Isolation, and Structural and Chemical Characterization of Outer Sheath Carrying a Polygonal Array from *Treponema phagedenis* Biotype Reiter

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(Accepted for publication, February 20, 1986)

**Abstract** An outer sheath was isolated from *Treponema phagedenis* biotype Reiter by our previously developed method (Masuda, K., and Kawata, T. 1982. *J. Bacteriol.* **150**: 1405-1413). The isolated outer sheath was observed as a triple-layered, closed vesicle carrying a polygonal array by electron microscopy. The outer sheath was mainly composed of protein (41.0%), phospholipid (38.7%), and carbohydrate (11.0%). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the isolated outer sheath in the presence of 2-mercaptoethanol (EtSH) gave one major protein band with an apparent molecular weight of about 69,000 and several minor protein bands. On the other hand, in the absence of EtSH, the major protein band disappeared but two new protein bands at positions of molecular weights of about 65,000 and 72,000 appeared. The SDS-PAGE profiles of the minor protein bands did not change with or without EtSH. Sodium deoxycholate (DOC)-solubilized materials from the isolated outer sheath were reassembled into thin membranous sheets carrying a roughly polygonal array upon removal of DOC by dialysis against Tris-HCl buffer in the absence of Mg$^{2+}$.

An outer sheath is the outermost membranous structure specific for spirochetal cells. The outer sheath has been isolated from several spirochetes including the genus *Treponema* (7, 13, 18), *Leptospira* (1, 20), and *Borrelia* (8). The outer sheath was shown to be mainly composed of protein, lipid, and carbohydrate (8, 13, 18, 20). However, detailed information concerning properties of these outer sheath components is extremely limited: especially protein species forming the outer sheath and their properties have not yet been studied except for our previous investigation of an oral treponeme (13).

The outer sheath of parasitic spirochetes appears to play an important role in the host-parasite interaction. In fact, it has been demonstrated that the outer sheath of *Leptospira* is important as an immunogen (1). Therefore, it may be worthwhile to characterize the spirochetal outer sheath components to clarify the host-parasite interaction.

*Treponema phagedenis* biotype Reiter is one of the treponemes for which the ultra-
structure, biology and biochemistry have been extensively studied. However, the outer sheath of this organism has been neither isolated nor characterized. This paper describes the isolation, and morphological and chemical characterization of the outer sheath from the organism. Reassembly of the isolated outer sheath is also presented.

MATERIALS AND METHODS

Organism and growth. T. phagedenis biotype Reiter was grown anaerobically at 37°C for 5 days in a modified Kawata's thioglycollate medium as described previously (12).

Isolation of the outer sheath. The outer sheath was isolated from the treponemal cells according to our previously developed method (13). Briefly, the outer sheath was isolated by disruption of the cells by 20 cycles of freezing and thawing followed by differential centrifugation. After contaminating flagella were dissociated and removed by treatment with 0.1 M sodium acetate-HCl buffer (pH 3.0), the crude preparation was subjected to centrifugation on a 30–45% linear sucrose density gradient. Then, fractions were collected from the bottom of the tubes and assayed for protein. Fractions containing major protein peaks were combined, diluted with 50 mM Tris-HCl buffer (pH 7.2) and centrifuged at 25,000 × g for 30 min to sediment the outer sheath. The resulting pellet was washed three times with the same buffer by centrifugation and used as the outer sheath preparation.

SDS-PAGE. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmlli (9) using a separation gel of 10% acrylamide. Samples were solubilized in the sample buffer by heating at 100°C for 3 min. RNA polymerase B from Bacillus stearothermophilus (Seikagaku Kogyo Co., Tokyo), which was dissociated by the action of SDS into five subunits with molecular weights of 180,000, 140,000, 100,000, 42,000, and 39,000, was used as a molecular weight marker. After electrophoresis the gel was stained for protein with Coomassie brilliant blue R-250 (5). Glycoprotein was detected with periodate-Schiff reagent (19).

Reassembly of outer sheath. The outer sheath preparation was treated with 50 mM Tris-HCl buffer (pH 8.0) containing 1% sodium deoxycholate (DOC) for 1 hr at 37°C and centrifuged at 70,000 × g for 1 hr. The resulting supernatant containing solubilized materials of the outer sheath was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) with or without 2 mM MgCl₂ as described previously (13). Reassembly was checked by examining the negatively stained preparations of the dialysates.

Chemical analyses. The outer sheath preparation was washed with distilled water by centrifugation, lyophilized, and then chemically analyzed. Protein and neutral sugar were assayed by the methods of Lowry et al (10) and Dubois et al (4), respectively. Aminosugar was determined according to the method of Rondle and Morgan (16) after hydrolysis of the outer sheath preparation in 4 N HCl for 8 hr at 100°C. Total lipid and phospholipid were estimated as described previously (13). Muramic
acid was analyzed on a Yanaco LC-5S amino acid analyzer after hydrolysis in 6 N HCl for 8 hr at 100 C.

Electron microscopy and optical diffraction. For thin sectioning, samples were fixed with 1% OsO₄ and embedded in epoxy resin, and thin sections were cut and then stained with lead citrate as described previously (13). Negative staining was performed with 2% ammonium molybdate (pH 7.0) or 4% uranyl acetate (pH 4.1). Specimens were examined with a Hitachi HU-11E electron microscope operating at 75 kV. Optical diffraction was performed as reported previously (13).

RESULTS

General Morphology of T. phagedenis

Electron microscopy of thin sections of the whole cells of T. phagedenis biotype Reiter revealed an outer sheath as the outermost membranous structure (Fig. 1).

Fig. 1. Electron micrograph of a thin section of whole cells of T. phagedenis biotype Reiter. An outer sheath (OS) is seen as the outermost triple-layered structure about 10 nm thick and surrounds the wall-membrane complex (WMC). Flagella (F) are seen in the space between the outer sheath and the wall-membrane complex in a cross section of the cells. Fluffy fibrils interconnecting between the outer sheath and wall-membrane complex are visible (arrows). Bars in all of the electron micrographs represent 100 nm.
The triple-layered outer sheath about 10 nm thick loosely covered the wall-membrane complex surrounding the protoplasmic cylinder. Flagella were seen in the space between the outer sheath and the wall-membrane complex in cross sections of the cells. Fluffy fibrils interconnecting between the outer sheath and the wall-membrane complex were also discernible as described previously (14).

Isolation and Fine Structure of the Outer Sheath

After disruption of treponemes by freezing and thawing, the outer sheath was isolated by differential centrifugation, and partially purified by acidic treatment to remove contaminating flagella. The crude preparation was further separated on linear sucrose density gradient by centrifugation, forming one major and two minor protein bands (Fig. 2). The major protein band composed the outer sheath. Thin sections of the purified outer sheath preparation revealed vesicles having the same triple-layered structure as seen in the whole cells (Fig. 3b). Negative staining showed that the outer sheath vesicles were covered roughly with a polygonal array (Fig. 3a). Although optical diffraction analysis was performed to resolve the structure of the polygonal array, definite diffraction spots could not be obtained, probably because the arrayed subunits might be fragile and disordered during preparation of the outer sheath. The minor heavy and light bands formed by sucrose density gradient centrifugation were composed of large cell debris, and small membranous and granular materials, respectively (data not shown).

Chemical Composition of the Outer Sheath Preparation

Table 1 shows the chemical composition of the outer sheath preparation. The

![Fig. 2. Isolation of the outer sheath by sucrose density gradient centrifugation. The crude preparation of the outer sheath which had been treated with acidic buffer to dissolve flagella was layered on the top of a 30–45% linear sucrose density gradient. After centrifugation at 38,000 × g for 2 hr, fractions were collected and assayed for protein.](image-url)
Fig. 3. Electron micrographs of the outer sheath preparation. (a) Negatively stained with uranyl acetate. The preparation is composed of membranous vesicles carrying a polygonal array. (b) Thin section. The preparation is composed of triple-layered, closed vesicles. The triple-layered structure about 10 nm thick is the same as that observed in the outer sheath of the whole cells.
outer sheath contained protein (41.0%), lipid (40.4%), and carbohydrate (11.0%). Phospholipid made up about 95.8% of the total lipid. The ratio of neutral sugar to aminosugar was about 2:3. When 2.5 mg of the outer sheath preparation was analyzed, no muramic acid was detected, indicating that the preparation was not contaminated with wall-membrane complex.

**SDS-PAGE of the Outer Sheath Preparation**

The outer sheath preparation was analyzed by SDS-PAGE to elucidate its protein constituents in the presence or absence of 2-mercaptoethanol (EtSH) (Fig. 4). In the presence of EtSH the outer sheath preparation gave one major protein band with an apparent molecular weight of about 69,000 (69K protein) and several very faintly stained protein bands. On the other hand, in the absence of EtSH, the major 69K protein band disappeared and new protein bands with apparent molecular

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**Table 1. Chemical composition of the outer sheath isolated from T. phagedenis biotype Reiter**

<table>
<thead>
<tr>
<th>Component</th>
<th>Dry weight (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>410.0</td>
</tr>
<tr>
<td>Total lipid</td>
<td>404.0</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>387.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>109.8</td>
</tr>
<tr>
<td>Neutral sugar</td>
<td>41.7</td>
</tr>
<tr>
<td>Aminosugar</td>
<td>68.1</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not detected.

![Fig. 4. SDS-PAGE of the outer sheath preparation. The preparation was analyzed in the presence (lane 2) or absence (lane 3) of EtSH. Lane 1 is a molecular weight marker, RNA polymerase B from *B. stearothermophilus*.](image-url)
Fig. 5. Reassembled products of the outer sheath. The DOC-solubilized outer sheath components were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) in the absence (a) or presence (b) of 2 mM MgCl₂. The dialysates were negatively stained with ammonium molybdate. (a) Irregularly shaped, membranous sheets were reassembled. Arrows show a roughly polygonal array in parts of the reassembled sheets. (b) Curved rod-like, granular and incomplete membranous materials were reaggregated.
weights of about 65,000 and 72,000 (65K and 72K proteins, respectively) appeared. In addition, a protein band possessing very high molecular weight, which could not enter the separation gel, also appeared in this case. This high molecular weight protein should be a homo- or hetero-multimer in which either or both 65K and 72K proteins might be polymerized through disulfide linkages. The profiles of faintly stained, minor protein bands seemed to be not affected by EtSH. Note of these protein species were stained with periodate-Schiff reagent, indicating the lack of detectable carbohydrate.

Reassembly of the Outer Sheath

Experiments on reassembly of the outer sheath were carried out to disclose its structural organization. When the outer sheath components solubilized with DOC were dialyzed against Tris-HCl buffer (pH 8.0), irregularly shaped, thin membranous sheets were reassembled in the absence of Mg$^{2+}$ (Fig. 5a). A roughly polygonal array was seen in parts of the membranous sheets reassembled. On the other hand, the reassembly of membranous sheets as incomplete membranous sheets, showed considerable interference due to the presence of Mg$^{2+}$, and curved rod-like and granular materials were formed (Fig. 5b). SDS-PAGE of both of the products reassembled in the absence and presence of MgCl$_2$, which had been recovered from the dialysates by sedimentation at 30,000 $\times$ g for 30 min, revealed the same profiles as those seen in the isolated outer sheath in the presence of EtSH (Fig. 6). In addition, based on the analysis in the absence of the reducing agent, the 65K and 72K proteins were also shown to be incorporated into both of the products.
DISCUSSION

The outer sheath of some species of Treponema possesses regular arrays (6, 13). Although *T. phagedenis* is one of the extensively studied treponemes, the presence of a regular array in the outer sheath has not been reported. The present study demonstrated that the outer sheath isolated from *T. phagedenis* biotype Reiter has a polygonal array. The polygonal array could not be observed in the outer sheath of negatively stained intact cells. Superimposition of the two outer sheath layers and protoplasmic cylinder would make it difficult to resolve the polygonal array. The vesicles of the isolated outer sheath carrying the polygonal array were the same triple-layered structure as seen in the intact cells and had no additional layer. Therefore, the polygonal array seems to be located in the triple-layered outer sheath in a similar manner to the hexagonal array in the outer sheath of *Treponema* strain E-21, an oral treponeme (13).

The outer sheath isolated from *T. phagedenis* biotype Reiter contained 41.0% protein, 40.4% total lipid, and 11.0% carbohydrate. The protein content of the outer sheath from biotype Reiter was lower than those (46.6–52.5%) from *Treponema* strain E-21 (13), *Borrelia hermsi* (8) and *Leptospira interrogans* serovar *pomona* (20). Conversely, the total lipid content of the outer sheath from biotype Reiter was considerably higher than those (22.5–30.7%) from the other three spirochetes.

In the present SDS-PAGE analysis of the outer sheath isolated from *T. phagedenis* biotype Reiter, the major 69K protein in the presence of EtSH appeared to be separated into two major 65K and 72K proteins in the absence of EtSH. Therefore, these 65K and 72K proteins seem to be major protein constituents of the outer sheath of biotype Reiter. We previously isolated the outer sheath from *Treponema* strain E-21 and showed that it contained one major protein with an apparent molecular weight of about 62,000 and a few minor proteins (13), but the SDS-PAGE profile was not affected by EtSH (unpublished data).

There have been a few studies on the surface proteins of spirochetal cells. Lactoperoxidase-catalyzed iodination strongly labeled seven to eight and three surface proteins of virulent *Treponema pallidum* and *Treponema pertenue*, respectively (17). The surface-exposed antigens of *T. pallidum* (11) and Lyme disease spirochete (3) were found to be proteins with apparent molecular weights of about 47,000 and 34,000, respectively, using their monoclonal antibodies. In addition, radioactive iodination of the cell surface of *B. hermsi* strongly labeled one protein with a molecular weight of about 41,000 and faintly many proteins (2). The spirochetal surface proteins were considered to be located in the outer sheath. These observations and our results suggest that the outer sheath possesses different protein compositions, depending on both the genus and the species of spirochetes.

The present study showed that the outer sheath components of *T. phagedenis* biotype Reiter, which had been solubilized with DOC, could be reassembled into membranous sheets by dialysis against Tris-HCl buffer in the absence of Mg²⁺. The outer sheath components solubilized with SDS from *T. phagedenis* biotype
Kazan 5 (7), *L. interrogans* serotype *canicola* (1), and *B. hermsi* (8) and those solubilized with DOC from *Treponema* strain E-21 (13) were reassembled into membranous vesicles on removal of the detergents by dialysis in the absence of Mg$^{2+}$. The solubilized outer sheath components from biotype Kazan 5 reaggregated into amorphous materials, but failed to reassemble into membranous vesicles on dialysis in the presence of Mg$^{2+}$ (7). We found here that the reassembly of the outer sheath components of biotype Reiter into membranous sheets showed considerable interference due to the addition of Mg$^{2+}$. These findings indicate that specific divalent cations including Mg$^{2+}$ are not necessary for the molecular organization of the outer sheath of *T. phagedenis*. On the other hand, reassembly of the outer membrane of *Escherichia coli* essentially requires Mg$^{2+}$ (15). Therefore, it can be concluded that the organization of the treponemal outer sheath differs substantially from that of the *E. coli* outer membrane as proposed previously (13).

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 59480165) from the Ministry of Education, Science and Culture of Japan.

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(Received for publication, January 17, 1986)