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Structural and Polypeptide Differences Between Envelopes of Infective and Reproductive Life Cycle Forms of *Chlamydia* spp.

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Significant differences in cysteine-containing proteins and detergent-related solubility properties were observed between outer membrane protein complexes of reproductive (reticulate body) and infective (elementary body) forms of *Chlamydia psittaci* (6BC). Elementary bodies harvested at 48 h postinfection possessed a 40-kilodalton major outer membrane protein and three extraordinarily cysteine-rich outer membrane proteins of 62, 59, and 12 kilodaltons, all of which were not solubilized by sodium dodecyl sulfate in the absence of thiol reagents. Intracellularly dividing reticulate bodies harvested at 21 h postinfection were severely deficient in the cysteine-rich proteins but possessed almost as much major outer membrane protein as did the elementary bodies. Most of the major outer membrane protein of reticulate bodies was solubilized by sodium dodecyl sulfate and was present in envelopes as monomers, although a proportion formed disulfide-cross-linked oligomers. By 21 to 24 h postinfection, reticulate bodies commenced synthesis of the cysteine-rich proteins which were found in outer membranes as disulfide-cross-linked complexes. The outer membranes of reticulate bodies of *Chlamydia trachomatis* (LGV434) also were found to be deficient in cysteine-rich proteins and to be more susceptible to dissociation in sodium dodecyl sulfate than were outer membranes of elementary bodies.

Chlamydiae are obligately intracellular parasitic bacteria which possess a characteristic developmental cycle (2, 15). The developmental cycle may be divided into three phases: (i) entry of infective forms (elementary bodies [EBs]) into host cells and their differentiation into reproductive forms (reticulate bodies [RBs]), (ii) multiplication of the RBs, and (iii) conversion of RBs into EBs and their release from host cells. The first phase is completed by 4 to 12 h postinfection. By 20 h postinfection, some of the RBs cease to divide and commence transformation to EBs; other RBs in the population continue to divide for up to 72 h after infection. Most of the infected cells lyse by 48 to 72 h, releasing a population of chlamydiae consisting mainly of EBs. The conversion of EBs to RBs and RBs to EBs is gradual and continuous; poorly defined intermediate forms exist.

EBs and RBs are defined primarily by morphological criteria. EBs are small, from 0.2 to 0.4 μm in diameter, and possess an electron-dense nucleoid; RBs are larger, 1 to 1.5 μm in diameter, and possess an evenly dispersed, reticulate cytoplasm (9, 25, 28). Also, EBs are denser and more resistant to mechanical and osmotic stress than are RBs (26, 28). In addition to infectivity (25, 28), EBs possess other biological functions not associated with RBs: cytotoxicity for mice (6) and macrophages in culture (3) and agglutination of erythrocytes (27). Along with their reproductive capacity, RBs show metabolic functions which are absent from EBs: the ability to transport ATP, GTP, and lysine (12) and to synthesize protein (11).

The cell envelopes of both developmental forms, consisting of an inner cytoplasmic membrane and an outer membrane, resemble those of gram-negative bacteria (9, 29). In contrast to the envelopes of gram-negative bacteria, the envelopes of both forms of chlamydiae lack or contain very little muramic acid (1, 10, 17, 26). Nonetheless, the possibility that chlamydiae possess peptidoglycan cannot be dismissed in that penicillin alters chlamydial growth by inducing the formation of swollen, abnormal RB forms (1, 19). Several morphological and chemical differences between the two life cycle forms have been reported. In electron micrographs, purified EB cell envelopes appear as folding, rigid membranes, whereas RB envelopes appear as thin, flattened structures (26); freeze-fracture patterns of the two forms also are different (16, 18). Tamura and Manire (26) reported that EB envelopes have a higher phospholipid content than do RB envelopes and that RB envelopes lack both cysteine and methionine; diferences in polypeptide content of the envelopes also have been reported (30). Failing to detect muramic acid in *Chlamydia psittaci* cell walls and noting that osmotically fragile RB envelopes lack cysteine, Tamura and Manire (26) suggested that S-S bonding might play a role in the formation of rigid membranes of EBs.

Recent evidence of a role for disulfide bonds in maintaining the structure of EBs was provided by Hatch et al. (13), who reported that sodium dodecyl sulfate (SDS) in the absence of a reducing agent fails to solubilize outer membrane proteins of *C. psittaci* (6BC) EBs, and Newhall and Jones (22), who observed disulfide bond-cross-linked complexes in EB outer membranes of an oculogenital strain of *Chlamydia trachomatis* (UW94, serotype F); RBs were not examined in either of these studies. Observations based on metabolic studies independently support the view that formation of cysteine-containing structures is essential in RB-EB transformation. Stirling et al. (24), in studies on *C. trachomatis* (DK-20, serotype E) in cycloheximide-treated cells, found that RB division occurs in medium lacking cysteine apparently as efficiently as in medium containing cysteine; however, conversion of RBs to EBs late in the developmental cycle is retarded severely in the absence of cysteine. The effect appears specific for cysteine deprivation

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with strains of both *C. trachomatis* and *C. psittaci* (T. P. Hatch, I. Allen, and J. H. Pearce, unpublished data).

The purpose of the present study was to seek evidence for the involvement of cysteine-containing polypeptides in structural differences between EBs and RBs which could play a role in EB rigidity. It was found that the outer membrane proteins of EB forms of *C. psittaci* (6BC) and *C. trachomatis* (LGV434) include three cysteine-rich proteins which are dissociated by SDS only in the presence of reducing agents. In contrast, outer membranes of the osmotically fragile RB form of these organisms are deficient in the three cysteine-rich proteins, and the major outer membrane protein (MOMP) of RBs is highly susceptible to dissociation by SDS alone.

MATERIALS AND METHODS

Growth of organisms. *C. psittaci* (6BC) was grown in 929 L cells, and *C. trachomatis* (LGV434, serotype L2) was grown in 229 HeLa cells (13). RBs and EBs were harvested at 21 and 48 h postinfection, respectively, by disrupting infected host cells in a Mettler ultrasonic cleaning bath. All chlamydial preparations were purified as described previously (12). RBs and EBs were defined on the basis of their sedimentation to the 30–35% and 35–40% Renografin (E. R. Squibb & Sons, Princeton, N.J.) interfaces, respectively, of 30, 35, and 40% Renografin step gradients (13). Radiolabeled *C. psittaci* organisms were prepared from 2×10^7 L cells which were incubated from the time of infection in medium 199 containing 5% fetal calf serum and 5 μCi of either L-[^{35}S]cysteine or L-[^{35}S]methionine per ml. Radiolabeled *C. trachomatis* organisms were prepared from HeLa cells which were incubated from the time of infection in Eagle minimal medium containing 5% fetal calf serum and 10 μCi of either L-[^{35}S]cysteine or L-[^{35}S]methionine per ml.

Preparation of outer membranes. Sarkosyl-prepared outer membranes were obtained by incubating purified EBs and RBs in 5 ml of Dulbecco phosphate-buffered saline (pH 7.4) containing 2% sodium lauryl sarcosinate (Sarkosyl; Sigma Chemical Co., St. Louis, Mo.) at 37°C for 1 h, shearing the released DNA by ultrasonic vibration (10 s at a setting of 5 on a model 185 Sonifier Cell Disruptor; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), and collecting the pellet after centrifugation at 40,000 rpm in an SW50.1 rotor for 30 min. SDS-prepared outer membranes were obtained as described above except that the chlamydiae were incubated in 2% SDS in the presence or absence of 5% (vol/vol) 2-mercaptoethanol or 20 mM dithiothreitol. Auto-oxidation of disulfide bonds was prevented by including 50 mM iodoacetamide in the extraction mixtures and by suspending the outer membrane preparations in electrophoresis solubilization buffer containing 5 mM iodoacetamide.

SDS-PAGE. Samples were heated for 3 min at 100°C in electrophoresis solubilization buffer containing 62.5 mM Tris-hydrochloride (pH 6.8), 2% SDS, 5% 2-mercaptoethanol (or 20 mM dithiothreitol), 10% glycerol, 5 mM iodoacetamide, and 0.001% bromophenol blue. In some experiments, the reducing agent was omitted. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in one dimension at a constant current of 30 mA in 7.5 to 15% acrylamide slab gels containing 0.1% SDS in the discontinuous Tris-glycine system described previously by Laemmli (14). The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30:0.8 in both the separating gel and the 4% stacking gel. Gels were stained with Coomassie brilliant blue R by the method of Fairbanks et al. (7), dried, and subjected to autoradiography, using Kodak NS-5T X-ray film. Gel bands corresponding to darkened areas on the X-ray film were excised with a razor blade, rehydrated, incubated for 2 days at 37°C in a toluene-based scintillation cocktail containing 3% Protosol (New England Nuclear Corp., Boston, Mass.), and counted. For two-dimensional SDS-PAGE, proteins were first resolved on a cylindrical gel (5 by 110 mm) consisting of 5% polyacrylamide (cross-linked with 2.6% bisacrylamide) and 0.2% SDS. The gel and electrode buffers were 40 mM sodium acetate–80 mM Tris–4 mM EDTA adjusted to pH 8.3 with glacial acetic acid. After electrophoresis in the first dimension, the gel was soaked for 30 min in 25 ml of 0.125 M Tris-hydrochloride (pH 6.8) containing 0.2% SDS and 10% (vol/vol) 2-mercaptoethanol. The gel was embedded at the top of the second-dimensional slab gel, which consisted of a 1-cm stacking gel containing 1% agarose, 10% (vol/vol) 2-mercaptoethanol, and 0.1% SDS in 0.125 M Tris-hydrochloride (pH 6.8) and an 11-cm 7.5 to 15% polyacrylamide gradient gel (described above), and was then electrophoresed. The relative molecular weights of chlamydial proteins were determined by using the following markers purchased from Pharmacia Fine Chemicals, Piscataway, N.J.: bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and RNase A (13,700).

Host-free protein synthesis. Purified 21-h-old RBs (250 μg of protein) were incubated for 1 h at 37°C in a 1-ml mixture containing 10 nmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0), 25 μmol of $MgCl_2$, 150 μmol of KCl, 700 nmol of ATP, 5.6 nmol of creatine phosphate (Sigma), 15 μg of phosphocreatine kinase (Sigma), 480 nmol of dithiothreitol, 75 nmol of unlabeled amino acids, and 10 μCi of either [^{35}S]cysteine (133 Ci/mol) or ^{14}C-labeled amino acid mixture (133 Ci/mol) per ml. The reaction was terminated by the addition of 5 ml of Dulbecco phosphate-buffered saline (pH 7.4), and the RBs were pelleted at 10,000 × *g* for 20 min and washed once in phosphate-buffered saline.

Alkylation of sulfhydryl groups with [^{14}C]iodoacetamide. Sarkosyl-prepared outer membranes from 21-h-old RBs (0.50 mg of protein) and 48-h-old EBs (1.0 mg of protein) were suspended in 300 μl of 62.5 mM Tris-hydrochloride (pH 8.0) containing 2% SDS. Each preparation then was split into two equal parts; 1.5 μmol of dithiothreitol was added to one part; and all samples were heated to 100°C for 3 min. After the preparations were cooled to 4°C, they were incubated for 1 h with [^{14}C]iodoacetamide (2 Ci/mol; 25 mM); the reactions were stopped by the addition of excess 2-mercaptoethanol (10% [vol/vol]) and by heating the preparations to 100°C for 3 min. The alkylated preparations (20 μl of each) were subjected to one-dimensional SDS-PAGE, and outer membrane proteins were excised and counted by liquid scintillation spectrometry.

Isotopes. L-[^{35}S]cysteine (950 Ci/mmol), L-[^{35}S]methionine (1,000 Ci/mmol), and [^{14}C]iodoacetamide (54 Ci/mol) were purchased from Amersham Corp., Arlington Heights, Ill. Reconstituted ^{14}C-labeled protein hydrolysate (Schwarz mixture; Schwarz/Mann, Spring Valley, N.Y.) had an average specific activity of 214 Ci/mol and contained alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

RESULTS

Comparative resistance of EB and RB forms of *C. psittaci* (6BC) to extraction by detergents and reducing agents. Sodium lauryl sarcosinate (Sarkosyl) extraction has been used to prepare outer membranes of *Escherichia coli* (8) and EBs of

C. trachomatis (4) and *C. psittaci* (13). For reference the SDS-PAGE profile of Sarkosyl-insoluble proteins of *C. psittaci* EBs is shown in Fig. 1B. Major features include a cluster of proteins with relative molecular weights in excess of 85,000, a broad, diffusely staining band with an average molecular weight of ca. 60,000, the 40-kilodalton (kd) MOMP (13), and a peptide of low molecular weight (12,000); the diffuse band could be resolved into a 62 kd-59 kd doublet (see Fig. 2). As was demonstrated previously (13), extraction of whole *C. psittaci* EBs with 2% SDS at 37°C for 1 h yielded a residue with a protein profile (after solubilization with SDS–mercaptoethanol) almost identical to the profile of EB residue after extraction with 2% Sarkosyl (cf. Fig. 1B and C); extraction of EBs with 2% SDS containing 5% mercaptoethanol almost completely solubilized all EB proteins (Fig. 1D). It is unlikely that mercaptoethanol was solubilizing EB envelope proteins by chelating divalent cations (see reference 21), because 20 mM EDTA in 2% SDS failed to solubilize EB outer membrane proteins, whereas 20 mM dithiothreitol, a reducing agent which is not believed to be a chelator, was as effective as mercaptoethanol.

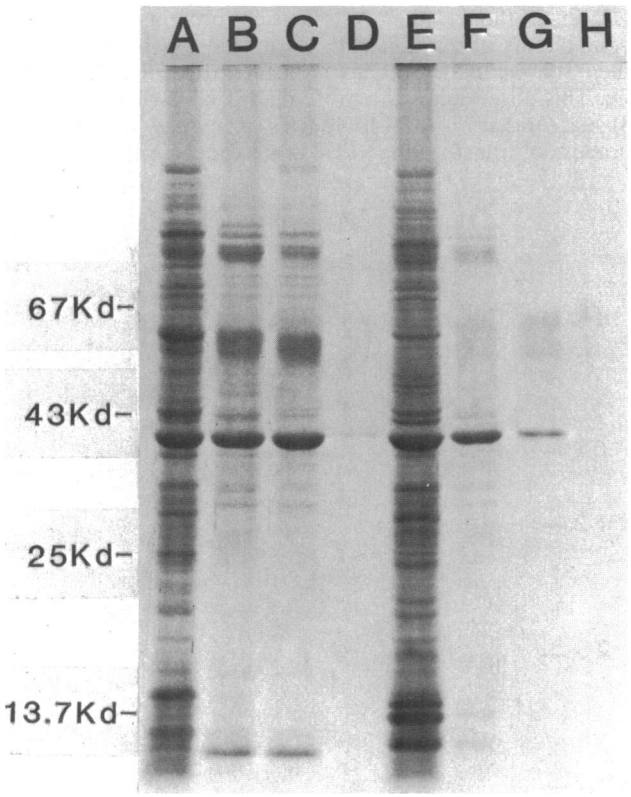

FIG. 1. SDS-PAGE profiles of residues of *C. psittaci* EBs and RBs after extraction with detergents. EBs and RBs were harvested and purified from 5×10^7 cells at 48 and 21 h postinfection, respectively. Each purified preparation (1 mg of protein) was divided into four equal parts which were extracted for 1 h at 37°C with phosphate-buffered saline, 2% Sarkosyl, 2% SDS, or 2% SDS–5% (vol/vol) 2-mercaptoethanol. Residues were suspended in 200 μl of solubilization buffer containing 5% mercaptoethanol and heated to 100°C for 3 min; 20 μl was subjected to one-dimensional SDS-PAGE. Lanes: (A) EBs extracted with PBS; (B) EBs extracted with Sarkosyl; (C) EBs extracted with SDS; (D) EBs extracted with SDS–mercaptoethanol; (E) RBs extracted with phosphate-buffered saline; (F) RBs extracted with Sarkosyl; (G) RBs extracted with SDS; (H) RBs extracted with SDS–mercaptoethanol.

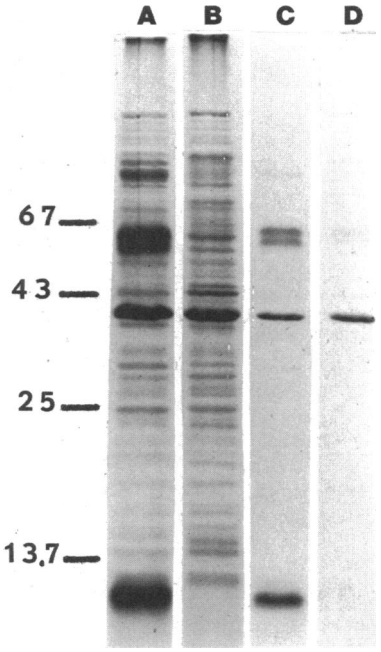

FIG. 2. SDS-PAGE profile of *C. psittaci* EB proteins labeled in vivo with [^{35}S]cysteine and [^{35}S]methionine. EBs were harvested and purified from 2×10^7 L cells which had been incubated in medium 199 containing 5% fetal calf serum in the continuous presence of 5 μCi of either [^{35}S]cysteine or [^{35}S]methionine per ml from the time of infection as described in the text. Untreated EBs (20 μg of protein) and their residues afer SDS extraction (2.5 μg of protein) were solubilized under reducing conditions and subjected to one-dimensional SDS-PAGE. An autoradiogram of the gel is shown. Lanes: (A) whole EBs, [^{35}S]cysteine (28,240 cpm loaded); (B) whole EBs, [^{35}S]methionine (23,323 cpm loaded); (C) SDS-extracted EBs, [^{35}S]cysteine (9,726 cpm loaded); (D) SDS-extracted EBs, [^{35}S]methionine (4,346 cpm loaded).

RB residues after Sarkosyl extraction (RBs harvested at 21 h postinfection) yielded an SDS-PAGE profile which included the 40-kd MOMP but was deficient in the 62 kd-59 kd doublet and almost completely lacked the 12-kd peptide (Fig. 1F). The exact nature of the Sarkosyl-extracted RB residue is not known but is referred to here as RB outer membranes because it contained the MOMP. The lower amounts of the doublet and 12-kd proteins were not due to their selective extraction by Sarkosyl, since these proteins were not present even in the SDS-PAGE profile of whole RBs (Fig. 1E). Although SDS extracted little MOMP from whole EBs, it solubilized most, although not all, of the MOMP from whole RBs (Fig. 1G). As was the case with EBs, SDS containing the reducing agent solubilized nearly all of the proteins of RBs of *C. psittaci* (Fig. 1H).

Cysteine-containing polypeptides in outer membrane preparations of EB and RB forms. The data of Stirling et al. (24) suggest that synthesis of cysteine-rich proteins might be required during transformation of RB to EB forms. To assess the distribution of cysteine and its availability for disulfide bond formation in outer membrane preparations, *C. psittaci* was grown in L cells in the presence of [^{35}S]cysteine or [^{35}S]methionine, and incorporation of these isotopic amino acids into EB proteins was examined by SDS-PAGE (Fig. 2). Although both [^{35}S]cysteine and [^{35}S]methionine were incorporated into the MOMP, proportionately more cysteine than

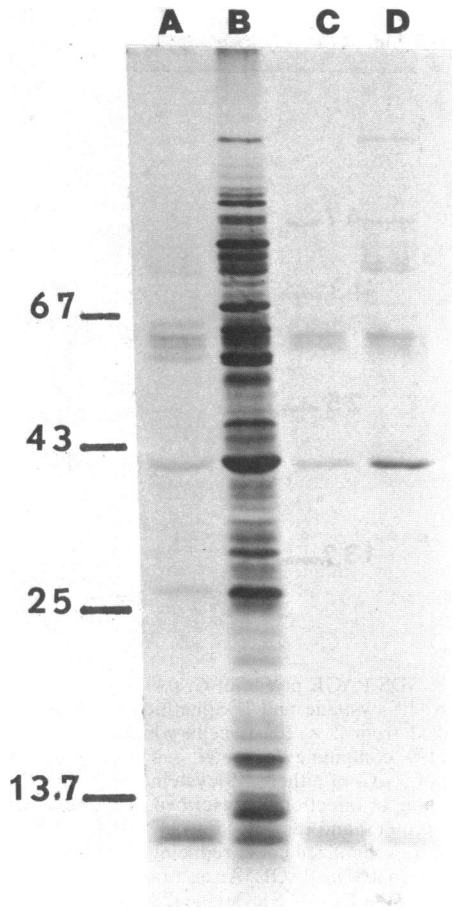

FIG. 3. Incorporation of [^{35}S]cysteine or ^{14}C-labeled amino acid mixture into host-free *C. psittaci* RB proteins. RBs were harvested and purified at 21 h postinfection and incubated for 1 h with 10 μCi of either [^{35}S]cysteine (133 Ci/mol) or ^{14}C-labeled amino acid mixture (133 Ci/mol) per ml in the host-free protein synthesis system. Whole RBs (21 μg of protein) and outer membrane preparations (1.5 μg of protein; residues after Sarkosyl extraction) were subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. Lanes: (A) whole RBs, [^{35}S]cysteine; (B) whole RBs ^{14}C-labeled-amino acids; (C) outer membranes, [^{35}S]cysteine, (D) outer membranes, ^{14}C-labeled amino acids.

methionine was incorporated into the 62 kd-59 kd doublet and the 12-kd peptide of EBs, indicating not only that the outer membrane proteins of EBs contain cysteine but also that the doublet and the 12-kd peptide are rich in cysteine relative to methionine. In an experiment similar to the one shown in Fig. 2, [^{35}S]cysteine and [^{35}S]methionine (added at the time of infection) were found to be incorporated mainly into one outer membrane protein, the MOMP, of 21-h-old RBs (data not shown). This is probably because the MOMP, but not the doublet and the 12-kd peptide, is synthesized early in the development cycle.

Synthesis of cysteine-rich proteins by RBs of *C. psittaci* (6BC). It is possible to induce the metabolically active RB forms of *C. psittaci*, which have been isolated from host cell material (host-free RBs), to synthesize proteins. EB forms, in contrast, do not synthesize protein in the host-free system (11). The host-free system has two advantages over the in vivo host-parasite system: (i) incorporation of amino acids is not complicated by host factors such as transport, protein turnover, and pool sizes and (ii) conditions for high-level incorporation of isotope may be readily established. The host-free system therefore was used to calculate the relative cysteine content of *C. psittaci* outer membrane proteins by incubating 21-h-old host-free RBs for 1 h with [^{35}S]cysteine or a mixture of 13 ^{14}C-labeled amino acids (lacking cysteine). Interestingly, although the 21-h-old RBs were deficient in the doublet and the 12-kd peptide (Fig. 1E and F), they were capable of synthesizing the cysteine-rich proteins in the host-free system (Fig. 3). The ratio of [^{35}S]cysteine to the ^{14}C-labeled amino acid mixture incorporated into the outer membrane proteins shown in Fig. 3 was 1.04 for the doublet proteins, 0.30 for the MOMP, and 2.17 for the 12-kd peptide; the ratio for total RB cell proteins was 0.31. Thus, relative to ^{14}C-labeled amino acid incorporation, 3.5 times (1.04/0.30) as much [^{35}S]cysteine was incorporated into the doublet proteins and 7.2 times (2.17/0.30) as much [^{35}S]cysteine was incorporated into the 12-kd peptide as was incorporated into the MOMP.

When L cells which had been infected with *C. psittaci* for 21 h were pulsed with [^{35}S]cysteine for 3 h, as much or more radioactivity was incorporated into the doublet and the 12-kd peptide as was incorporated into the MOMP (Fig. 4G and H), even though the cysteine-rich proteins were minor components of 24-h-old RBs as judged by the intensity of their staining with Coomassie brilliant blue R (Fig. 4C and D). This observation and those made on host-free RBs (Fig. 3) suggest that 21- to 24-h-old RBs (many of which are in the process of transforming to EBs) are capable of synthesizing

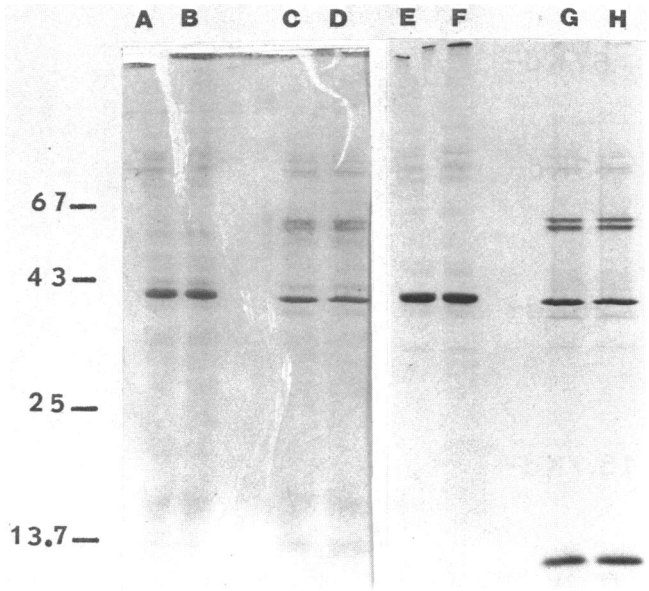

FIG. 4. Incorporation of [^{35}S]cysteine into outer membrane proteins of *C. psittaci* RBs. [^{35}S]cysteine (10 μCi/ml) was added to 2 × 10^7 L cells in medium 199 containing 5% fetal calf serum at 21 h postinfection. At 24 h postinfection, RB outer membrane preparations (residues after Sarkosyl extraction) were solubilized in SDS in the presence or absence of 20 mM dithiothreitol and subjected to one-dimensional SDS-PAGE (36,056 cpm was applied to each lane). Lanes A through D are the Coomassie brilliant blue R-stained gel, and lanes E through H are the autoradiogram of the gel. Lanes A and B, outer membrane preparations solubilized in the absence of dithiothreitol; lanes C and D, outer membrane preparations solubilized in the presence of dithiothreitol.

the cysteine-rich proteins. When the RB outer membrane preparation described above was electrophoresed under nonreducing conditions, almost no radioactivity was associated with bands migrating with relative molecular weights of ca. 60,000 and 12,000, whereas the amount of radioactivity associated with the 40-kd MOMP band was approximately the same whether or not the preparation was reduced before electrophoresis (Fig. 4E and F). These [^{35}S]cysteine-labeled outer membrane proteins were examined further by two-dimensional SDS-PAGE (Fig. 5). When electrophoresis was

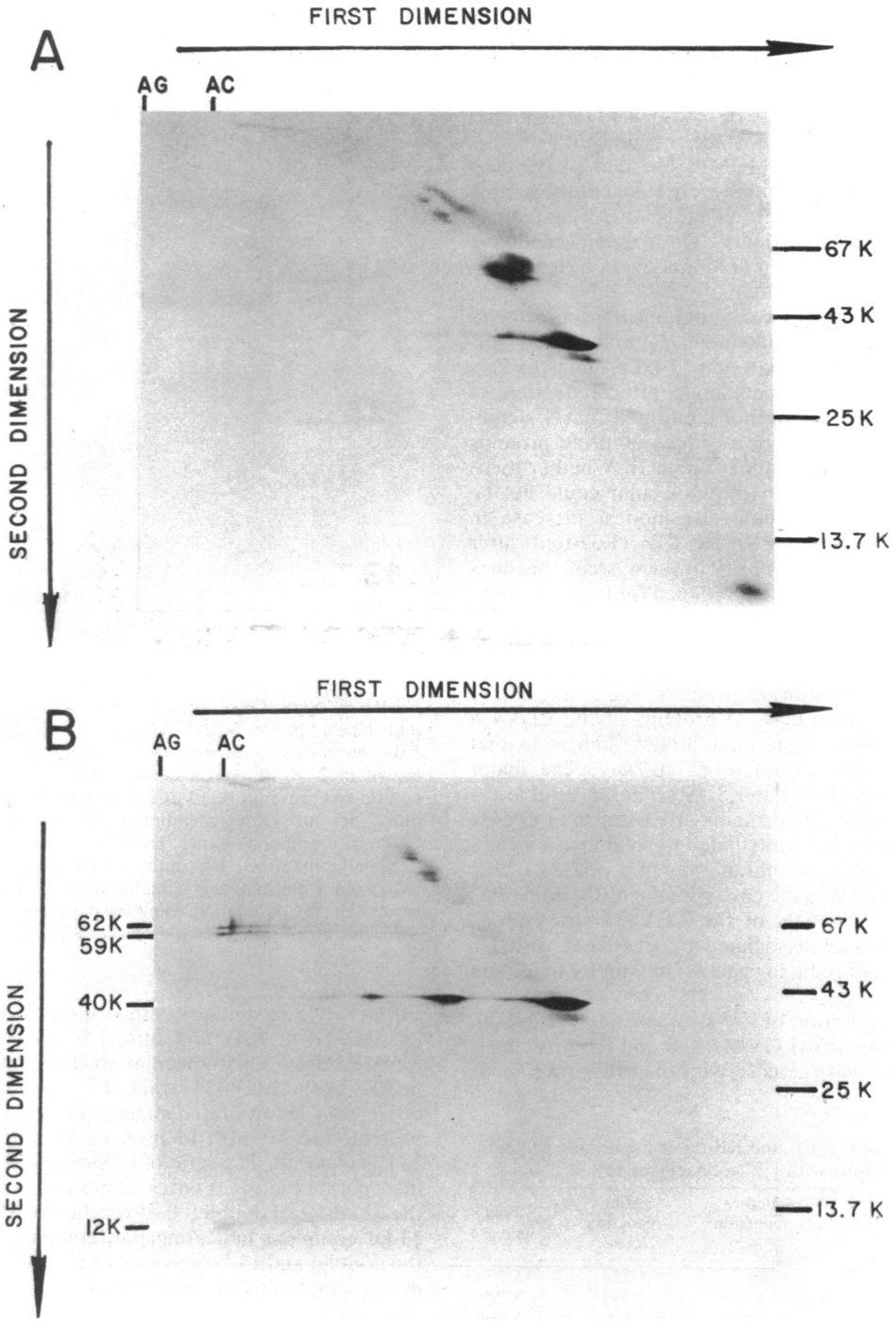

FIG. 5. Two-dimensional SDS-PAGE profile of [^{35}S]cysteine-labeled RB outer membrane protein preparations. The preparations (72,112 cpm) shown in Fig. 4 were subjected to two-dimensional SDS-PAGE, and an autoradiagram was prepared as described in the text. (A) Sample was reduced with 20 mM dithiothreitol before migration in the first dimension and reduced again before migration in the second dimension. (B) Sample was electrophoresed without reduction in the first dimension and then reduced before migration in the second dimension. The direction of migration during SDS-PAGE is indicated for each dimension. AG and AC represent the polyacrylamide-agarose and the polyacrylamide origins, respectively, in the first dimension gel.

carried out under reducing conditions in both the first and second dimensions, radioactivity was associated with proteins with approximate molecular weights of 90,000, 60,000, 40,000, and 12,000 along a diagonal (Fig. 5A). When electrophoresis was carried out under nonreducing conditions in the first dimension and under reducing conditions in the second dimension, most of the radioactivity along the diagonal was associated with the 40-kd MOMP (Fig. 5B). A proportion of the MOMP also was located below the diagonal, forming a trail extending from the diagonal to the origin of the first-dimensinal gel. Distinct spots along the trail which migrated with relative molecular weights of ca. 80,000, 160,000, and 240,000 in the first dimension most likely represent disulfide-cross-linked oligomers of the MOMP. The radioactive doublet proteins and the 12-kd peptide were found at the origins of the stacking and running gels and along streaks extending from the diagonal to the origins. These complexes may represent disulfide-cross-linked homopolymers or heteropolymers consisting of these proteins.

Disulfide bonding in *C. psittaci* outer membrane proteins detected with iodoacetamide alkylation. The increased susceptibility of the doublet, 40-kd, and 12-kd proteins of EBs to alkylation with [^{14}C]iodoacetamide after reduction of disulfide bonds with dithiothreitol confirmed that a significant proportion of the cysteine residues of these proteins were engaged in disulfide bonding (Table 1). Whether these disulfide bonds were inter- or intramolecular could not be determined by this experiment. The modest increase in alkylation of the MOMP of 21-h-old RBs (1.37-fold) after reduction suggests that the majority of the cysteine residues in the MOMP of RBs existed in a reduced form. In contrast, the cysteine residues in the small amount of doublet and 12-kd proteins present in RBs appeared to be engaged in disulfide bonds.

***C. trachomatis* outer membrane proteins.** The susceptibility to dissociation with detergents of proteins of the LGV434 strain of *C. trachomatis* was qualitatively similar to that observed with the 6BC strain of *C. psittaci*. The major difference was that Sarkosyl and SDS rendered soluble ca. 25% of the outer membrane proteins of EBs of the LGV434 strain, whereas these treatments failed to solubilize a significant proportion of outer membrane material of EBs of the 6BC strain (Fig. 1). As was the case with the 6BC strain of *C. psittaci*, the MOMP of RBs of the LGV434 strain of *C. trachomatis* largely was dissociated by treatment with 2% SDS (in the absence of reducing agents) but not by treatment with 2% Sarkosyl.

The in vivo incorporation of [^{35}S]cysteine and [^{35}S]methionine into *C. trachomatis* (LGV434) EB and RB proteins is shown in Fig. 6. As was the case with the 6BC strain of *C. psittaci*, [^{35}S]cysteine was incorporated mainly into the 40-kd MOMP in RBs and into a 62 kd-59 kd doublet, the MOMP, and a low-molecular-weight complex of ca. 13,000 in EBs of the LGV434 strain of *C. trachomatis*. [^{35}S]methionine was incorporated mainly into one outer membrane protein, the MOMP, in both developmental forms of the LGV434 strain. The ratio of [^{35}S]cysteine to [^{35}S]methionine incorporated into EB outer membrane proteins was 1.75 for the 62 kd-59 kd doublet, 0.45 for the MOMP, and 8.09 for the 13-kd complex, indicating that compared with the MOMP the doublet and 13-kd proteins of the LGV434 strain are rich in cysteine relative to methionine.

TABLE 1. Alkylation of EB and RB outer membrane protein preparations with [^{14}C]iodoacetamide[a]

Life cycle form	Protein (kd)	Before reduction (cpm)	After reduction (cpm)	Fold increase
EB (48-h-old)	62	967	3,254	3.37
	59	451	2,352	5.22
	40	589	1,589	2.70
	12	752	5,528	7.35
RB (21-h-old)	62-59	273	597	2.12
	40	412	565	1.37
	12	169	581	3.44

[a] The experimental protocol is described in the text.

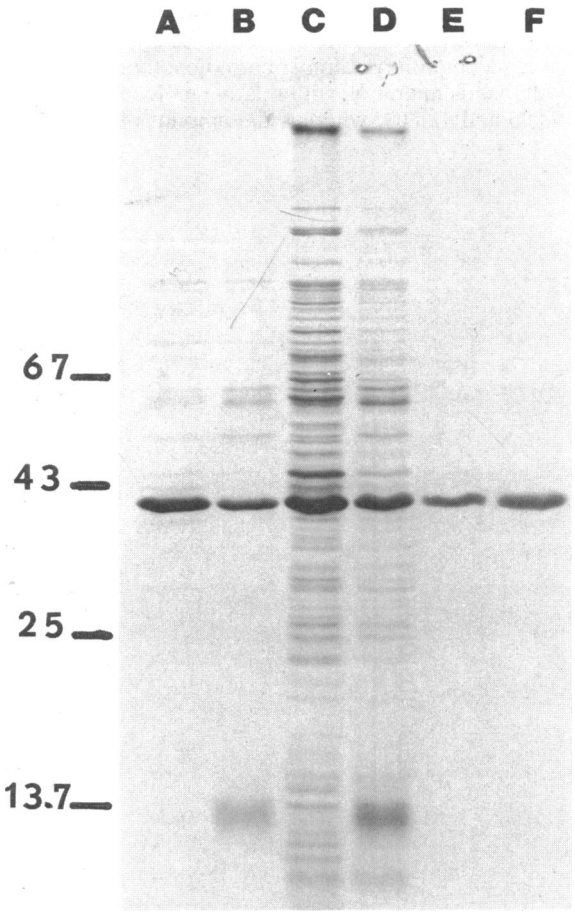

FIG. 6. SDS-PAGE profiles of *C. trachomatis* (LGV434) proteins labeled in vivo with [^{35}S]cysteine or [^{35}S]methionine. RBs and EBs, harvested at 21 and 48 h postinfection, respectively, were grown in 2 × 10^7 HeLa cells in Eagle minimal medium in the continuous presence of 10 μCi of either [^{35}S]cysteine or [^{35}S]methionine per ml. Outer membrane preparations were obtained by Sarkosyl extraction. Lanes: (A) EB outer membrane preparations, [^{35}S]methionine; (B) EB outer membrane preparations, [^{35}S]cysteine; (C) whole EBs, [^{35}S]methionine; (D) whole EBs, [^{35}S]cysteine; (E) RB outer membrane preparations, [^{35}S]methionine; (F) RB outer membrane preparations, [^{35}S]cysteine.

DISCUSSION

The observation that SDS plus reducing agents but not SDS alone dissociated EB outer membrane proteins of *C. psittaci* (6BC) suggests that disulfide bonds play an important role in maintaining the structural integrity of the EB form of this strain. Although the exact topographical rela-

tionship among EB outer membrane proteins was not analyzed by two-dimensional SDS-PAGE because of the failure of these proteins to enter gels under nonreducing conditions, it is tempting to speculate that these proteins are present in outer membranes as supra-macromolecular complexes cross-linked by disulfide bonds.

Several important differences were noted between EB and RB outer membrane preparations in structure and polypeptide content. (i) The MOMP was found to be present in approximately equal amounts in the outer membranes of both developmental forms; however, in RBs it was rendered soluble by treatment with SDS in the absence of a reducing agent. (ii) The 21-h-old RBs were found to be deficient in three cysteine-rich proteins with molecular weights of 62,000, 59,000, and 12,000. (iii) In 21-h-old RBs, the quantitatively minor cysteine-rich proteins were found by two-dimensional SDS-PAGE to be present in high-molecular-weight, disulfide-cross-linked complexes, whereas the MOMP was present mainly, but not exclusively, in monomeric form. It also was noted that the commencement of synthesis of the cysteine-rich proteins in RBs occurred at a time in the developmental cycle (21 to 24 h postinfection) when RBs initiate the process of reorganizing into EBs. Although other models are conceivable, it is possible that the MOMP, drawn from a preexistent pool of monomers, forms disulfide-cross-linked polymeric complexes with itself, the newly synthesized doublet or 12-kd protein, or a combination of these as RBs differentiate to EBs. Correspondingly, the delay in the conversion of RBs to EBs in medium lacking cysteine observed by Stirling et al. (24) may reflect the inability of RBs to synthesize cysteine-rich outer membrane proteins and to form high-molecular-weight, disulfide-cross-linked polymers under conditions of cysteine deficiency.

In contrast to our observations of EBs of *C. psittaci* (6BC), Newhall and Jones (22) reported that all of the MOMP in EBs of an oculogenital strain of *C. trachomatis* (UW94, serotype F) was rendered soluble by heating the material to 100°C for 2 min in 2.5% SDS in the absence of a reducing agent. The MOMP in SDS-soluble material was identified by these investigators in monomer, dimer, trimer, and higher multimeric forms by two-dimensional SDS-PAGE. It also was noted that material associated with the origin of the first-dimensional gel under nonreducing conditions was resolved into proteins with apparent molecular weights of 60,000 and 40,000 when electrophoresed under reducing conditions in the second-dimensional gel. These observations are qualitatively similar to those which we report here on the RB form of *C. psittaci* (6BC) (Fig. 5). It is possible that the outer membrane proteins of *C. trachomatis* are considerably less extensively cross-linked by disulfide bonds than are the outer membrane proteins of EBs of *C. psittaci* (6BC). Our observation that outer membrane proteins of *C. trachomatis* (LGV434) are partially soluble in SDS suggests that the cross-linkage of LGV 434 outer membrane proteins may be intermediate in relation to *C. psittaci* and oculogenital strains of *C. trachomatis*.

Tamura and Manire (26) failed to detect cysteine and methionine in RB outer membranes preparations of *C. psittaci* (CAL10). We report that the MOMP, which contains both cysteine and methionine, is a prominent feature of both RB and EB outer membranes. However, we also found that the cysteine content of proteins in our RB outer membrane preparations was considerably less than that of the EB outer membrane proteins since RB preparations were deficient in three cysteine-rich proteins. It is possible that the failure of Tamura and Manire to detect cysteine and methionine in RB outer membrane preparations was related to their method of extraction which included exposure to 0.5% SDS for 2 h at 37°C, a treatment which in our hands extracted a large proportion of the proteins in the RB but not the EB envelopes.

Tamura et al. (30) noted that the SDS-PAGE profile of RB outer membrane preparations of *C. psittaci* (CAL10) lacked, or was deficient in, several proteins found in the SDS-PAGE profile of EB outer membrane proteins. Two proteins, with reported molecular weights of 57,500 and 13,800, may correspond to the 62-kd-59kd doublet and the 12-kd peptide reported here. The cysteine content of the Cal10 proteins and their possible role in EB structure was not examined. Matsumoto and Manire (20) observed lattices of hexagonally arrayed structures on the inner side of EB outer membranes of *C. psittaci* (CAL10) which were present in only trace amounts in RB outer membranes (prepared by a method which included extraction with SDS) and suggested that this lattice provided the structural rigidity found in EBs. Tamura et al. (30) reported that these hexagonal arrays, extracted with hot formamide from EB outer membranes, were composed of three proteins with molecular weights of 43,700 (the MOMP), 28,500, and 13,800. Recently, Chang et al. (5) confirmed the presence of hexagonally arrayed lattice structures on the inner side of *C. trachomatis* outer membranes and observed the structures in both EBs and RBs, noting them to be more sensitive to detergent digestion in RBs. In light of the observations reported here, it is possible that the hexagonal arrays are extensively cross-linked by S-S bonds in EBs (hence detergent resistant) and partially cross-linked in RBs (hence detergent sensitive).

The function of disulfide-cross-linked complexes in chlamydial outer membranes is not known. Their abundance in EB outer membranes which are rigid and their near absence in RB outer membranes which are nonrigid suggest that disulfide bonding may confer rigidity to chlamydial outer membranes. This possibility was suggested first by Tamura and Manire (26) and more recently by Newhall and Jones (22). Likewise, the differential susceptibility of EBs and RBs to osmotic lysis may be related to the degree of disulfide bonding between outer membrane proteins in the two life cycle forms. To date, however, evidence which directly supports either of these possibilities has not been presented. Sarov and Becker (23) observed that EBs of an LGV434 strain of *C. trachomatis* incorporated nucleotides into RNA more efficiently if they were pretreated with reducing agents. This observation suggests that extensive cross-linking of EB outer membrane proteins may render EBs impermeable to nutrients required for RB multiplication.

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