The Subunit b of the F₀F₁-type ATPase of the Bacterium Mycoplasma pneumoniae Is a Lipoprotein*

The DNA sequence analysis of the F₀F₁-ATPase operon of the bacterium Mycoplasma pneumoniae predicted that the subunit b, encoded by the gene atpF, is a lipoprotein of the murine lipoprotein type of Escherichia coli. Here we experimentally verify this prediction by metabolic labeling of subunit b with [¹⁴C]palmitic acid and by in vivo interfering with the processing of the prelipoprotein form of subunit b by the antibiotic globomycin, a specific inhibitor of the signal peptidase II. Our results suggest that the subunit b of the F₀F₁-ATPase of M. pneumoniae is anchored at the cytoplasmic membrane by an N-terminal lipid modification in addition to its transmembrane domain. The lipoprotein nature of subunit b and its proposed membrane topology seems to be characteristic for mycoplasmas, since among all sequenced bacterial atpF genes, only those from Mycoplasma gallisepticum and Mycoplasma genitalium code for a conserved lipoprotein consensus sequence.

The bacterial membrane-bound F₀F₁-type ATPase serves two purposes. The enzyme complex catalyzes the synthesis of ATP in response to an electrochemical proton gradient and generates a transmembrane proton gradient by hydrolyzing ATP (1). Mycoplasmas differ from the majority of the bacteria by the lack of a cytochrome-containing electron transport chain; therefore their F₀F₁-ATPase function seems to be restricted to maintaining a proton gradient (2). DNA sequence analyses of the complete F₀F₁-ATPase operons of the three mycoplasma species Mycoplasma gallisepticum (3), Mycoplasma genitalium (4), and Mycoplasma pneumoniae (5) indicated the presence of the same eight homologous subunits as in the corresponding operons of Escherichia coli (6) and Bacillus subtilis (7). Therefore, by analogy, we assume that in mycoplasmas the F₀ complex is formed by the five subunits a, b, γ, δ, and ε and the F₁ complex by the subunits a, b, and c. Thus, the F₀ complex would be inserted in the cytoplasmic membrane of mycoplasmas and interact with the F₁ complex, which would be oriented toward the cytosol. Comparative sequence analyses show that of the orthologs in the three mycoplasma species, E. coli and B. subtilis, the genes atpA (subunit a) and atpD (subunit β) share the highest similarities, about 50–70% identity at the amino acid level, whereas the other genes are less well conserved and differ in length (5). The most prominent example for a structural difference is the subunit b encoded by the gene atpF. The DNA sequence-based analysis predicts that the subunit b of the three different mycoplasma species has the specific features of a lipoprotein of the murine lipoprotein type of E. coli (5). These include a signal peptide with positively charged amino acids close to the N-terminal end and an accumulation of hydrophobic residues within the signal peptide followed by a cysteine at position 20–35 of the putative prelipoprotein. This cysteine is part of the conserved sequence of the prelipoprotein modification/processing site and will become the N-terminal amino acid in the mature protein after the signal peptide has been cleaved off by signal peptidase II (8). The processed protein is associated with the membrane by the attachment of a diacyl-glycerol moiety to the SH₂-group of the cysteine prior to cleavage (9,10). Searching the data bases for subunits b with a lipoprotein signature revealed that this motif was absent not only in E. coli and B. subtilis but in all other available bacterial atpF sequences. This suggests that the lipoprotein character of the subunit b is a specific trait of mycoplasmas. Their most differentiating characteristic compared with other bacteria is the complete lack of a cell wall (11). Being surrounded only by a cytoplasmic membrane might therefore require additional means to anchor certain proteins to the membrane. A relatively high number of lipoproteins have been identified experimentally in several mycoplasmas (12), and the large number predicted from analyses of the DNA sequences of complete mycoplasma genomes support this hypothesis (4,13).

As the DNA sequence-based prediction is not full proof, we decided to examine whether the subunit b of the F₀F₁-ATPase of M. pneumoniae is indeed a lipoprotein. The following experimental approaches were taken to prove the lipoprotein character (8) of the F₀F₁-ATPase subunit b of M. pneumoniae: (i) metabolic labeling of the proposed lipoprotein with [¹⁴C] palmitic acid and identification of the [¹⁴C]-labeled subunit b by a specific antibody; (ii) inhibition of the signal peptidase II by globomycin (18) and identification of the uncleaved prelipoprotein.

**EXPERIMENTAL PROCEDURES**

**Organisms and Growth Conditions—**M. pneumoniae M129 (broth passage 21) (ATCC 29342) cultures were grown at 37 °C in 50 ml Falcon tissue flasks containing modified Hayflick medium supplemented with 20% horse serum (Boehringer Mannheim). After 48 h, attached cells were washed twice with phosphate-buffered saline (0.15 M NaCl, 10 mm sodium phosphate, pH 7.4), scrapped off, collected by centrifugation at 6000 × g for 10 min at 4 °C, and stored at −70 °C. E. coli strain JM101 was used for propagation of plasmids, and E. coli M15 transformed with the plasmid pSU400 was used for expression of fusion proteins in E. coli (15).

**Preparation of Antiserum against Subunit b—**The region of the atpF gene coding for a peptide extending from amino acid residue 123 to 207 (Fig. 1B) was amplified by polymerase chain reaction and ligated into the frame to the mouse dihydrofolate reductase gene in the expression vector pQE40 (Qiagen). This vector allows the regulated expression in E. coli of a fusion protein with six N-terminal histidine residues. The fusion protein was purified by immobilized metal chelate affinity chroma-

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RESULTS AND DISCUSSION

The sequence alignments of three mycoplasma species and E. coli as a representative of a Gram-negative and B. subtilis of a Gram-positive bacterium reveal that only the subunit b proteins of M. gallisepticum, M. genitalium, and M. pneumoniae are N-terminally extended by a signal peptide with a cleavage site analogous to the consensus sequence for prolipoprotein modification and processing (Fig. 1A). In addition hydrophobicity plots predict two closely spaced transmembrane segments for the mycoplasmal subunits as compared with only one in B. subtilis and other bacteria (Fig. 1B). In accordance, the uncleaved subunit b of M. pneumoniae is 37 amino acids, but the processed form is only 9 amino acids longer than the ortholog in B. subtilis.

Mycoplasmas are not able to synthesize long-chain fatty acids by themselves (2, 12, 19). When grown under laboratory conditions they depend on a supplement of 10–20% serum, which contains also the fatty acids required for acylation of proteins. Since palmitic acid has been frequently found in mycoplasma lipoproteins and lipids (20), we labeled M. pneumoniae by adding $^{14}$C-palmitic acid to the culture medium of growing bacteria. Protein extracts from the labeled cells were analyzed by SDS-PAGE and autoradiography. At least 25 labeled proteins were detected under these experimental conditions (data not shown). The deduced amino acid sequence of subunit b predicts a molecular weight of 24,020 for the subunit b unambiguously, the complete 14C-labeled protein extract was immunoprecipitated with subunit b specific antibody in the precipitated form (Fig. 2A, lane 1). This indicates either that subunit b is synthesized in low amounts or that $^{14}$C-palmitic acid is not efficiently incorporated. To identify subunit b unambiguously, the complete $^{14}$C-labeled protein extract was immunoprecipitated with subunit b specific antibodies (see “Experimental Procedure”), and the precipitate was analyzed by SDS-PAGE and Western blotting. A protein with an apparent molecular weight of 21,000 could be detected after immunostaining with the anti-subunit b antibody in the protein extract (Fig. 2A, lane 1) and in the immunoprecipitated sample (Fig. 2A, lane 2). By subsequent autoradiography of the membrane, a signal could be detected at the same position (Fig. 2B, lane 2), providing convincing evidence that the subunit b is acylated with palmitic acid.

The biosynthetic pathway of the murein lipoprotein of E. coli, also known as Braun’s lipoprotein, is considered to be repre-
The immunoprecipitation-enriched fraction (lane 1) and of the immunoprecipitation-enriched fraction (lane 2); B, exposure of the nylon membrane from the immunoblot analysis to a X-ray film. For the enriched fraction (lane 2), three times as much cell extract was used as for the total cell extract (lane 1).

FIG. 2. [14C]Palmitic acid labeling of subunit b of M. pneumoniae. The labeled proteins were analyzed by SDS-10% PAGE before and after immunoprecipitation with anti-subunit b antiserum. A, immunoblot with anti-subunit b antiserum of total cell extract (lane 1) and of the immunoprecipitation-enriched fraction (lane 2); B, exposure of the nylon membrane from the immunoblot analysis to a X-ray film. For the enriched fraction (lane 2), three times as much cell extract was used as for the total cell extract (lane 1).

sentative for the bacterial lipoproteins (8, 21). The penultimate step is the cleavage of the signal peptide from the diacylglycerol-prolipoprotein by the signal peptidase II. This enzyme can be specifically inhibited by the antibiotic globomycin (18). Preliminary growth inhibition tests showed that globomycin at a concentration of 150 μg/ml greatly inhibits growth of M. pneumoniae. A concentration of 50 μg/ml was used as it permitted growth of the bacteria but inhibited the signal peptidase II. After growth of M. pneumoniae in the presence of globomycin for 16 h, an additional band with an apparent molecular weight of 23,000 could be detected by SDS-PAGE and immunoblotting with anti-subunit b antibodies (Fig. 3). Within the limits of SDS-PAGE for molecular weight estimation, this increase in molecular weight could be attributed to the additional 27 amino acids of the prolipoprotein, which was not further processed to the mature lipoprotein due to inhibition of the signal peptidase II by globomycin.

Inhibition of signal peptidase II action by globomycin and the specific labeling of subunit b with [14C]palmitic acid proved that the subunit b of the F0F1-ATPase of M. pneumoniae is a lipoprotein. Based on the presence of the lipoprotein motif and the almost identical hydrophobicity plots, which indicate two transmembrane segments near the N terminus, we concluded that the subunit b of the F0F1-ATPase of M. genitalium and M. gallisepticum is also a lipoprotein.

The structural predictions for subunit b of M. pneumoniae and its proposed function as a protein that interconnects F0 and F1 in the enzyme complex strongly suggest it to be an integral membrane protein oriented toward the cytosol of the cell. According to the present model, the N-terminal part of subunit b interacts with subunit a in the membrane, and its C-terminal region interacts with subunit δ, which is in contact with the F1 headpiece consisting of the subunits α and β (22). Here we show by partition of proteins during phase separation in solutions of the nonionic detergent Triton X-114 that subunit b is recovered exclusively in the detergent phase (Fig. 4D). This phase is supposed to contain only integral proteins (16). The cytosolic protein elongation factor G (G07_orf688) was partitioned in the aqueous phase (Fig. 4B), and the Triton X-114-insoluble protein P65 (F10_orf405) (Fig. 4A) was recovered in the Triton X-114-insoluble fraction (Fig. 4); both of these proteins lack a transmembrane segment. Finally, PsaH (K05_orf705), with two predicted transmembrane segments partitioned in the detergent phase (Fig. 4C), like subunit b, supporting the assumption that subunit b is an integral membrane protein (13, 23). Treatment of intact M. pneumoniae cells with trypsin and proteinase K also showed that subunit b was not accessible to these proteases whereas the surface-exposed protein P1 (24) was cleaved, and the cytosolic protein elongation factor G was not affected under the same experimental conditions (data not shown). From these data we conclude that the C-terminal part of subunit b is oriented toward the cytosol.

Based on the structural prediction for the subunit b and the experimental data provided in this paper, we propose a model for the processing and arrangement of subunit b in the cell membrane of M. pneumoniae (Fig. 5), which should also be valid for M. genitalium and M. gallisepticum. This model improves a previous proposal (3) for the membrane topology of the subunit b of M. gallisepticum, which had not taken into account the lipoprotein character of the protein.

What could be the possible advantage for the unusual lipoprotein structure of subunit b in M. pneumoniae? The most obvious effect is the stronger anchoring of this protein in the membrane through the additional acyl chains. This might be important, since mycoplasmas do not have the rigid murein layer forming a network around the cytoplasmic membrane.
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A lipopeptide from Mycoplasma fermentans was found to have a free N terminus (27), supporting the lack of an N-acyl transferase in this bacterium, whereas evidence was provided that lipoproteins from M. gallisepticum have indeed O-ester-bound and amide-linked acyl chains (28). However, the degree of acylation of the lipoproteins remains uncertain in M. gallisepticum as in other mycoplasmas.

Another unresolved question is the origin of the lipoprotein motif in subunits b of mycoplasmas. According to a widely accepted theory, these bacteria originated from Gram-positive bacteria by genome reduction (29). Since Gram-positive and all other bacteria so far analyzed do not carry the lipoprotein motif in the subunit b of the $F_0F_1$-ATPase, one can assume that mycoplasmas adopted this motif during the process of genome reduction either from another lipoprotein gene of the same cell or they received it by horizontal gene transfer. Ultimately, the importance of the lipid modification for a functional subunit b of the $F_0F_1$-ATPase can only be decided experimentally by transforming M. pneumoniae with an $atpF$ gene coding for a subunit b devoid of the lipid anchor and analyzing the biological activity of the resulting $F_0F_1$-ATPase.

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Fig. 4. Subcellular localization of subunit b. Treatment of M. pneumoniae cells with Triton X-114 resulted in three fractions (15): the Triton X-114-insoluble fraction (lane 2), Triton X-114-soluble detergent phase (lane 3), and Triton X-114-soluble aqueous phase (lane 4). Whole cell extracts were analyzed as control in lane 1. The individual subfractions were separated by 12.5% SDS-PAGE, transferred to nylon membranes, and immunostained with monospecific antibodies against P65 (A), elongation factor G (B), FtsH (C), and subunit b (D).

Fig. 5. Schematic model for the processing and the membrane topology of subunit b of M. pneumoniae. The precursor of the subunit b of M. pneumoniae traverses the membrane twice. The signal peptide will be cleaved off by signal peptidase II after the cysteine C in position 28 has been modified by the attachment of a diacyl-glycerol moiety. The second transmembrane segment ensures that the C-terminal part of subunit b is oriented toward the cytosol showing the same orientation as in E. coli (1, 22).

which protects bacterial cells against osmotic and mechanical stress (11). Among the 46 predicted lipoproteins in M. pneumoniae (13), we found 9 lipoproteins that are predicted to have at least one more transmembrane segment in addition to the signal peptide. However, in all these instances, the distance between the lipoprotein processing site and the additional transmembrane segment is greater than in subunit b, allowing formation of larger surface-exposed protein loops.

The number of acyl chains attached to the N-terminal cysteine of the subunit b remains unclear. The maximal number observed in the murein lipoprotein of E. coli is three, two by an O-ester formation with the glycerol moiety and one by an amide linkage to the free NH$_2$ moiety. The amide linkage is catalyzed by a N-acyltransferase (25), but the gene for this enzyme has not been found in the completed genome sequences either of M. genitalium (4) or M. pneumoniae (13, 26). Thus it is uncertain whether an N-acyltransferase and its end product, N-acyl cysteine, are present in these species. The few experimental data from other mycoplasmas on this topic indicate that in one species an N-acyltransferase is present but absent in another.

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![Diagram of subcellular localization of subunit b.](image-url)
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