JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 5039–5047
0021-9193/89/095039-09$02.00/0

Vol. 171, No. 9

Topology and Acylation of Spiralin

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Received 13 February 1989/Accepted 10 June 1989

Of the 51 polypeptides detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the plasma membrane of the helical mollicute *Spiroplasma melliferum*, 21 are acylated, predominantly with myristic (14:0) and palmitic (16:0) chains. This is notably the case for spiralin, the major membrane protein of this bacterium, which contains an average of 0.7 acyl chains per polypeptide, attached very probably by ester bonds to alcohol amino acids. The amphiphilicity of spiralin was demonstrated by the behavior of the protein in charge-shift electrophoresis, its incorporation into liposomes, and its ability to form in the absence of lipids and detergents, globular protein micelles (diameter, ≈15 nm). The presence of epitopes on the two faces of the cell membrane, as probed by antibody adsorption and crossed immunoelectrophoresis, and the strong interaction between spiralin and the intracytoplasmic fibrils show that spiralin is a transmembrane protein. The mean hydropathy of the amino acid composition of spiralin (−0.30) is on the hydrophilic side of the scale. Surprisingly, the water-insoluble core of spiralin micelles, which is the putative membrane anchor, has a still more hydrophilic amino acid composition (mean hydropathy, −0.70) and is enriched in glycine and serine residues. Taking into account all these properties, we propose a topological model for spiralin featuring a transbilayer localization with hydrophilic domains protruding on the two faces of the membrane and connected by a small domain embedded within the apolar region of the lipid bilayer. In this model, the membrane anchoring of the protein is strengthened by a covalently bound acyl chain.

As the smallest and simplest cells known thus far (32, 33), mollicutes (trivial name, mycoplasmas) are of fundamental interest in cell biology. Indeed, the mycoplasma cell is bounded by only the plasma membrane, and its genome, which is the smallest cellular genome recorded to date, contains a low proportion of guanine and cytosine (33). Mollicutes are thus the bacteria which illustrate best the concept of the minimal cell (33). According to recent phylogenetic studies based on sequence comparisons of rRNAs and tRNAs, the mollicutes arose by degenerative evolution from the *Clostridium* spp. (46). Most, if not all, mollicutes are parasites, and a large number of species are pathogenic for plants, animals, or humans (32). Moreover, several species are also frequent contaminants of eucaryotic cell cultures (28).

Spiroplasmas are a group of mollicutes characterized by a helical cell morphology and motility (7). Spiralin, the major membrane protein of the phytopathogenic species *Spiroplasma citri*, has been purified to homogeneity from three distinct strains (52, 53). This amphiphilic protein cannot be solubilized without recourse to detergents (47) and forms homo-oligomers in the spiroplasmal membrane (48). A similar protein has also been purified from the membrane of the honeybee spiroplasma *Spiroplasma melliferum* (53). The four spiralins analyzed to date share very similar amino acid compositions characterized by the lack of methionine and tryptophan and a high polarity index (53). Since spiralin contributes to about 22% (wt/wt) of the total integral membrane protein fraction in *S. citri* and *S. melliferum* and since its molecular mass is rather small (26 to 28 kilodaltons [kDa]) (52, 53), the spiroplasmal membrane is crowded with copies of the spiralin polypeptide. It is thus probable that this component plays a prominent part in the physiology, structure, or both of these microorganisms.

We show here that the *S. melliferum* spiralin exhibits very pronounced amphiphilic properties and contains a lipophilic membrane anchor. This protein is acylated and forms, in the absence of detergents and lipids, small, water-soluble protein micelles. Furthermore, topological investigations show that spiralin is exposed on both faces of the spiroplasma membrane, which is evidence for a transmembrane localization.

MATERIALS AND METHODS

Cell culture, preparation of membranes, and purification of spiralin and cytoplasmic fibrils. *S. melliferum* B88 cells were grown and plasma membranes were prepared as previously described (53). Spiralin was purified by agarose suspension electrophoresis after selective extractions with detergents of membranes depleted of extrinsic proteins (51). The original purification procedure (52) was modified in the first detergent extraction which was performed with 20 mM Sarkosyl (sodium lauroyl sarcosinate) for 1 h at room temperature instead of 5% Tween 20 for 24 h at 4°C. The cytoplasmic protein fibrils were recovered in the Sarkosyl-sodium deoxycholate (DOC)-insoluble material.

For immunoadsorption experiments (see below), membrane vesicles were obtained from cells lysed by osmotic shock (50) and were washed four times by dispersion and centrifugation (40,000 × *g*, 4°C, 1 h) in 50 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl.

Growth conditions for acylation studies. BSR medium (10 to 30 ml) (3) was supplemented with (i) 2 mCi of ^{14}C-labeled fatty acid (specific activity, 50 to 60 mCi/mmol) per liter, (ii) 10 mCi of [^{3}H]glycerol (3.0 Ci/mmol) per liter, or (iii) 6 mCi of [^{3}H]cholesterol (48 Ci/mmol) (Amersham International plc) per liter from ethanolic stock solutions. After complete solubilization of the additives (1 h of stirring at 22°C), the

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labeled media were sterilized by filtration (0.2 μm). Thereafter, 7 mCi of a ^{3}H-labeled sterile amino acid mixture (Amersham TRK 440) per liter was added to most media. Before harvest, the cultures were supplemented with 20 mM EDTA, the concentration of which was maintained during cell lysis and the preparation of membranes to inhibit endogenous phospholipase activity (11).

Lipid extraction of membranes and growth medium. To quantify free fatty acids in the BSR (3) growth medium, this one was extracted by a Bligh and Dyer procedure as modified by Kates (22). Free fatty acids were then separated by thin-layer chromatography on silica gel H plates (Merck), converted to their methyl esters, and analyzed by gas-liquid chromatography as described previously (29). The major fraction of the radioactive lipid labels is incorporated into the membrane lipid fraction. The membranes were therefore extracted three times with 15 ml of chloroform-methanol (2:1, vol/vol) and centrifuged at 15,000 × *g* for 15 min at 5°C. The amount of membrane lipid extracted was estimated by liquid scintillation counting (see below).

Preparation of proteoliposomes and spiralin detergent-free aggregates. Incorporation of spiralin into liposomes made with egg yolk lecithin was achieved by detergent elimination (17). The proteoliposomes were centrifuged in a 0 to 20% (wt/vol) sucrose gradient in NEM buffer (pH 7.5), over a 0.5-ml cushion of 50% sucrose, at 180,000 × *g* for 24 h at 20°C. Fractions (0.2 ml) were collected and spiralin was detected by immunoelectrophoresis (21), and phospholipids were detected by the colorimetric method of Hallen (14).

To obtain detergent-free protein aggregates, 2 ml of NEM buffer (pH 8.0) containing 26 mM sodium DOC, 0.12 M 2-mercaptoethanol, and 1 mg of purified spiralin was dialyzed as described above, except that the protein solution did not contain lipids. The turbid preparation obtained upon extensive removal of the detergent was centrifuged at 100,000 × *g* for 30 min at 4°C and washed once with NEM buffer (pH 7.5).

Proteolytic digestion of spiralin. For dissection of spiralin micelles, crystalline TPCK (tosyl-L-phenylalanyl chloromethyl ketone)-trypsin was added to 1 ml of 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mg of spiralin detergent-free aggregates, 10 mM $CaCl_2$, and 2 mM NaN_3 to obtain a trypsin/spiralin ration of 1:20 (wt/wt). After 24 h at 37°C, the preparation was centrifuged at 180,000 × *g* at 4°C, washed twice with Tris hydrochloride buffer (pH 8.0), and analyzed by electron microscopy and polyacrylamide gel electrophoresis (PAGE) (see below).

Localization of different acyl chains on the spiralin molecule was analyzed by incomplete digestion of spiralin with endoproteinase Glu-C (Boehringer) from *Staphylococcus aureus* V8. Lipid-extracted membrane proteins labeled with either [^{14}C]-14:0 or [^{14}C]-16:0 were separated by sodium dodecyl sulfate (SDS)-PAGE as described below. The spiralin bands were cut out from fresh, wet, and weakly stained gels and immersed in gel slice buffer (125 mM Tris [pH 6.8] 0.1% [wt/vol] SDS, 1 mM EDTA, 0.3% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol) two times for 1 h each time at 22°C. The gel slices were then fitted into the wells of a new SDS gel containing 15% (wt/vol) acrylamide (pH 6.8) in the stacking gel. Wells were then filled with gel slice buffer and 5 ng of protease (see above) per well. The gel was run for 17 h at 80 V and 22°C. After subsequent staining and destaining, the gel was shrunk, immersed in enhancer, and dried (45).

PAGE. Spiralin tryptic peptides were separated by SDS-urea-PAGE (39) and stained with Coomassie brilliant blue R250 after fixation with formaldehyde (38). For the detection of acylated proteins, labeled and nonlabeled membrane proteins were separated by using a discontinuous SDS-PAGE system as described before (29). Lipid-extracted membrane protein pellets were solubilized in 100 to 300 μl of SDS-PAGE sample cocktail by boiling for 3 min. The concentration of acrylamide in the separation gel was 11.1% with 1% cross-linking. Gels were stained with Coomassie brilliant blue R250 and dried (45).

Quantification of labeled proteins. Radioactively labeled proteins were detected by autoradiography for 5 to 20 days. Occasionally, an enhancer (Amplify; Amersham) was incorporated into gels for the detection of ^{3}H-labels. The identity of spiralin was verified after electroblotting onto nitrocellulose filters and detection with anti-spiralin monospecific antibodies by previously published procedures (29). Likewise, the *S. melliferum* membrane proteins were recognized after electroblotting with anti-membrane antibodies. Quantification of individual (labeled) proteins with an attached fatty acid was performed as described previously (29).

Hydrolysis of acyl-labeled spiralin. To determine the type of association between spiralin and the bound lipid, [^{14}C]16:0-labeled spiralin was electroeluted from a fresh, wet, and weakly stained polyacrylamide gel containing lipid-extracted membrane proteins (see above). After being freeze-dried, the pure spiralin was treated with 0.1 M KOH in 90% (vol/vol) methanol or 1 M hydroxylamine (pH 9.9) followed by extraction and separation of the obtained free fatty acids and fatty acid esters by thin-layer chromatography as described by Dahl et al. (9). Quantification was provided by liquid scintillation counting.

Immunoadsorption of anti-spiralin antibodies and crossed immunoelectrophoresis. Anti-spiralin antibodies were elicited in two rabbits by subcutaneous inoculation, twice a week, of purified spiralin. Each inoculum contained 10 μg of spiralin in 0.5 ml of 0.15 M NaCl emulsified with 0.5 ml of Freund incomplete adjuvant. After 3 months of immunization, the rabbits were bled and the two serum samples were pooled. Immunoglobulins were purified by ion-exchange chromatography (15).

For topological investigations by immunoadsorption, increasing amounts of *S. melliferum* intact cells, membrane vesicles obtained by osmotic shock, proteoliposomes, spiralin micelles, or membranes solubilized with sodium DOC were added to 0.5-ml fractions of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10% sorbitol, and 10 mg of antibody. Each antigen sample contained a known amount of spiralin (see Fig. 2). After 2 h at 4°C with frequent stirring, the mixtures were centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatants containing unadsorbed antibodies were recovered and analyzed by crossed immunoelectrophoresis by the method of Laurell (25) in 1% agarose gels (Indubiose A37, IBF, France) made in Veronal buffer (pH 8.6) containing 13 mM sodium DOC. Each antigen sample contained 5 μg of pure spiralin solubilized with 0.1 M sodium DOC in the presence of 0.24 M 2-mercaptoethanol. The electrophoresis was run for 18 h at room temperature. After being washed and dried, the gels were stained with Coomassie brilliant blue R250 in acetic acid-methanol-water (1:4:5, vol/vol/vol) (21).

Charge-shift electrophoresis (19). Spiralin micelles were solubilized in the presence of 2-mercaptoethanol with (i) 1% Triton X-100, (ii) 1% Triton X-100–0.5% sodium DOC, and (iii) 1% Triton X-100–0.02% CTAB (cetyl trimethyl ammonium bromide). After overnight equilibration, the samples were electrophoresed for 70 min (current, 15 mA) at 10°C in 1% agarose gels in samples of Veronal buffer (pH 8.5) (ionic

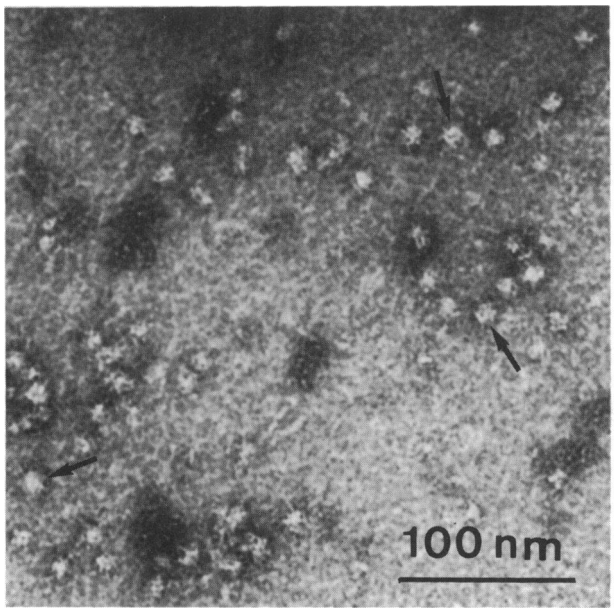

FIG. 1. Morphology of spiralin protein micelles. Spiralin from *S. melliferum* B88 membranes was purified in the presence of sodium DOC, and protein micelles were prepared by detergent elimination. The material was stained with 2% uranyl acetate (pH 4.5) for examination by electron microscopy. The arrows point out individual micelles.

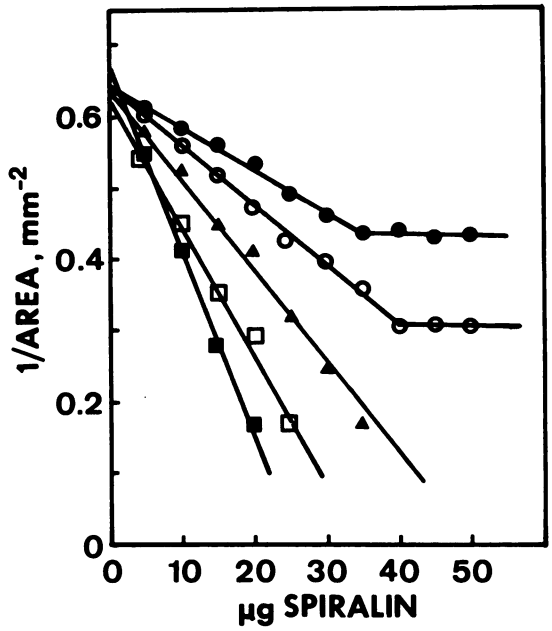

FIG. 2. Adsorption of anti-spiralin antibodies. Anti-spiralin antibodies were progressively adsorbed with intact cells (●), membrane vesicles obtained from cells lysed by osmotic shock (○), spiralin micelles (■), proteoliposomes made with spiralin and phospholipids (▲), and membranes solubilized with sodium DOC (□). Unadsorbed anti-spiralin antibodies were titrated by crossed immunoelectrophoresis as described in Materials and Methods, and the areas subtended by the immunoprecipitates were measured. Each point on the curves is a mean of two determinations.

strength, 0.03) containing (i) 0.5% Triton X-100, (ii) 0.5% Triton X-100–0.25% sodium DOC, and (iii) 0.5% Triton X-100–0.01% CTAB. The position of spiralin in the gels was determined by crossed immunoelectrophoresis as described previously (47).

Amino acid analysis. Peptide samples were hydrolyzed for 24 and 72 h at 110°C in 6 N HCl in thoroughly evacuated ampoules. The amino acid content was determined with an Alpha Plus LKB amino acid analyzer. The values for threonine and serine were extrapolated to zero time, assuming zero- and first-order kinetics, respectively. Tryptophan and methionine were not determined, since these amino acids were not detected in *S. melliferum* spiralin (53).

Electron microscopy. Spiralin micelles and aggregates of the water-insoluble domain of the protein were negatively stained with 2% aqueous uranyl acetate (pH 4.5) on collodion- and carbon-coated copper grids (300 mesh).

RESULTS

Binding of detergents by spiralin. In contrast to phycoerythrin from *Ceramium rubrum*, a water-soluble pigmented protein, the electrophoretical mobility of the *S. melliferum* spiralin was extremely dependent on the detergent combinations used in agarose gels. In the presence of Triton X-100 alone, spiralin exhibited a very slow anodic mobility equal to 3.8% of the mobility of phycoerythrin. The addition of the anionic detergent sodium DOC of Triton X-100 induced a very sharp positive shift in the mobility (+46.2%), while the addition of the cationic detergent CTAB resulted in a strong negative shift (−50%). These differences in the electrophoretical mobilities are necessarily the consequence of the binding of large amounts of detergent (18, 19). The fact that this binding occurred under nondenaturing conditions is evidence of the amphiphilic nature of the *S. melliferum* spiralin.

Incorporation of spiralin into liposomes and formation of protein micelles. The material obtained after removal by dialysis of sodium cholate from a solution containing spiralin and phospholipids gave a single band with a buoyant density of 1.06 in isopycnic ultracentrifugation in a sucrose gradient. The major part of the lipids and of the protein were localized in this band, while pure liposomes, in a control experiment, were found in the top of the gradient. Furthermore, it was not possible to release spiralin from the liposomes by increasing the ionic strength with 0.5 and 1 M NaCl. It may thus be concluded that spiralin associates with phospholipids to form true proteoliposomes by anchoring within the lipid bilayer.

When spiralin was depleted of detergent in the absence of lipids, globular particles with a mean diameter of 15 nm and very similar to the protein micelles of *Bacillus licheniformis* membrane penicillinase (37) were obtained (Fig. 1). No lipid and only traces of detergent were detected within these complexes, which could be solubilized with Triton X-100, octyl glucoside, or bile salts but not by only increasing the ionic strength. One may thus conclude that sodium DOC-solubilized spiralin molecules aggregated upon removal of the detergent to form protein micelles and that the main driving force of the phenomenon was hydrophobic interaction.

Sidedness of spiralin epitopes. To determine the position of spiralin with respect to the lipid bilayer of the *S. melliferum* membrane, an electroimmunochemical method combining the adsorption of antibodies and crossed immunoelectrophoresis (20) was used. The results were interpreted on the basis of the principles formulated by Owen (31). Figure 2 shows that it was possible to extensively adsorb anti-spiralin antibodies with spiralin micelles, with proteoliposomes con-

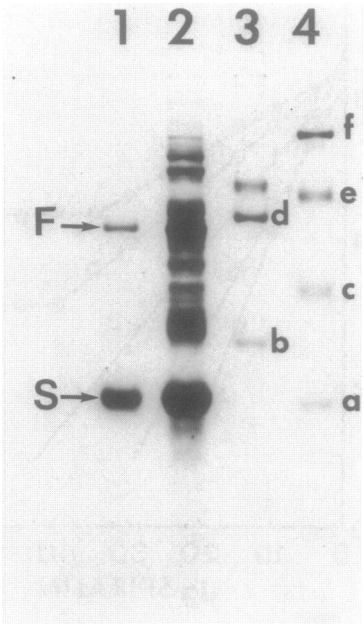

FIG. 3. Analysis by SDS-PAGE of the fibrils obtained by detergent extraction of the *S. melliferum* membrane. Samples analyzed: lane 1, fibrils; lane 2, *S. melliferum* membrane proteins; and lanes 3 and 4, molecular mass markers (a, carbonic anhydrase, 30 kDa; b, lactate dehydrogenase, 36 kDa; c, ovalbumin, 43 kDa; d, catalase, 60 kDa; e, serum albumin, 67 kDa; and f, phosphorylase *b*, 94 kDa). All the samples were treated with 0.6 M 2-mercaptoethanol. The gel was 8% T and 3.6% C. The buffer used was 40 mM Tris–20 mM CH_3COONa (pH 7.4) containing 2 mM EDTA and 1% SDS. Proteins were stained with Coomassie brilliant blue R250. S, Spiralin; F, fibril protein.

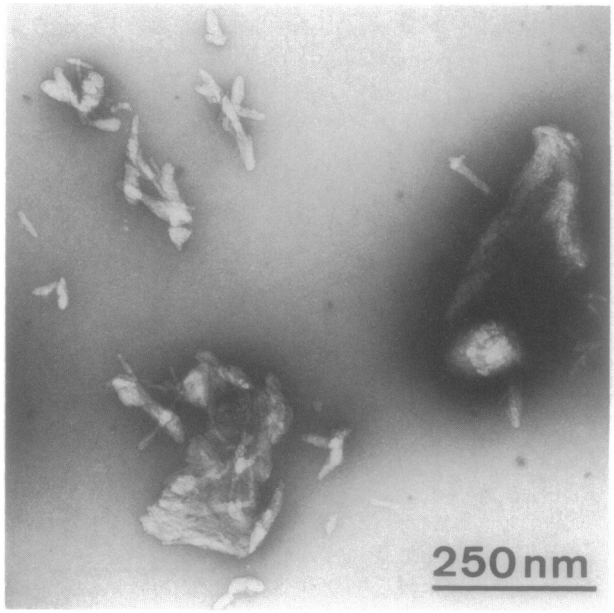

FIG. 4. Morphology of the water-insoluble material obtained by the digestion of spiralin micelles with trypsin. Spiralin micelles were digested overnight with trypsin, and the water-insoluble material was recovered by centrifugation, washed, and stained with 2% uranyl acetate (pH 4.5) for examination by electron microscopy.

taining purified spiralin, or with detergent-solubilized membranes. This indicates that in these three cases most, if not all, spiralin epitopes were accessible to antibodies. With intact cells, it was possible to adsorb only a fraction of the anti-spiralin antibody population. Indeed, since a plateau was reached, this means that at least several epitopes of spiralin were not accessible to antibodies *in situ*. A similar phenomenon was observed when the adsorption of anti-spiralin antibodies was performed with membrane vesicles obtained by osmotic lysis of spiroplasma cells. It may be concluded from this observation that the vesicles obtained by osmotic shock were essentially of the right-side-out type. However, the slope of the curve in that case was steeper than when the adsorption was performed with intact cells, suggesting that additional spiralin epitopes were accessible on the surface of the vesicles. This was probably caused by the unmasking of epitopes shielded in intact cells by exogenous proteins. Indeed, mycoplasmas are capable of adsorbing serum proteins of the culture medium during their growth (36), and extensive washing of the membranes releases most of these proteins. In addition, the accessibility of spiralin epitopes is probably greater in isolated vesicles than in intact cells because the curvature of the membrane is more pronounced in the former than in the latter.

One possible interpretation of these experiments is that spiralin is anchored in the plasma membrane because of a covalently bound acyl chain (see below), the polypeptide being exposed on exclusively the outer face of the membrane. If this interpretation is true, the observed shielding of spiralin epitopes in the membrane could be explained by interactions between spiralin molecules or between spiralin and other molecules (proteins or lipids). Another interpretation which seems more plausible is that spiralin is a transmembrane polypeptide with distinct epitopes localized on the cytoplasmic face and the outer face of the *S. melliferum* plasma membrane. Using intact cells or membrane vesicles permits the adsorption of the antibodies directed against only the epitopes localized on the outer face. Solubilization of spiralin with a nondenaturing detergent makes most, if not all, epitopes, including those exposed on the inner face of the membrane, accessible to antibodies, as for spiralin micelles. In the case of proteoliposomes, the quantitative adsorption of antibodies may be explained by a symmetrical incorporation of the protein.

Interactions of spiralin with cytoplasmic fibrils. When the *S. melliferum* membrane was extracted with 20 mM Sarkosyl and then 0.2 M sodium DOC, the detergent-insoluble material contained the cytoplasmic fibrils. In the electron microscope, after negative staining, this material proved composed of tightly entangled fibers, characterized by the shape, periodic structure, and dimensions reported earlier (43). The analysis of this fraction by SDS-PAGE (Fig. 3) showed that it contained only two polypeptides: the fibril protein (57 kilodaltons [kDa]), as expected, and spiralin (28 kDa). Variation of pH in the 4 to 10 range, treatment by different nondenaturing detergents in which spiralin is soluble (bile salts, octyl glucoside, Triton X-100, and Sarkosyl), exposure to high salt concentrations (e.g., 1 M NaCl or KCl), EDTA, or urea up to 8 M did not disrupt the protein complexes. The dissociation occurred, however, upon solubilization of the fibrils with SDS.

Proteolytic dissection of spiralin micelles. The water-insoluble fraction obtained after the digestion of spiralin micelles with trypsin was composed of large, amorphous aggregates, heterogeneous in size (Fig. 4), the shape of which was quite

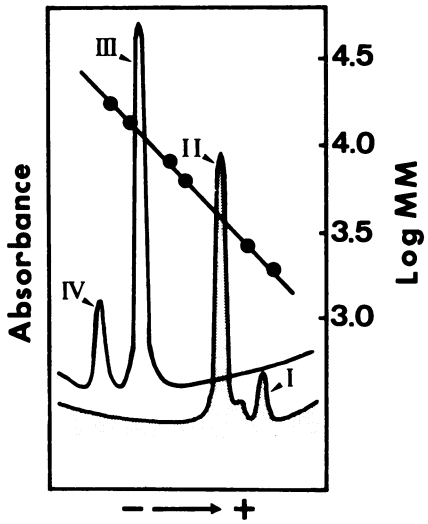

FIG. 5. Electrophoretical analysis of the peptides obtained by treatment of spiralin micelles with trypsin. The soluble and insoluble tryptic fractions were separated by centrifugation, and their peptide compositions were determined by SDS-urea-PAGE. The gels were stained with Coomassie brilliant blue R250 after fixation with formaldehyde and analyzed with a densitometer. The following markers (Pharmacia Fine Chemicals, Uppsala, Sweden) were used as reference for molecular mass estimation: myoglobin (17.2 kDa), myoglobin I and II (14.6 kDa), myoglobin I (8.2 kDa), myoglobin II (6.4 kDa), myoglobin III (2.6 kDa), and myoglobin I-14 (1.7 kDa). Shaded peaks show peptides of the water-insoluble fraction: peptide I (2 kDa) and peptide II (3.9 kDa); unshaded peaks show peptides of the soluble fraction: peptide III (13.5 kDa) and peptide IV (25 kDa).

different from that of spiralin micelles. When analyzed by SDS-urea-PAGE, this material proved to contain a major polypeptide of 3.5 kDa and a minor one of 2 kDa (Fig. 5). In the soluble tryptic fraction, a major band containing a 13.5-kDa polypeptide coming from spiralin and a minor band corresponding to trypsin were detected. In comparison to intact spiralin, the water-insoluble fraction was enriched in glycine and serine residues (Table 1). The amino acid composition of spiralin has a mean hydropathy of −0.31 (Table 2), similar to that of hydrosoluble proteins (24), outer membrane proteins (6, 30), or bacterial acylated membrane proteins (29). Surprisingly, the mean hydropathy of the water-insoluble fraction is still more hydrophilic (−0.70).

Acylation of spiralin. Of the 51 membrane polypeptides resolved by SDS-PAGE, 21, including spiralin, contained bound lipid label after extraction with chloroform-methanol and boiling in the SDS-PAGE cocktail (Fig. 6). The identity of spiralin and the other membrane proteins was verified by detection with monospecific antibodies after electroblotting (data not shown; see Materials and Methods). Lipid labels can also easily be detected in the protein bands on the blot filters. As can be seen in Fig. 6, there is a strong preference for the incorporation of myristic (14:0) or palmitic (16:0) acid in the proteins. No incorporation of glycerol or cholesterol was detected in the proteins, and no acyl proteins were found in the cytoplasmic fraction obtained after cell lysis for any of the labels used.

The amounts of fatty acids incorporated into membrane proteins and lipids are shown in Table 2. All fatty acids except stearic acid were incorporated into membrane lipids in substantial amounts. However, as indicated from the autoradiograms, only myristic and palmitic acyl chains were incorporated in the protein (Table 2). The amounts of these

TABLE 1. Amino acid composition of spiralin from the *S. melliferum* B88 membrane and of the water-insoluble tryptic fraction

Amino acid	Composition (mol %)[a]		Deviation (%)[b]
	Spiralin	Fraction	
Asx	11.37	8.22	−27.7
Thr	10.85	6.55	−39.6
Ser	5.47	23.77	+334.6
Glx	9.42	3.81	−59.6
Pro	3.10	3.13	+1
Gly	5.86	14.57	+148.6
Ala	13.99	8.63	−38.3
Cys	0	0	0
Val	11.30	4.56	−59.6
Met	0	0	0
Ile	5.32	3.17	−40.4
Leu	4.40	3.83	−13.0
Tyr	2.18	2.39	+9.6
Phe	2.36	1.92	−18.6
His	0.60	1.78	+196.7
Lys	13.12	13.10	−0.2
Arg	0.65	0.54	−16.9
Trp	0	0	0

[a] The amino acid composition of the spiralin from *S. melliferum* B88 was determined earlier (53).

[b] Deviation is the percent difference in amino acid composition between the water-insoluble fraction and intact spiralin. The symbols + and − indicate that the fraction contains more or less, respectively, of the corresponding amino acid.

two on the proteins were 1.3 and 3.7% (mol/mol), respectively, compared with their amounts in the lipid fraction. A major fraction of the protein-bound acyl chains was found on spiralin. The molar ratio of acyl chains per spiralin molecule added up to 0.7 (Table 2). Similar or slightly larger (≈1.0) ratios were observed for six membrane acyl proteins with molecular weights smaller than that of spiralin. For proteins with larger molecular weights, the ratios were smaller than that of spiralin. Interestingly, a 16-kDa protein (Fig. 6, bottom) had a modification ratio close to 2.0 (data not

TABLE 2. Fatty acid incorporation into *S. melliferum* membrane lipids and proteins

Free fatty acid in growth medium	Amt of fatty acid or acyl chain				
	Growth medium (μM)[a]	Added label (μM)[b]	In lipids (μmol/liter of culture)[c]	On proteins (μmol/liter of culture)[d]	Acyl chain/spiralin (mol/mol)[e]
Myristic acid (14:0)	1.8	37.0	8.55	0.111	0.17
Palmitic acid (16:0)	37	34.5	8.46	0.313	0.47
Stearic acid (18:0)	22	33.3	2.21	0.013	0.02
Oleic acid (18:1c)	15	35.7	5.70	0.009	0.02[f]
Linoleic acid (18:2c)	76	35.1	5.85	0.016	0.02

[a] Amounts of free fatty acids (non-labeled) in the growth medium as determined by gas-liquid chromatography (see Materials and Methods).

[b] Amounts of radioactive fatty acids added. Each label was tested in separate experiments.

[c] Total amounts of fatty acids (as lipid acyl chains and free acids) in the membrane lipid fraction after growth (27 h at 32°C), determined by liquid scintillation counting.

[d] Amounts of acyl chains bound to membrane proteins in cells grown and analyzed as described in footnote *c*. Approximately two-thirds of these are found on spiralin.

[e] Molar ratio of acyl chain per spiralin obtained from fatty acid and amino acid labels (see Materials and Methods).

[f] Occasionally, 18:1c/spiralin ratios of 0.1 were found.

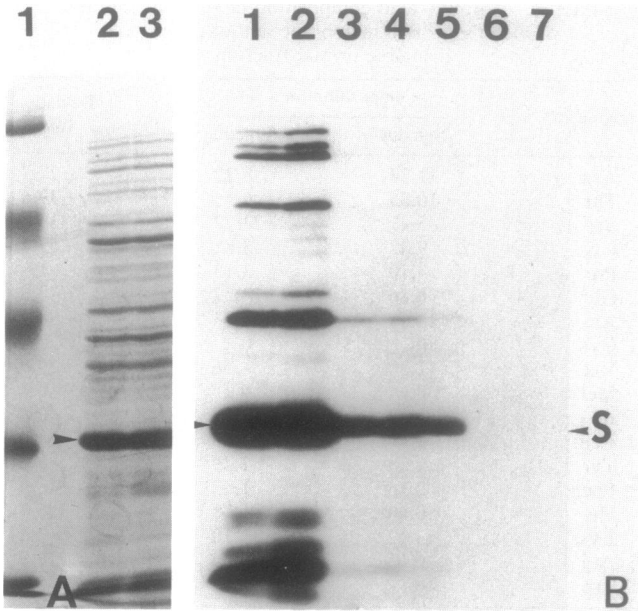

FIG. 6. Identification of acyl proteins in *S. melliferum* membranes. SDS-PAGE of membranes from cells grown with different radioactively labeled lipid precursors. (A) Coomassie brilliant blue-stained proteins; lane 1, molecular mass markers (see legend to Fig. 3); lanes 2 and 3 correspond to lanes 1 and 2 in panel B. (B) Fluororadiograms of membrane proteins labeled as follows: lane 1, [^{14}C]14:0; lane 2, [^{14}C]16:0; lane 3, [^{14}C]18:0; lane 4, [^{14}C]18:1C; lane 5, [^{14}C]18:2C; lane 6, [^{3}H]glycerol; and lane 7, [^{3}H]cholesterol (see Materials and Methods). Equal cell growth was obtained with these additives, and identical amounts of membrane protein were applied to all wells. Spiralin (S, see arrow heads) is the dominant protein in both gels.

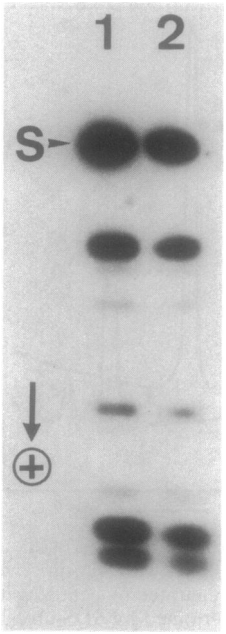

FIG. 7. SDS-PAGE of acyl chain-labeled spiralin incompletely hydrolyzed by *S. aureus* V8 protease. Autoradiogram of gel showing peptide fragments obtained after protease treatment (see Materials and Methods) of spiralin labeled with [^{14}C]16:0 (lane 1) and [^{14}C]14:0 (lane 2). Equal amounts of spiralin were used. S, Position of uncleaved spiralin, and the shortest peptide corresponds to a molecular mass of ≈8 kDa.

shown). The fibril protein of *S. melliferum* membrane was not modified with acyl chains.

In eucaryotic cells, proteins with only one acyl chain usually contain myristic or palmitic acid with different attachment mechanisms but not both. Obviously, in *S. melliferum*, individual membrane proteins can be modified with both these acyl chains (Fig. 6 and Table 2). Cleavage of spiralin, labeled with either [^{14}C]14:0 or [^{14}C]16:0, with protease V8 followed by SDS-PAGE clearly shows that these two acyl chain labels reside on the same spiralin peptide fragments down to the smallest fragments obtained (≈8 kDa) (Fig. 7). Hence, the acyl chains must be attached to the same part of the spiralin molecule.

The lack of incorporation of glycerol (Fig. 6) and the low molar ratio of bound acyl chains for spiralin (Table 2) indicate that the acyl chains are not attached as diacylglycerol units, as is common in other bacteria. In other systems, single acyl chains are usually attached to proteins by ester or amide bonds. Mild alkaline hydrolysis released the majority of [^{14}C]16:0 from purified (labeled) spiralin. With hydroxylamine at alkaline pH, only 5% of this amount was released. This strongly indicates that the majority of the acyl chains are attached to spiralin by ester bonds and not by thioester or amide bonds.

DISCUSSION

Spiralin topology and amphiphilic properties. Spiralin has been considered an integral membrane protein because it cannot be released from the *S. citri* membrane without the use of detergents (47, 52). We show here that *S. melliferum* spiralin exhibits, in charge-shift electrophoresis, sharp variations in electrophoretic mobility which indicate that the molecule contains a hydrophobic domain large enough to bind substantial amounts of detergent under nondenaturing conditions, a property specific to amphiphilic proteins (19, 40). The presence of a lipophilic domain in spiralin is confirmed by the ability to incorporate this protein into liposomes. It should be noted that the controls made by salt treatment of proteoliposomes show that spiralin is not simply adsorbed to the surface of the liposomes but actually anchored within the lipid bilayer. However, since spiralin is acylated (see below) and hydrophilic proteins may be attached to a membrane by one or several covalently bound acyl chains, embedded within the lipid bilayer (42, 54), one might argue that spiralin belongs to this particular class of membrane proteins. However, the presence of distinct epitopes on the two faces of the membrane (as determined by the immunotopological study performed by adsorption of antibodies and crossed immunoelectrophoresis) and the strong association between spiralin molecules and the cytoplasmic fibrils provide evidence for the transmembrane localization of spiralin and thus its integrated nature. Townsend and Plaskitt (44) arrived at the same conclusion by an immunoelectron microscopic approach but with some uncertainty because of the use of an anti-spiralin polyclonal serum which was not strictly monospecific.

The fact that spiralin molecules can aggregate to form protein micelles (Fig. 1) is further evidence for the integrated membrane position of spiralin. This property suggests also that the lipophilic domain of spiralin is small in comparison to the hydrophilic ones. Indeed, the ability to form protein micelles is typical of membrane proteins containing large hydrophilic domain(s) protruding outside the membrane and

a small hydrophobic anchor embedded within the lipid bilayer (37). In the case of spiralin, this hypothesis is supported by the mean hydropathy of the amino acid composition which is in the range of that of water-soluble proteins (24). *Escherichia coli* porins (6, 30) span the membrane several times and also have hydropathies expected for soluble proteins. However, the comparison with spiralin does not seem relevant because the outer membrane to which these proteins belong is quite different from plasma membranes. Our hypothesis is also supported by the fact that spiralin is highly immunogenic (52). Indeed, antigenicity and immunogenicity, being surface properties, are mainly dependent on the hydrophilicity of protein segments (1). Integral membrane proteins with large hydrophilic domains are thus expected to be more immunogenic than proteins deeply embedded within the lipid bilayer. We have actually observed that bacteriorhodopsin, a very hydrophobic membrane protein (23), was poorly immunogenic in contrast to spiralin (49).

Surprisingly, with a mean hydropathy of −0.70, the water-insoluble peptidic fraction of spiralin obtained by cleavage with trypsin has an amino acid composition still more hydrophilic than that of full-size spiralin. Therefore, the insolubility of this domain of the protein might be explained by its acylation and, possibly, unexpected conformational properties. This fraction is actually highly enriched with serine residues which are probable sites of spiralin acylation (see below).

Acylation of spiralin. The number of acylproteins in the *S. melliferum* membrane relative to the total number of membrane protein species, as deduced from Fig. 6, is substantially larger than for common bacteria (54). However, this number is very similar to the number in other mycoplasmas, i.e., *Mycoplasma capricolum*, *Acholeplasma laidlawii*, *Mycoplasma arginini*, and *Mycoplasma hyorhinis* (5, 9, 10, 29; E. Ruuth and Å. Wieslander, submitted for publication). With respect to the distribution of acylproteins, the dominance of spiralin is analogous to Braun's lipoprotein (an acylprotein) being the most numerous protein in *E. coli* (4).

Most proteins with just one acyl chain investigated so far contain either 14:0 or 16:0 (42). In *S. melliferum* (this work), *A. laidlawii*, and *M. arginini* (Å. Wieslander et al., submitted for publication), proteins can be modified with both these acyl chains. 16:0 is the major acyl chain on *S. melliferum* and *M. arginini* proteins, whereas 14:0 is preferred on *A. laidlawii* proteins under similar conditions. The two former organisms cannot synthesize fatty acids (e.g., see reference 12), whereas the latter organism has this capacity. Preferential incorporation of 16:0 into the membrane lipids from a serum-containing medium has previously been observed for *S. citri* (12, 34). On spiralin, 16:0 was enriched at least 20-fold over linoleic acid (18:2c), the most common free fatty acid in the growth medium (Table 2). However, a similar strong preference is not observed in the lipids (Table 2).

The extent of spiralin acyl chain modification, i.e., close to one acyl chain per molecule, indicates a modification mechanism different from that in common bacteria. This is supported by the lack of cysteine in spiralin from *S. melliferum* (Table 1). In common bacteria, an N-terminal cysteine in acylproteins is modified with three acyl chains and glycerol (54). It might be argued that acyl chains donated to the proteins by polar lipids of the serum fraction, known to be possible in *M. capricolum* (8), have escaped our detection since they are not labeled. However, exogenously incorporated polar lipids seem to be metabolically inert in spiroplasmas, whereas this is not the case in mycoplasmas (11, 35). The water-insoluble fraction remaining after digestion of spiralin with trypsin has several possible sites, i.e., amino acid residues containing a hydroxyl group (Table 2), where an acyl chain can be attached by an ester bond. This would explain the water-insoluble character of this part of the protein. The 21-kDa membrane-associated protein p21 of the *ras* oncogene family is water-soluble without its C-terminal ester-bound single 16:0 acyl chain but insoluble (or membrane attached) with the chain (13, 26).

An acylation mechanism different from that in other bacteria would yield an unacylated protein upon cloning of the gene in *E. coli*. However, cloned spiralin is found in, or attached to the membranes in *E. coli* (2). A major membrane acyl protein from *A. laidlawii* is not acylated by *E. coli* upon cloning (S. Nyström and Å. Wieslander, unpublished observation) but is still found in *E. coli* membranes (41). Hence, these acylproteins might not be solely dependent upon the acyl chain for a proper membrane attachment.

Hypotheses about the role of acylation. The role of membrane protein acylation is poorly understood except for the proteins anchored to membranes exclusively by an acyl chain (42, 54). In the case of spiralin, if we assume the absence of multiple acylations (i.e., more than one acyl chain per polypeptide), this means that in the *S. melliferum* membrane, each molecule of spiralin contains no more than one covalently bound acyl chain that may function as an anchor. On the other hand, if we assume multiple acylations of a fraction of spiralin, the other major fraction of spiralin molecules will stay integrated without the help of any acyl chain since, in *S. melliferum*, the whole spiralin fraction is bound to the cell membrane. Interestingly, in *E. coli*, less than 15% of the lipoprotein PBP3 (penicillin-binding protein 3) in the cytoplasmic membrane is modified with acyl chains (16). Whatever the actual situation, it is very probable that the covalent binding of hydrophobic chain(s) to spiralin strengthens the anchoring of the protein within the lipid bilayer. An attractive hypothesis would be that acylation also plays a role in the membrane integration of spiralin by a targeting mechanism. In other words, the fixation of one or more acyl chains during or just after elongation of the polypeptide chain might help the molecule to be irreversibly bound to the membrane and thus facilitate the proper integration into the lipid bilayer. In chloroplasts, for example, the 32-kDa herbicide-binding protein is transiently modified with 16:0, which probably acts as a lateral targeting signal in the thylakoid membrane (27). Because of the abundance of spiralin in the spiroplasmal membrane, the acyl chains covalently linked to this protein should afford a major contribution to the physical properties of the lipid bilayer.

ACKNOWLEDGMENTS

We thank Viola Tegman for excellent technical assistance, Annie Cavalier for help in electron microscopy, and Marie-Madeleine Gaillard for processing of the manuscript.

This work was supported by the Région Bretagne, the Langlois Foundation (Rennes), and the Swedish Natural Sciences Research Council.

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