination of pore size was not possible because of fluctuation of data from experiment to experiment. The cause of this variation is not known. It may mean that the pore size varies within a certain

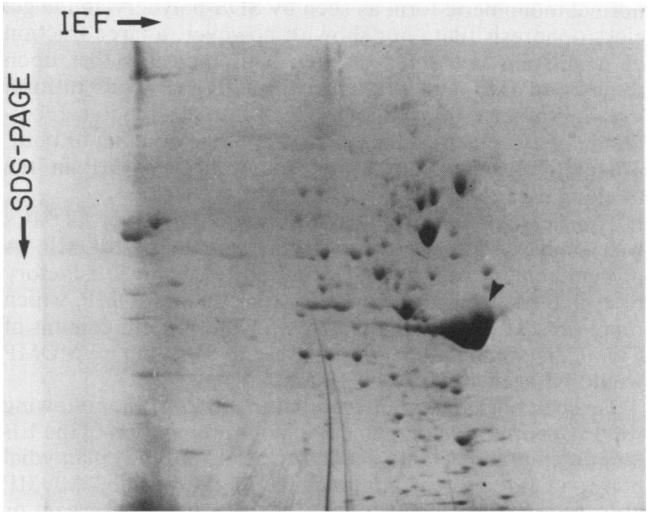

FIG. 4. Two-dimensional gel electrophoresis of *C. trachomatis* L2 EB proteins. Approximately 50 μg of protein was applied.

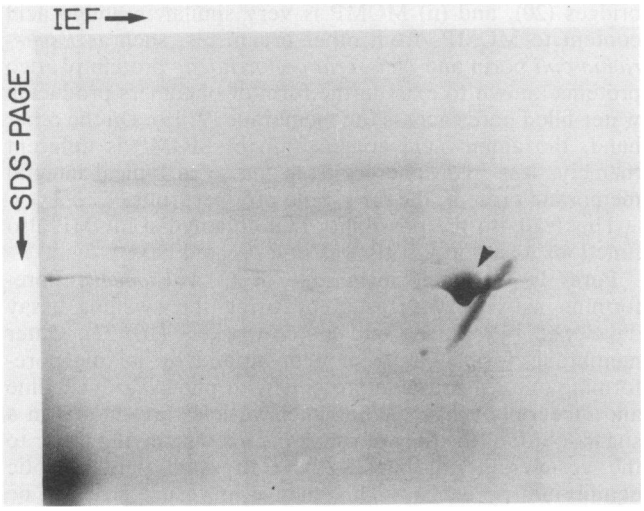

FIG. 5. Two-dimensional gel electrophoresis of purified MOMP. MOMP was purified as described in the text. A sample (3 μl) of the octylglucoside–DTT-soluble fraction was applied. The slash-shaped band near MOMP is a gel artifact.

range and that the ratio of large versus small pores is not reproduced consistently with each experiment.

When this experiment was done with MOMP purified with octylglucoside-DTT, a very similar result was obtained (data not shown). However, the efficiency at which MOMP formed pores was very low: 15 μg of purified MOMP was needed per swelling assay, roughly 200 times what would be needed for *E. coli* porin (less than 0.1 μg of purified porin routinely is used per swelling assay [22]). This is similar to, although more pronounced than, the situation with *Pseudomonas aeruginosa*, for which the pore-forming efficiency of porin was approximately 40 times less than that of *E. coli* porin (30).

There may be a simple explanation for the relative inefficiency of MOMP in forming pores. Removal of DTT and, to a lesser extent, of detergent by dialysis from the octylglucoside–DTT-purified MOMP led to the formation of large aggregates, which increased in size upon storage. This phenomenon was only partially reversible: boiling in SDS in the presence of 2-mercaptoethanol returned some MOMP to its normal monomeric form as seen by SDS-polyacrylamide gel electrophoresis (data not shown); however, a large fraction of it still did not enter the gel. This indicates that upon removal of DTT and octylglucoside, MOMP tends to form irreversibly cross-linked aggregates through random reoxidation of cysteine residues or hydrophobic bonding or both. This may hamper the testing of purified MOMP in the swelling assay.

To a lesser extent, the pore-forming activity of COMCs was similarly low, relative to the amount of MOMP. A protein content of 8 μg was required to obtain satisfactory rates in the swelling assay. This means that if MOMP, which represents at least 60% by weight of the protein content of COMC, is the functional porin, close to 5 μg of pure MOMP would be needed per swelling assay.

These results can be interpreted in either of the following ways: (i) pores are not abundant in our preparation of the EB outer membrane (COMCs) and MOMP is not the chlamydial porin, i.e., a protein contaminant in the purified MOMP preparation could then account for the activity observed, or (ii) MOMP is the chlamydial porin, albeit at a very low efficiency, which suggests, as it has been with *P. aeruginosa* (30), that most pores of the EB outer membrane are in a conformationally inactive state. We demonstrate that this is indeed the case in the next experiment.

Disulfide-mediated "opening and closing" of chlamydial outer membrane pores. As noted above, purified MOMP as well as COMCs were rather inefficient in producing pores. Although this is probably of artifactual origin in the case of purified MOMP, it clearly is not in the case of COMCs for which the method of preparation is very gentle: electron micrographs show that three-dimensional arrangement of the outer membrane is preserved after Sarkosyl extraction (4); thus, proteins are most likely in their original quaternary structures in COMCs. Because of the observation by others that MOMP and other outer membrane proteins are cross-linked by disulfide bridges (20) and our own supporting findings reported above, we tested the possibility that chlamydial pores become "open" upon breakage of disulfides by

TABLE 1. Comparison of the amino acid composition of MOMP with those of various membrane proteins from gram-negative bacteria[a]

Amino acid	Composition (total no. of residues) in[b]:			
	E. coli porin[c]	*Gonococcus* pI[d]	*lacY* gene product from *E. coli*[e]	Chlamydial MOMP
Neutral	215 (0.66)	222 (0.66)	327 (0.82)	215 (0.69)
Gly	45	46	36	35
Ala	30	40	35	44
Val	22	29	29	15
Leu	21	18	54	24
Ile	11	10	33	14
Ser	17	31	29	19
Thr	21	19	19	29
Phe	18	13	56	18
Tyr	25	13	14	7
Trp	[3]	[1]	[6]	ND
Cys	1	2	8	3
Met	4	1	14	7
Pro	[5]	[7]	[12]	ND
Charged[f]	113 (0.34)	116 (0.34)	72 (0.18)	96 (0.31)
Dicarboxylic[f]	81 (0.25)	77 (0.23)	44 (0.11)	70 (0.23)
Asx	54	41	22	40
Glx	27	36	22	30
Basic	32 (0.10)	39 (0.12)	28 (0.07)	26 (0.08)
Arg	12	11	12	13
His	2	6	4	13
Lys	18	22	12	
Hydrophobic[g]	127 (0.39)	123 (0.36)	221 (0.55)	122 (0.39)

[a] MOMP was assumed to contain Trp and Pro residues that would contribute 1,000 in terms of molecular weight by analogy with the other outer membrane proteins cited. Therefore, the amino acid composition given corresponds approximately to a molecular weight of 38,500 for MOMP. MOMP was purified by the method of Caldwell et al. (4) for this experiment.

[b] Numbers in parentheses represent the ratios of total number of residues in one class of amino acid over the total number of residues in the protein. For the sake of simplifying the comparison with MOMP amino acid totals and ratios, the data for Trp and Pro (values in brackets) were deleted from these calculations. ND, Not determined.

[c] The data are from reference 8.

[d] The data are from reference 12.

[e] The data are from reference 10.

[f] Charged and dicarboxylic amino acid classes include asparagine and glutamine here, as the analysis did not permit distinguishing between them and aspartate and glutamate, respectively.

[g] Hydrophobic amino acids used here are: Ala, Val, Leu, Ile, Phe, and Tyr.

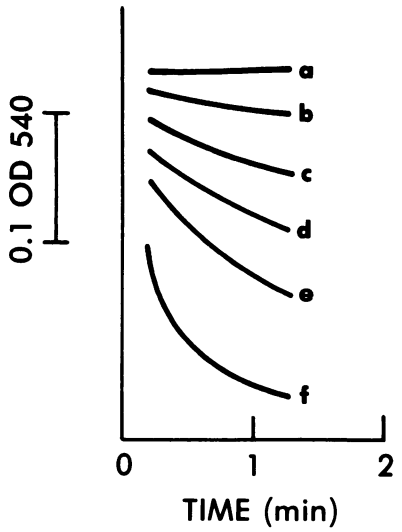

FIG. 6. Swelling of COMC-containing liposomes upon dilution in various sugar solutions. Liposomes were made by suspending 2 μmol of phosphatidylcholine and 0.05 μmol of dicetylphosphate in 0.6 ml of 15% dextran T-40 in 5 mM Tris-hydrochloride buffer (pH 7.4) in the presence of COMCs (8 μg of protein). Portions (50 μl) of this suspension were diluted in 0.6 ml of isotonic sugar solution in 5 mM Tris-hydrochloride (pH 7.4). Results obtained after dilution into stacchyose (a), sucrose (b), *N*-acetyl-D-glucosamine (c), D-glucose (d), L-arabinose (e), and buffer alone (f) are shown. Optical densities at 10 s were comprised between 0.65 and 0.80.

a reducing agent. Treatment of COMCs by DTT before reconstitution into liposomes did not result in a significantly higher efficiency of pore-forming activity (data not shown). This is probably due, as for purified MOMP, to random reoxidation of free sulfhydryls during liposome preparation. If, however, treatment of COMCs with DTT was followed immediately by the blockage of free sulfhydryls by carboxymethylation with iodoacetamide, efficiency of pore formation was drastically improved (Fig. 7), i.e., the amount of protein needed per swelling assay was reduced by nearly a factor of 10. This is now close to the results obtained for organisms such as *E. coli*.

DISCUSSION

In this study, we showed that chlamydial outer membrane proteins can be solubilized at a high yield by a variety of relatively mild detergents, provided that this is done in the presence of a reducing agent. The nonionic detergent octylglucoside was chosen to carry out purification of MOMP because it is easily removed by dialysis and is least likely to interfere in assays of the biological activities of MOMP, as the micelle is small and uncharged. The resistance to solubilization of MOMP by mild anionic detergents, such as deoxycholate, probably is the result of the electrostatic repulsion between natural aggregates of MOMP, an acidic protein, and negatively charged detergent micelles. Naturally occurring aggregates of MOMP most likely correspond to the anionic sites on the EB surface identified by Schiefer et al. (28). The purification procedure reported here represents an alternative to the procedure of Caldwell et al. (4), which involves the use of SDS, a strong denaturing anionic detergent.

The key element of our purification procedure is the requirement of the presence of a reducing agent for efficient solubilization of the proteins in the membrane. This parallels the finding by Newhall and Jones that several proteins of the EB outer membrane are cross-linked by disulfide bonds to form large aggregates (20), and also the recent study by Hatch et al. (11), who demonstrated the importance of DTT in the solubilization of *Chlamydia psittaci* outer membranes by SDS. It also clearly demonstrates the essential role that these bonds play in the maintenance of the EB outer membrane structural integrity: disulfide bonding is at least partially responsible for the formation of a proteinaceous skeleton that confers to the EB outer membrane the characteristic rigid spherical shape one also finds in the COMCs. In light of the fact that chlamydiae appear to be lacking the peptidoglycan layer found in other gram-negative bacteria, this disulfide-linked proteinaceous network appears to fulfill the same role played by the peptidoglycan in other bacteria, i.e., it confers shape and stability to variations in osmotic pressure as the EB is released from the host cell. At the same time, it probably acts as the limiting factor on membrane growth as long as the chlamydial cell is in the EB form. Subsequently, when an EB infects a host cell and develops into an actively multiplying RB, one of the first steps must be the breakage of these bonds to allow membrane growth and subsequent chlamydial growth.

The amino acid analysis of MOMP shows several important points. (i) MOMP has the ability to form as many as three disulfide bonds, as it contains three cysteine residues per molecule. (ii) MOMP is very similar in general amino acid composition to major outer membrane proteins from other gram-negative bacteria such as *N. gonorrhoeae* or *E. coli*. Other characteristics of MOMP that are shared by other major outer membrane proteins include (i) its abundance in the membrane, (ii) its acidic pI, (iii) its molecular weight range, (iv) its presence in the membrane in the form of

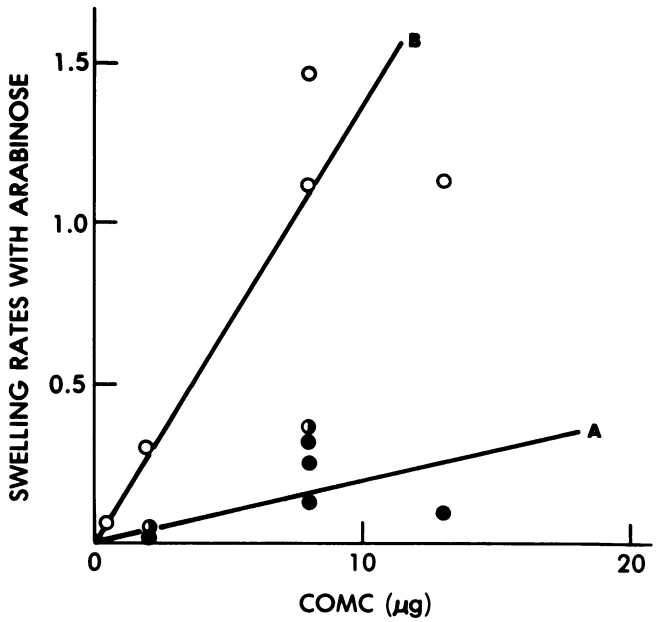

FIG. 7. Swelling rates of liposomes reconstituted with COMCs. Specified amounts of COMCs were reconstituted with 2 μmol of phosphatidylcholine and 0.05 μmol of dicetylphosphate. The rates of diffusion of L-arabinose into the liposomes were determined as $d(1/OD)/dt$ from the optical density changes between 10 and 20 s obtained in the swelling assay. The rates are approximate because the first 10 s of the swelling reaction could not be recorded. The data shown in this figure were obtained in four separate experiments. A, untreated COMCs (●) and COMCs treated with DTT only (◑); B, COMCs treated with DTT and iodoacetamide (○).

oligomers, and (v) its relatively low content of hydrophobic amino acid, i.e., between that of soluble proteins and that of a typical integral membrane protein such as the *lacY* gene product of *E. coli*. The other major outer membrane proteins cited in Table 1 have been shown to be involved in the formation of water-filled channels across the membrane (8, 16). Their amino acid content reflects the greater exposure of the surfaces of the proteins to an aqueous environment due to the presence of the transmembrane channel.

Tamura and Manire (29) have reported the absence of cysteine and methionine residues in the membranes of RBs from *C. psittaci*, although these residues are present in the EBs (17). This appears to be a very unlikely ocurrence in *C. trachomatis*, since MOMP represents roughly 60% of total envelope proteins in both reticulate and elementary bodies (data not shown; H. D. Caldwell, personal communication). Hatch et al. also reached this conclusion on the basis of the incorporation of radiolabeled cysteine and methionine in MOMPs of *C. psittaci* and *C. trachomatis* (11).

In the second phase of this study, we demonstrated the presence of water-filled pores in the outer membrane of *C. trachomatis*. Diffusion of sugars across the reconstituted membrane was clearly size dependent. However, because of the limitations of the swelling assay used here, we were unable to determine accurately the exclusion limit of the pore. Nevertheless, it is large enough to allow diffusion of nucleotide triphosphates as an energy source and as precursors of RNA synthesis, two expected early requirements of chlamydial growth. Such pores may correspond to the depressions or craters that have been observed by electron microscopy in several laboratories (7, 14, 18).

Chlamydial pores have the unique property of being developmentally controlled, i.e., the pore-forming specific activity is drastically enhanced under specific conditions. We could activate pore formation in vitro by treating COMCs first with a reducing agent (DTT) to break disulfide bonds and then with iodoacetamide to block the free sulfhydryl groups produced by reduction (iodoacetamide treatment probably is required to prevent random reoxidation of cysteine residues occurring under our assay conditions). This explains the earlier finding of Sarov and Becker (26) that incorporation of labeled nucleotide triphosphates into chlamydial RNA is dependent on pretreatment of EBs with 2-mercaptoethanol.

We have already discussed the essential role that disulfide bonding of the outer membrane proteins plays in maintaining the EB structural integrity. It now appears that disulfide bonding also affects pore-forming activity. One can then formulate a simple model on the roles of disulfide bonding in the physiology of chlamydial development. In the EB stage, proteins of the outer membrane, including porins, are cross-linked by disulfide bonds so that (i) the outer membrane is mostly impermeable to hydrophilic solutes, and (ii) the outer membrane is rigidified significantly to render the EB osmotically stable. After the EB is phagocytosed, it is exposed to intraphagosomal reducing conditions, probably in the form of reduced glutathione. Development into the RB form is initiated upon reduction of the outer membrane disulfide bonds, i.e., pores become "open" and uptake of ATP and nutrients commences, and the membrane becomes flexible. Possibly, chlamydial growth will stop as the level of ATP and reducing power, in the form of NADPH or reduced glutathione or both, decrease below a certain limit. This will result simultaneously in a decrease in metabolic activity, oxidation of free sulfhydryls into disulfides, closing of the outer membrane pores, and outer membrane rigidification, all characteristic features of the EB stage.

As the physiological changes caused by simple chemical oxidation of cysteine residues into cystine appear sufficient to block metabolic activity and growth, it is tempting to speculate that the reverse reaction, i.e., reduction, also is sufficient to initiate development. Confirmation of this hypothesis awaits demonstration in a cell-free system of the effects of reduction on EB development. Preliminary results of such experiments showed some success (T. Hackstadt and H. D. Caldwell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D34, p. 64). In time, the major benefit of these experiments, besides added knowledge of chlamydial development at the molecular level, could be initiating chlamydial growth in a cell-free system.

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LITERATURE CITED

1. **Ames, G. F.-L., and K. Nikaido.** 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry **15**:616–623.
2. **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.
3. **Becker, Y.** 1978. The Chlamydia: molecular biology of procaryotic obligate parasites of eucaryotes. Microbiol. Rev. **42**:274–306.
4. **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.
5. **Caldwell, H. D., C. C. Kuo, and G. E. Kenny.** 1975. Antigenic analysis of Chlamydia by two-dimensional immunoelectrophoresis. I. Antigenic heterogeneity between *C. trachomatis* and *C. psittaci*. J. Immunol. **115**:963–968.
6. **Caldwell, H. D., and J. Schachter.** 1982. Antigenic analysis of the major outer membrane protein of *Chlamydia* spp. Infect. Immun. **35**:1024–1031.
7. **Chang, J.-J., K. Leonard, T. Arad, T. Pitt, Y.-X. Zhang, and L.-H. Zhang.** 1982. Structural studies of the outer envelope of *C. trachomatis* by electron microscopy. J. Mol. Biol. **161**:579–590.
8. **Chen, R., C. Kramer, W. Schmidmayr, and U. Henning.** 1979. Primary structure of the major outer membrane protein I of *E. coli* B/r. Proc. Natl. Acad. Sci. U.S.A. **76**:5014–5017.
9. **Douglas, J. T., M. D. Lee, and H. Nikaido.** 1981. Protein I of *Neisseria gonorrhoeae* outer membrane is a porin. FEMS Lett. **12**:305–309.
10. **Ehring, R., K. Beyrenther, J. K. Wright, and P. Overath.** 1980. In vitro and in vivo products of *E. coli* lactose permease gene are identical. Nature (London) **283**:537–540.
11. **Hatch, T. P., I. Allan, and J. H. Pearce.** 1984. Structural and polypeptide differences between envelopes of infective and reproductive live cycle forms of *Chlamydia* spp. J. Bacteriol. **157**:13–20.
12. **Heckels, J. E.** 1981. Structural comparison of *Neisseria gonorrhoeae* outer membrane proteins. J. Bacteriol. **145**:736–742.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **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.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Luckey, M., and H. Nikaido.** 1980. Specificity of diffusion channels produced by phage λ receptor protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:167–171.
17. **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.
18. **Matsumoto, A.** 1982. Morphology of *C. psittaci* elementary bodies as revealed by electron microscopy. Kawasaki Med. J. **8**:149–157.
19. **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.
20. **Newhall, W. J. V., and R. B. Jones.** 1983. Disulfide-linked oligomers of the major outer membrane protein of chlamydiae. J. Bacteriol. **154**:998–1001.
21. **Nikaido, H.** 1979. Permeability of the outer membrane of bacteria. Angew. Chem. **18**:337–350.
22. **Nikaido, H., and E. Y. Rosenberg.** 1981. Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of *E. coli*. J. Gen. Physiol. **77**:121–135.
23. **Nikaido, H., and E. Y. Rosenberg.** 1983. Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. **153**:241–252.
24. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
25. **Renkin, E. M.** 1954. Filtration, diffusion, and molecular sieving through porous cellulose membranes. J. Gen. Physiol. **38**:225–243.
26. **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.
27. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Annu. Rev. Microbiol. **34**:285–309.
28. **Schiefer, H. G., H. Krauss, and U. Schummer.** 1982. Anionic sites on Chlamydia membranes. FEMS Lett. **15**:41–44.
29. **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.
30. **Yoshimura, F., L. Shaltiel Zalman, and H. Nikaido.** 1982. Purification and properties of *Pseudomonas aeruginosa* porin. J. Biol. Chem. **258**:2308–2314.