Biosynthesis and Disulfide Cross-Linking of Outer Membrane Components during the Growth Cycle of *Chlamydia trachomatis*

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The synthesis and accumulation of *Chlamydia trachomatis* outer membrane proteins within infected HeLa 229 host cells were monitored by assessing the uptake of [^{35}S]cysteine into chlamydial proteins during the 48-h growth cycle of a lymphogranuloma venereum strain, L_2/434/Bu. Synthesis of the major outer membrane protein, a protein that accounts for about 60% of the outer membrane protein mass of elementary bodies (EB), was first detected between 12 and 18 h after infection. The uptake of [^{35}S]cysteine into the 60,000-molecular-weight doublet (60K doublet) and 12.5K cysteine-rich proteins was not observed until 30 h after infection, when the intracellularly dividing reticulate bodies were beginning to transform into infectious EBs. By using a more sensitive immunoblotting method in conjunction with monoclonal antibodies specific for the 60K doublet proteins, synthesis of these proteins was detected even earlier, by 18 h after infection. These data suggest that the time and extent of synthesis of these outer membrane proteins are regulated by processes that coincide in time with the transformation of reticulate bodies into EBs. Additional studies were performed to determine the extent of disulfide cross-linking of outer membrane proteins during the growth cycle. Both the major outer membrane protein and the 12.5K protein became progressively cross-linked to about 60% during the last 24 h of the growth cycle, whereas the 60K doublet proteins were extensively cross-linked during most of the cycle. These data may indicate an intracellular cross-linking mechanism, possibly enzymatic, that exists in addition to an auto-oxidation mechanism that occurs upon host cell lysis and exposure to the extracellular environment.

Substantial changes in the morphological and biochemical characteristics of chlamydiae occur during their growth within a host cell phagosome. The growth cycle is initiated by the elementary body (EB), which can survive extracellularly but is metabolically dormant and possesses infectious properties that lead to attachment and ingestion. Upon ingestion, phagosome-lysosome fusion is prevented by a process that may involve the outer membrane, since purified cell envelopes can produce this effect (6). Once ingested, the EB transforms into a reticulate body (RB), which is about two to three times larger than the EB. The RB is metabolically active and is responsible for the formation of progeny RBs. About midway through the growth cycle (24 to 36 h, depending on the strain), some of the RBs begin a process of condensation during which they transform back into infectious EBs. By the end of the growth cycle, this asynchronous process is complete for most of the chlamydial particles; the chlamydiae are released, and the EBs are free to begin another infection cycle.

Much recent work with chlamydiae has focused on the cell envelope, since many of the pathogenic properties of these unique organisms seem to be associated with this structure. Both morphological forms possess an outer membrane and a structure corresponding to an inner membrane (5, 19). The EB is resistant to disruption by osmotic and mechanical forces compared with the highly fragile RB (18). This difference in structural stability appears to be due to differences in protein-protein cross-linking by disulfide bonds in which the outer membrane proteins of the EB are extensively cross-linked, whereas those of the RB are not (4, 9, 10, 14). Interestingly, a structure analogous to peptidoglycan is apparently absent in both forms (1, 7). Another difference between RB and EB outer membranes is that the RB is deficient in two polypeptides that are present in the EB and

which have apparent molecular masses of 60 and 12 kilodaltons (kDa) (10). Relative to other chlamydial proteins, the 60,000-molecular weight (60K) and 12K proteins have a higher cysteine content and have been referred to as "cysteine-rich" proteins (10). In *Chlamydia trachomatis*, the 60K protein exists as a single polypeptide in strains within the trachoma biovar and as a doublet in strains within the lymphogranuloma venereum (LGV) biovar (2). Another difference between the biovars is the presence of a 12-kDa protein in the trachoma strains and a slightly larger (ca. 12.5 kDa) protein in the LGV biovar. In addition to molecular mass, these polypeptides also differ between the biovars in terms of their intrinsic charge (2). Thus, these polypeptides are possibly involved in the transition of RBs and EBs and may be responsible in part for known biological differences between chlamydial species and biovars.

To better understand the role these proteins play in the evolution of outer membrane structure during the growth cycle, experiments were performed that evaluated the biosynthesis of chlamydial outer membrane proteins with respect to time, and when these proteins become cross-linked.

MATERIALS AND METHODS

Growth and harvest of chlamydiae. *C. trachomatis* strain L_2/434/Bu was used to infect HeLa 229 cell monolayers in 75-cm^2 culture flasks with standardized inocula that yielded greater than 80% infection of the host cells. After incubation for 2.5 h at room temperature (2.5 h postinfection), the inocula were removed, 20 ml of growth medium was added, and the cultures were incubated at 37°C. The growth medium was 90% Eagle minimum essential medium–10% fetal calf serum (pH 7.3) supplemented per milliliter with 4 mg of glucose, 1 μg of cycloheximide, 100 μg of streptomycin, and

100 µg of vancomycin. All flasks in each experiment were seeded identically with the same number of HeLa cells. Of a total of 16 Chlamydia-infected and 2 mock-infected 75-cm² HeLa monolayers per experiment, one-half were also supplemented with 6 µCi of L-[³⁵S]cysteine (New England Nuclear Corp., Boston, Mass.) per ml. Every 6 h postinfection up to 48 h, one nonlabeled and one [³⁵S]cysteine-labeled HeLa monolayer were harvested. The mock-infected samples were harvested at 48 h. One-half hour before harvest, the growth medium was removed and the monolayers were rinsed twice for 15 min with 10 ml of Hanks balanced salt solution containing 10 mM iodoacetamide. HeLa cells from each flask were harvested into 25 ml of the same buffer by using 0.5-cm-diameter glass beads and centrifuged at 4°C for 30 min at 31,000 × g. Each pellet was suspended to a final volume of 0.5 ml in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)–145 mM NaCl–10 mM iodoacetamide (pH 7.4) and stored at −70°C until analysis. Samples radiolabeled with [³⁵S]cysteine were assessed for radioactivity by scintillation counting in a model LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Measurement of [³⁵S]cysteine uptake. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (13). A 10-µl sample from each of the time points during chlamydial infection and the 48-h mock-infected sample were solubilized by boiling for 5 min in 90 µl of 62.5 mM Tris (pH 6.8)–2% (wt/vol) SDS–5% (vol/vol) 2-mercaptoethanol, and 25 µl was resolved on a 14% polyacrylamide gel. After fixation and staining of the gel with Coomassie brilliant blue R-250 in isopropanol-acetic acid (15), the gel was photographed and then incubated in 150 ml of Enlightning (New England Nuclear, Boston, Mass.) for 30 min before being dried under vacuum at 60°C. Fluorograms were obtained by exposing X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. After fluorography, areas of the gel corresponding to the positions of the major outer membrane protein (MOMP) and the 12.5K and 60K outer membrane proteins for each sample were excised and radioactivity was determined as previously described (15).

Measurement of protein turnover. Pulse-chase experiments were performed with a 4-h pulse, starting either at 21 or 26 h postinfection. Infection and growth conditions were as described above. Cultures were pulsed with 25 µCi of L-[³⁵S]cysteine per ml for 4 h at 37°C. The chase was initiated by rinsing the monolayers twice with Hanks balanced salt solution and by incubating them in growth medium without radiolabel. Monolayers were harvested immediately after the pulse and at 36 and 48 h postinfection. Samples were analyzed by SDS-PAGE and fluorography by the methods outlined above.

Immunoblotting. Immunoblotting was performed by a standard protocol (13). Samples from each of the time points were resolved by SDS-PAGE on 14% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. For experiments to quantitate MOMP, the membranes were probed with a species-specific anti-MOMP monoclonal antibody (3) kindly provided by Pertti Terho. To quantitate the 60K doublet, membranes were probed with a pool of three murine monoclonal antibodies that recognize three different epitopes on each of the proteins (12). Bound antibody was localized by incubation with ¹²⁵I-labeled protein A, followed by autoradiography. Areas on the nitrocellulose membrane corresponding to MOMP and the 60K doublet were excised, and the associated radioactivity was

measured in a gamma counter. Protein-specific radioactivity was determined by subtracting the background radioactivity from an equivalent area of the same lane that showed no obvious reactions or abnormal background binding. The relative amount of antigen present in each sample was determined by reference to a standard titration curve of antigen versus bound radioactivity. This curve was generated for each experiment by the evaluation of twofold dilutions of the 48-h Chlamydia-infected sample out to 1:1,024. Dilutions were with the 48-h mock-infected sample to maintain an equivalent amount of HeLa cell material in each sample and to more closely mimic the transfer and immunoblotting conditions of the experimental samples. The amount of antigen present in the 48-h Chlamydia-infected sample was defined as 100%.

Measurement of protein cross-linking. For experiments to quantitate the extent of protein-protein disulfide cross-linking of MOMP and the 12.5K protein, [³⁵S]cysteine-labeled samples from each time point were solubilized (i) as described above in the presence of 2-mercaptoethanol and (ii) in the same buffer lacking 2-mercaptoethanol but containing 40 mM iodoacetamide and were resolved on the same gel. ³⁵S counts per minute (cpm) associated with the areas corresponding to the monomeric positions of these proteins was measured for the reduced (cpm_{red}) and the alkylated (cpm_{alk}) samples as described above, and the percentage of cross-linking was calculated at each time point as (cpm_{red} − cpm_{alk})/cpm_{red} × 100%. In this equation, cpm_{red} represents the total amount of the protein in the sample and cpm_{alk} represents the portion that is not cross-linked. Cross-linking of the 60K doublet proteins was estimated by quantitating monomeric protein in reduced and in alkylated non-[³⁵S]cysteine-labeled samples by immunoblotting with monoclonal anti-60K antibodies. The percentage of cross-linking was calculated with the same equation given above but substituting ¹²⁵I cpm bound to the proteins in reduced and in alkylated samples.

RESULTS

Accumulation of chlamydial outer membrane proteins within infected HeLa cells during the growth cycle. The net accumulation of various chlamydial proteins after the infection of HeLa cells was assessed by SDS-PAGE analysis of whole infected cells at various times postinfection. In comparison to mock-infected controls, Chlamydia-infected HeLa cells possessed a number of unique proteins (Fig. 1). Most easily identified was MOMP, which was initially observed at 24 h and was present in increasing amounts for the remainder of the growth cycle. The 60K outer membrane protein doublet was difficult to distinguish from the HeLa cell background, which increased during the course of the experiment due to protein sythesis that was not inhibited by cycloheximide. However, in the last half of the growth cycle, bands at the expected position of these proteins were observed in higher amounts than in mock-infected controls. Similar results were obtained for bands having estimated masses of 76 and 29 kDa. Another protein that was clearly identified in Chlamydia-infected cells but not in mock-infected cells had an apparent mass of 15 kDa. The 15K protein was first distinguishable at 30 h and accumulated to higher amounts up to the end of the cycle. Although all of these proteins, as well as others that appeared to be Chlamydia-associated, were found to increase in relative abundance during the growth cycle, a protein with an apparent

mass of 86 kDa was first detected at 18 h, increased in abundance until 30 h, and then was maintained at a constant amount for the rest of the cycle.

Uptake of [³⁵S]cysteine into proteins of Chlamydia-infected HeLa cells. To better distinguish chlamydial proteins from HeLa cell proteins than was possible in gels stained with Coomassie blue and to allow for the direct quantitation of accumulated chlamydial proteins, chlamydia- and mock-infected HeLa cells were grown in the continuous presence of [³⁵S]cysteine and harvested every 6 h as before. Cysteine was chosen since MOMP and the 60K and 12.5K outer membrane proteins have been shown to be extensively cross-linked by disulfide bonds (11) and to incorporate [³⁵S]cysteine (10). This was particularly important for the detection of the 12.5K protein, which is not easily detected by Coomassie blue staining. Samples from each of the time points were resolved by SDS-PAGE and fluorography (Fig. 2). At 48 h postinfection, the mock-infected HeLa cells demonstrated very little uptake of [³⁵S]cysteine into cellular proteins. In contrast, significant uptake was observed into proteins of chlamydia-infected cells. A small amount of radiolabel was detected in MOMP at 18 h but not earlier, even when longer exposures were used. Uptake into MOMP increased rapidly from 18 to 30 h and then more gradually for the remainder of the cycle. The 60K outer membrane protein doublet was first visible by fluorography at 24 h and increased in abundance thereafter. Excluding MOMP, the component with the most associated radioactivity at 48 h was the 12.5K protein. This protein was not detectable until 30 h, after which there was a progressive increase in the uptake of [³⁵S]cysteine. An identical result was obtained for a component with an estimated mass of 10 kDa. While the synthesis and accumulation characteristics of the 12.5K and 10K proteins are very similar, these proteins differ in other characteristics. For example, the 10K protein is soluble in Sarkosyl, whereas the 12.5K is not (2). In addition, the 10K protein is not cross-linked into oligomeric complexes by disulfide bonds as is the 12.5K protein (data not shown). Analysis of other components by this method, including the 86K, 76K, and 15K proteins, confirmed the results obtained with Coomassie blue staining.
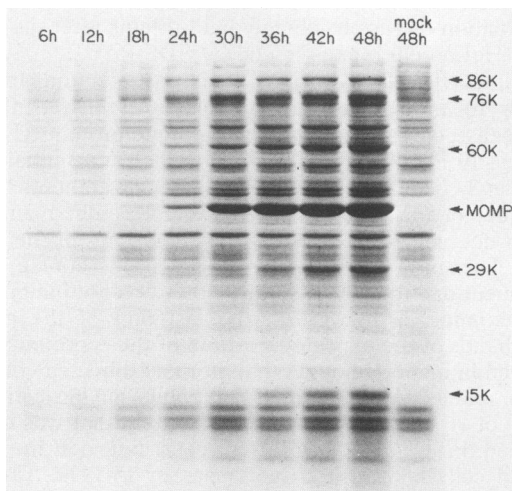

FIG. 1. Coomassie blue-stained gel of HeLa cells infected with strain L₂/434/Bu and harvested at 6-h intervals throughout the 48-h growth cycle. The uninfected HeLa cell protein profile at 48 h is labeled as mock 48 h. Arrows indicate the positions of some of the easily distinguishable chlamydial proteins.
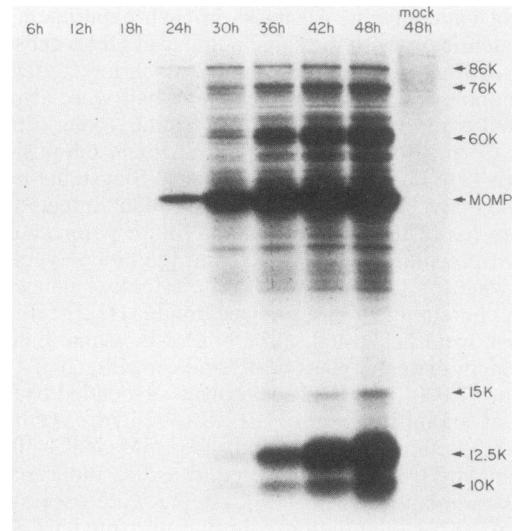

FIG. 2. Fluorogram of polyacrylamide gel of Chlamydia-infected HeLa cells grown in the continuous presence of [³⁵S]cysteine. The gel used to obtain this fluorogram is the same as that shown in Fig. 1.

The amount of radioactivity incorporated into MOMP and the 60K and 12.5K outer membrane proteins was measured for each time point (Fig. 3). Based on a detection limit of about 35 cpm above the background, protein synthesis was not detected until 18 h for MOMP and until 30 h for the 60K and 12.5K proteins. MOMP accumulated rapidly between 12
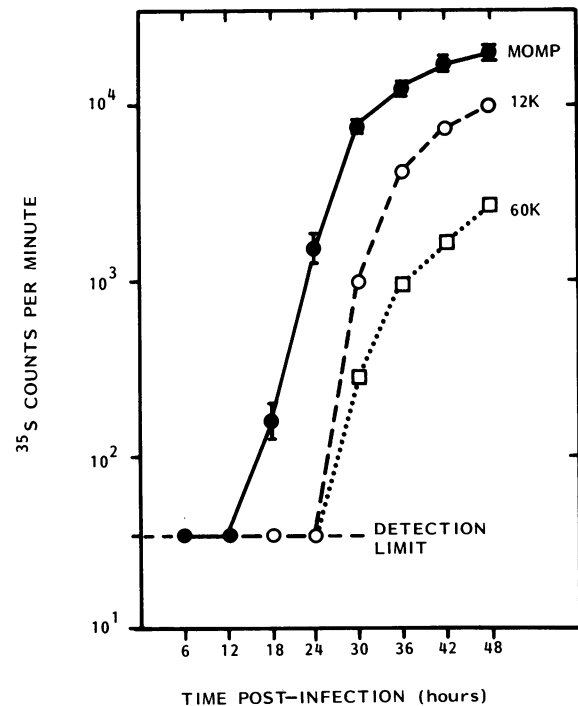

FIG. 3. Uptake of ³⁵S counts per minute into three chlamydial outer membrane proteins as a function of time during the 48-h growth cycle. The detection limit in these experiments was about 35 cpm above the background. The error bars, shown for the MOMP, represent 1 standard deviation of the mean of two measurements. The magnitudes of the standard deviations for the 60K and 12.5K proteins were similar to that for the MOMP at the same time point.

TABLE 1. Turnover of [^{35}S]cysteine-labeled chlamydial proteins

Protein	Pulse (h)	Associated ^{35}S cpm[a] ($\pm$SD) at:			
		25 h	30 h	36 h	48 h
MOMP	21-25	5,082 $\pm$ 167	—[b]	5,723 $\pm$ 182	5,319 $\pm$ 215
MOMP	26-30	—	9,433 $\pm$ 180	9,635 $\pm$ 282	9,257 $\pm$ 304
60K doublet	26-30	—	1,087 $\pm$ 218	1,228 $\pm$ 147	1,095 $\pm$ 176
12.5K	26-30	—	1,892 $\pm$ 230	1,855 $\pm$ 138	1,712 $\pm$ 151

[a] Mean of duplicate samples.
[b] —, Not determined.

and 30 h and more slowly thereafter. For the 60K and 12.5K proteins, the most rapid accumulation was between 24 and 36 h. From 30 to 36 h and generally until 48 h, the net rate of accumulation of these two proteins was substantially greater than that of MOMP as indicated by the differences in slopes.

Turnover of [^{35}S]cysteine-labeled proteins. To assess the possibility that chlamydial proteins may be degraded after their synthesis, a pulse-chase experiment was designed to evaluate the turnover of the MOMP made early in the growth cycle (21- to 25-h pulse) and of the MOMP and the 60K and 12.5K proteins made midway through the cycle (26- to 30-h pulse). The radioactivity associated with these components was measured immediately after the pulse and at two times during the chase (Table 1). No significant turnover of these proteins was observed. The small increase seen at 36 h for the 21- to 25-h MOMP probably indicates the uptake of the [^{35}S]cysteine that was not removed during the rinse. The results suggest that the uptake of [^{35}S]cysteine into proteins during continuous labeling can be taken as a measure of net biosynthesis.

Detection of newly synthesized proteins with monoclonal antibodies. The [^{35}S]cysteine labeling experiments suggested that the 60K and 12.5K proteins may not be synthesized before 24 h postinfection. The data seem particularly convincing for the 12.5K protein, since this protein was located in an area with low backgrounds. However, the ability to quantitate accurately ^{35}S cpm associated with the 60K protein was hampered by the background from the HeLa cell and other chlamydial proteins. To provide an alternative method for the quantitation of the 60K protein doublet, as well as MOMP, gels similar to that shown in Fig. 1 were transferred to nitrocellulose membranes and probed with MOMP- and 60K-specific monoclonal antibodies by immunoblotting. The amount of each protein present at the different time points was expressed as a percentage of the final 48-h value and plotted as a function of time (Fig. 4). The quantitation of MOMP by immunoblot was nearly identical to that derived by measuring [^{35}S]cysteine uptake, suggesting that this immunological method may serve as a reliable means of detecting relative amounts of antigen. Indeed, the immunoblot method appeared sensitive enough to detect MOMP at 6 and 12 h. This antigen presumably represents the MOMP from EBs at the beginning of the infection.

A similar analysis was performed with a pool of three monoclonal antibodies specific for the 60K protein. Again, this method was able to detect the antigen present early in the growth cycle. No change in the amount of antigen was seen from 6 to 12 h. The antigen present at these times is likely from the infecting EBs. A small increase in the amount of 60K antigen was detected from 12 to 18 h. A larger increase was observed between 18 and 24 h, and the maximum rate of 60K synthesis occurred between 24 and 30 h. These data suggest that the 60K doublet proteins are syn-

thesized before 24 h postinfection, although the relative amounts are fairly low (about 1% of the total that will eventually be made). In addition, compared with MOMP, the 60K doublet proteins appeared to be synthesized at a lower rate between 12 and 24 h.

Extent of protein-protein cross-linking by disulfide bonds. To determine when in the growth cycle the outer membrane proteins became cross-linked, the extent of disulfide bonding to form oligomeric complexes was measured at different times postinfection (Fig. 5). Results for MOMP and the 12.5K protein were derived by measuring ^{35}S cpm associated with monomeric protein in reduced versus alkylated samples. For the 60K doublet, the amount of monomeric proteins in these same samples was estimated by immunoblotting. Before 30 h, MOMP was found predominantly in the monomeric form. The apparent absence of cross-linking at 18 h for MOMP and the 60K doublet in the experiment shown probably reflects the inability of the methods to detect differences between the reduced and alkylated samples for the small amounts of material present at that time. After 30 h, the proportion of MOMP in cross-linked complexes progressively increased to about 60% at the end of the growth cycle. The increase and extent of cross-linking of the 12.5K protein were nearly parallel to those of MOMP. In
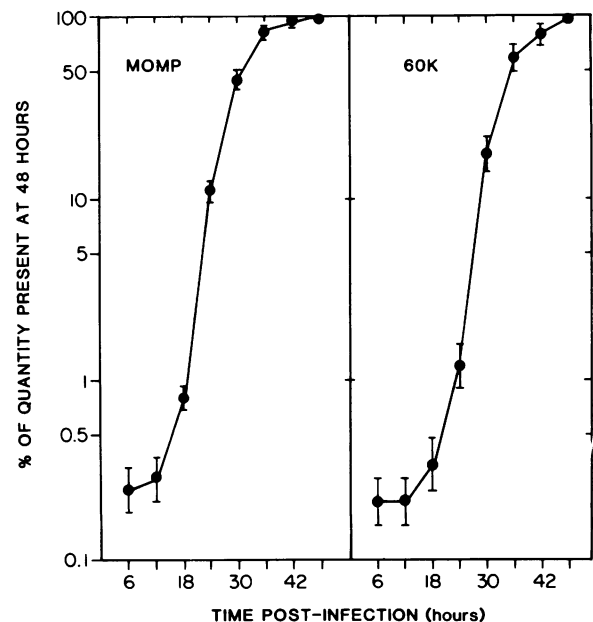

FIG. 4. Quantitation of MOMP and 60K doublet proteins by immunoblotting. The amount of each protein present at different times in the growth cycle was expressed as a percentage of the amount present at 48 h. The error bars represent 1 standard deviation of the mean of three independent measurements.

contrast, the 60K protein appeared to become cross-linked much earlier, with about 60% of the protein present in cross-linked complexes by 30 h and about 80% by 48 h.

DISCUSSION

Previous studies indicate that the cell envelopes of the infectious, sporelike EBs of both *Chlamydia psittaci* and *C. trachomatis* are structurally stable as a result of extensive cross-linking among membrane proteins by disulfide bonds rather than a network of peptidoglycan, which is apparently absent in chlamydiae (1, 4, 7, 9, 10, 14). Studies of RBs, which have relatively fragile cell envelopes, indicate that these intracellular dividing forms do not have their membrane proteins cross-linked to a significant degree (10, 14). These findings suggest that the formation of a rigid and stable cell envelope occurs during the condensation of RBs into EBs. Additional work has shown that the EB possesses three outer membrane proteins that are present in disulfide cross-linked macromolecular complexes: MOMP, the 60K protein (trachoma biovar) or 60K doublet (LGV biovar and *C. psittaci*), and the 12K protein (trachoma biovar) or 12.5K protein (LGV biovar and *C. psittaci*) (2, 10, 11). In contrast, RBs have significantly decreased amounts of the 60K and 12K outer membrane proteins relative to those of EBs (10). Thus, there is a correlation between structural stability, extent of disulfide cross-linking, and the presence of certain outer membrane proteins. The purpose of the present study was to determine the time course of biosynthesis of chlamydial outer membrane proteins of strain L₂/434/Bu and the times when these proteins become crosslinked.

Synthesis of MOMP and the 60K doublet was detected by 18 h postinfection but not before 12 h. This finding is consistent with the observation that during the first 12 h of infection, the EBs must first undergo a morphological transformation into RBs before beginning the multiplication phase. Electron micrographs show that the size and therefore surface area of the organism increases during the transition from EB to RB. In the absence of detectable outer membrane protein synthesis, perhaps the membrane becomes larger as the result of incorporating newly made phospholipids or other lipid components.

By 24 h, the RBs have gone through a number of divisions and electron microscopy has demonstrated the presence of small inclusions containing RBs and only infrequently an EB or condensing form. On the basis of the data described here, about 10% of the MOMP that had been made by 48 h after infection had been synthesized by this time, and more than 90% of this MOMP was in a non-cross-linked form. Pulse-chase experiments indicated that this MOMP was not degraded during the next 24 h. In contrast, no 12.5K and only about 1% of the 60K doublet proteins had been made by 24 h. These latter results are different from the results of Hatch et al. (11) with a related organism, *C. psittaci*, that indicate the synthesis of significant amounts of analogous proteins by 24 h postinfection. Thus, the timing and rates of synthesis of analogous proteins in the two bacteria appear to be very different. Nevertheless, by showing that these proteins are also not detectable in significant amounts in whole infected cells bearing predominantly RBs, the present results confirm previous work that indicates the absence of the 12.5K and 60K doublet proteins in purified RBs of *C. trachomatis* L₂/434/Bu (10).

About 70% of the MOMP is synthesized during the 12-h interval from 24 to 36 h, whereas only about 20% is made during the final 12 h of the growth cycle. Thus, most of the
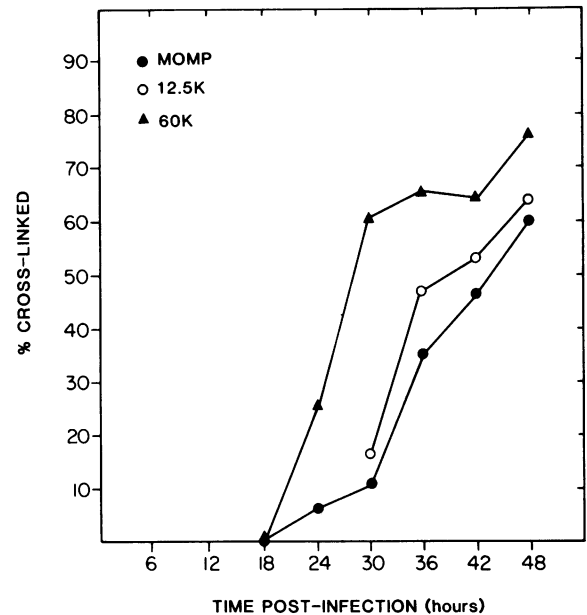

FIG. 5. Extent of disulfide cross-linking of three outer membrane proteins determined by measuring the amount of monomeric protein present at each time point. The results shown are from a single experiment. The absolute percentage of cross-linking varied from experiment to experiment by 15 to 20%. The relative differences, however, were the same in different experiments (for example, between the 60K doublet and MOMP at 30 h).

MOMP is synthesized during the period of rapid multiplication of RBs. Synthesis then tapers off late in the cycle during the condensation of RBs into EBs. Because of the asynchronous nature of chlamydial growth, this synthesis may be by RBs that are still growing and dividing. Since the surface area of the RB is as much as 16 times greater than that of the EB and since near the end of the cycle the chlamydiae have almost completed MOMP production, the concentration of MOMP per unit of surface area must increase dramatically during the conversion of RB into EB. However, this conclusion assumes that the MOMP that is made during the cycle remains associated with the chlamydiae. Another possibility is that some of the MOMP is released from the RB outer membrane, possibly within the blebs that have been reported by many investigators (17, 20).

In contrast to MOMP, which increased about sevenfold from 24 to 36 h, the 60K doublet increased by over 50-fold during the same period. In addition, the 12.5K protein increased from undetectable levels at 24 h to about 50% of its ultimate 48-h level. These results and the finding that none of these proteins appear to be degraded once made suggest the presence of independent controls at the level of transcription or translation of the genes encoding the MOMP and the 60K and 12.5K proteins. The regulation of these or other processes that control the time-dependent biosynthesis of chlamydial outer membrane proteins appears to be a crucial aspect of the overall mechanisms that may govern division and the maturation of RBs into EBs.

The results described here are similar in some ways but different in others from the observations made by Hatch et al. (11). Many of the differences are probably attributable to the difference in the organisms evaluated. The detailed analysis by Hatch et al. (11) was derived predominantly with a strain of *C. psittaci*, an organism that is quite different from

the *C. trachomatis* strain used in the present study. In terms of methodologies, a major difference between the two studies is that the present study monitored the appearance of chlamydial proteins and antigens within intact, infected host cells, whereas the study by Hatch et al. (11) assessed the incorporation of MOMP, 60K doublet, and the 12K protein into Sarkosyl-insoluble extracts of infected cells. Such extracts are thought to represent chlamydial outer membranes but may contain only a portion of the total protein that has been synthesized. In addition, some MOMP in purified EBs is soluble in Sarkosyl (2). Thus, the present data give an indication of total synthesis.

Another difference between the studies was in the estimation of the extent of disulfide cross-linking, particularly of MOMP. In the 6BC strain of *C. psittaci*, MOMP is not cross-linked until the end of the growth cycle, when the host cell lyses and the chlamydiae are released into an oxidizing environment (11), whereas the 60K doublet and 12K proteins are present in cross-linked complexes throughout the cycle. In the present study of an LGV strain of *C. trachomatis* which used iodoacetamide to prevent auto-oxidation or auto-reduction of sensitive disulfide bonds, the MOMP in the total cellular sample appeared to become progressively cross-linked from 30 to 48 h postinfection. Another difference is that the present data suggest that the cross-linking of the 12.5K protein followed that of the MOMP very closely, while the 60K doublet was more extensively cross-linked, particularly between 24 and 36 h. Thus, while the MOMP of *C. psittaci* may indeed become cross-linked as a result of the auto-oxidation of disulfide bonds after cell lysis, the data described here are consistent with the presence of an intracellular mechanism of cross-linking in *C. trachomatis*. A similar mechanism could also be postulated to explain the extensive cross-linking of the 60K doublet and 12K proteins in the *C. psittaci* strain studied by Hatch et al. (11). One possible mechanism might be the presence of a disulfide interchange enzyme similar to that described by Roth and Koshland (16). Such an enzyme may be synthesized or become active only in RBs that have begun the transformation to EBs. The presence of both mechanisms in *C. trachomatis* might explain why the extent of cross-linking increases from 60 to 70% in intracellular chlamydiae to greater than 90% in purified chlamydial preparations (data not shown), although other explanations are also possible.

In addition to MOMP, the 60K doublet, and 12.5K outer membrane protein, this study also provided some information on the synthesis of other chlamydial proteins. For example, the 15K and 29K proteins were made in relatively small amounts and, like the 12.5K protein, were made mainly during the last 24 h of the growth cycle. These two proteins correspond to the 18K and 32K proteins that Hackstadt (8) has shown to bind eucaryotic cell surface proteins, based on the reactivity of both sets of polypeptides with monoclonal antibodies (T. Hackstadt and W. J. Newhall, unpublished data), and are probably equivalent to the 18K and 31K proteins shown to have similar adhesion characteristics by Wenman and Meuser (21).

The synthesis of chlamydial outer membrane proteins appears to be a very important aspect in the development of infectious progeny. Also, the presence of an extensively disulfide-cross-linked outer membrane protein matrix is a relatively unique structural substitute for peptidoglycan. This and other studies have begun to detail when and how the chlamydial cell wall structure and composition evolve. Such information may prove useful in further studies of virulence, pathogenesis, and latency and possibly will aid in the development of strategies to inhibit cell wall synthesis and interrupt the chlamydial growth cycle.

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