The Flagellar Membrane of *Ochromonas danica*

**ISOLATION AND ELECTROPHORETIC ANALYSIS OF THE FLAGELLAR MEMBRANE, AXONEMES, AND MASTIGONEMES***

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The isolation and purification of the flagellar membrane of the phytoflagellate, *Ochromonas danica*, is described. The procedure is simple, mild, rapid, and it produces a pure membrane preparation. The method additionally permits the isolation of clean preparations of axonemes and mastigonemes from a single flagella preparation. Each component was studied by electron microscopy and acrylamide gel electrophoresis. The isolated flagella preparation was nearly free of other cellular organelles as judged by phase contrast and electron microscopy. The purified membrane preparation consisted of small vesicles (300 to 1500 Å in diameter) with a trilamellar pattern about 80 Å thick. Isolated membrane was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, displaying five major protein bands, five minor protein bands, and some protein which remained at the origin. The five major protein components had apparent molecular weights of 54,000, 47,000, 35,000, 31,000, and 28,000. All mastigoneme protein components are glycoproteins as judged by periodic acid-Schiff staining. The mastigoneme preparation contained three major protein bands. Two of them were revealed as doublets and migrated with an average velocity corresponding to 83,000 daltons, the other major protein band migrated with a velocity corresponding to 54,000 daltons. A heavy carbohydrate band is seen near the bromphenol blue tracking dye. The axoneme preparation showed one major protein band having an apparent molecular weight of about 54,000 and some proteins having high molecular weights which remained on top of the polyacrylamide gel.

Biological membranes play a crucial role in almost all cellular activity. Insights are slowly evolving about their structure and about the mechanisms of membrane-associated reactions. Protozoa, being unicellular, possess the same advantages found in bacteria with respect to ease of experimental manipulation, availability of mutants, potential for batch culture under controlled conditions, and growth on chemically defined media. The cytoplasmic membrane of many protozoa devoid of cell wall is the only outer barrier of the cell. The phytoflagellate *Ochromonas danica* contains a substantial quantity of polar lipids, making it a potential attractive model system for the study of membrane structure.

Our interest in the isolation of flagellar membrane stems from three considerations. First, many continuities between different membrane systems have been reported (1). To obtain a pure cytoplasmic membrane preparation, free from contamination of intracellular membrane, has proven extremely difficult. On the other hand, flagella, which are devoid of any internal membrane systems, may be readily detached and isolated by gentle differential centrifugation. Such a procedure would permit the isolation of a very pure membrane preparation. Second, the problem of membrane structure must be considered in relation to the problems of membrane function and membrane biosynthesis. Flagella may be amputated, and the synchrony of regeneration which follows may provide us with a probe with which to approach problems of organellar development and membrane biogenesis (2). Third, the flagellar membrane has two interesting aspects. It is on the one hand, continuous with the cell membrane of all organisms in which it has been studied, and yet it is a component of a specialized organelle. Presumably the membrane of the flagella plays some functional role. The surface membrane of these species varies widely, whereas the flagella ultrastructure remains the same. It is likely, therefore, that the flagellar membrane, which serves as a cell surface membrane on the exterior, including such functions as gamete recognition and agglutination (3, 4), is also a functionary of some sort in flagellar action. In the case of *Ochromonas danica* this bimodal function makes the system especially amenable to structural studies, since the unique lipids of the membrane have characteristics which
can be exploited by physical techniques. These substances are derivatives of 1,14-docosanediol-1,14-disulfate and 1,15-tetra-

cosanediol-1,15-disulfate, with from zero to six chlorine atoms

In order to provide a basis for a detailed investigation of

attached to the flagellar membrane are extrflagellar hairs,
called the mastigonemes. The mastigonemes of *O. danica*

EXPERIMENTAL PROCEDURE

**Cultures**—Ochromonas danica was grown in the chemically defined

**Flagella Detachment and Isolation (Scheme B)**—Cultures of *O.

danica were harvested 5 days after inoculation by centrifugation for

**Fractionation of Flagella**—Flagella pellets were resuspended in

**Isolation of Axonemes**—The above pellet was thoroughly re-

**Isolation of Mastigonemes**—A centrifugation force of 113,700 x g

**Negative Staining (Electron Microscopy)**—The mastiginem pellicle

**Light Microscopy**—Phase contrast light microscopy was conducted

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—The

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**Light Microscopy**—Phase contrast light microscopy was conducted with

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—The proteins were incubated at 37° for 2 hours in 0.01 M sodium phosphate buffer, pH 7.0, 1% in sodium dodecyl sulfate and 1% in mercaptoethanol prior to electrophoresis. The protein concentration was normalized 0.5 mg/ml. Gels containing 10% acrylamide/0.27% N,N'-methylene-

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marked, and the gels were placed in tubes filled with staining solution prepared by dissolving 1.25 g of Coomassie brilliant blue in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. Staining was at room temperature for 6 hours. The gels were destained electrophoretically or simply by sitting for a long period of time in destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of water). Assuming an even swelling of the gels, mobility was calculated as the ratio of the distance of protein migration to the distance of dye migration. The mobilities were plotted against the known molecular weights of marker protein on a semilogarithmic scale. The marker proteins used were myoglobin (17,800), chymotrypsinogen A (25,000), pepsin (35,000), ovalbumin (43,000), and albumin (68,000). Gels were stained for carbohydrate using the periodic acid-Schiff procedure (18).

RESULTS

Isolated Flagella—Flagella preparations were examined under phase contrast light microscopy and electron microscopy (Fig. 1). They were nearly free of other cellular contamination. Isolated flagella are very delicate. In order to maintain the integrity of the flagellar membrane, flagella were washed with fresh culture medium. The flagella preparation represented about 1% of the dry weight of the cells.

Detachment of Flagellar Membranes from Axonemes—When flagella were suspended in distilled water or Tris/Mg/EtSH buffer, most of the flagellar membrane became released and detached from the axonemes as revealed by electron microscopy (Fig. 2). Sodium acetate buffer having the same ionic strength and pH as the medium also caused clear although less complete detachment of flagellar membrane.

Appearance of Flagellar Membrane—Most of the flagellar membrane appeared as large membrane sheets when it was originally released from flagella as shown in Fig. 2. The membrane preparation obtained following the outlined procedure consisted mostly of small vesicles (approximately 500 to 1500 Å in diameter) as shown in Fig. 3. Many vesicles showed a flattened appearance after the final step in the preparative procedure (113,700 × g for 1 hour at 4°C). More than 20% of the membrane vesicles were converted into little membrane sheets after centrifugation at 250,000 × g for more than 3 hours at 4°C. The flagellar membrane preparation appeared to be pure and showed a trilamellar structure (Fig. 3). The membrane thickness is about 80 Å. Because flagella have no internal membranes and because axonemes, mastigonemes, and extramastigonemfilaments are easily identified by their typical appearance in electron microscopy, it is safe to use this method as a criterion of flagellar membrane purity. Membrane density determined by sucrose density gradient centrifugation varies slightly from 1.11 to 1.15. Repeated electron microscopic examinations of centrifugation runs with the membrane in a variety of conditions (small vesicles, large vesicles, or sheets) shows that variation in sedimentation behavior relates to the form of the membrane in the preparation.

Electron Microscopy of Other Flagellar Components—The axoneme preparation is shown in Fig. 4. There is some contamination from intact flagella and flagellar membrane. The mastigonemefpreparation (Fig. 5) contains tubular mastigonemes (tm) and fibrous mastigonemes (f/m). The tubular mastigoneme shafts are 200 Å in diameter in confirmation of the work of Bouck (14). Tubular mastigonemes have hairs called extramastigonemfilaments. The fibrous mastigonemes were described by Bouck to be 1 to 3 μm in length. Unlike the tubular mastigonemes they have no extramastigonemfilaments. Indeed, they
Electrophoretic Analysis of the Flagellar Components—The protein composition of the various fractions was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific proteins could be assigned to certain flagella fractions. By comparing the protein bands and the areas of the appropriate regions of the densitometric scans of the gels, the relative purity of the various fractions can be determined. Five marker proteins were used to determine the molecular weight of flagellar proteins. Proteins or protein subunits with molecular weights above 120,000 and below 10,000 could not be examined in our system.

Electrophoretic Analysis of Whole Flagella and Flagellar Membrane—The whole flagella and flagellar membrane protein patterns stained with Coomassie blue and the corresponding densitometric scans are shown in Fig. 6. Some protein remained on top of each gel. Any protein not solubilized by sodium dodecyl sulfate or having a molecular weight substantially larger than 120,000 would remain at the top and was not investigated. Flagellar membrane showed five major protein subunits having molecular weights of 54,000, 47,000, 35,000, 31,000, and 28,000, and five minor protein bands, in addition to traces of other proteins.

Electrophoretic Analysis of Mastigonemes—The electrophoretic analysis in Fig. 7 shows that the mastigoneme preparation contained three major protein bands. Two of them were revealed as a doublet and migrated with an average velocity
Electrophoretic Analysis of Axonemes—The axoneme preparation showed one major protein band having a molecular weight of about 54,000, several minor protein bands, and protein which remained on top of the polyacrylamide gel (Fig. 8).

DISCUSSION

Isolation of Flagella and Their Components—Ochromonas danica was deflagellated by means of a Vortex mixer at 4°. This is the mildest deflagellation method known to us. A simple and mild isolation procedure was then employed to yield a pure flagellar membrane preparation, an axonemes preparation, and a pure mastigonemes preparation. O. danica mastigonemes had earlier been isolated after treatment with Sarkosyl (14). Chlamydomonas flagellar mastigonemes, membrane, and axonemes were isolated in Rosenbaum’s laboratory after addition of a nonionic detergent such as Triton X-100, Nonidet P-40, or Sarkosyl (19). The use of detergent to isolate the membrane may well have affected the quality of the membrane preparation. Cilia axonemes were isolated by Gibbons after digitonin treatment (20, 21). In the isolation procedure herein described, the membrane was obtained without the introduction of any detergent.

O. danica is normally cultured at pH 4.5. The organism does not grow above pH 6, and the method used by Rosenbaum and Child (2) for amputating Chlamydomonas flagella by adjusting the pH to 4.5 was not effective. Furthermore, several attempts to wash flagella in buffer appeared to alter the preparation. It was not anticipated that the resuspension of the flagella in Tris buffer at pH 7.5 would dislodge the membrane from the axoneme. It should be noted that suspension in distilled water had a similar effect, although the whole organism is highly motile when suspended in distilled water.

Flagellar Membrane—The two basic requirements of high yield and high purity of fractionation of membranes cannot be reconciled at present. For our purpose of studying membrane architecture, we took heavy losses in order to obtain high purity. Precipitate 5 (Scheme 1), containing large membrane vesicles and mastigonemes, was discarded because we could not free the large membrane vesicles from the contamination of mastigonemes. Purified O. danica flagellar membrane consists of five major proteins (subunits) (Fig. 6) having apparent molecular weights of 54,000, 47,000, 35,000, 31,000, and 28,000, and contains some protein which remained on top of sodium dodecyl sulfate-acrylamide gel. The 54,000 band is too close in its apparent molecular weight to axoneme tubulin to exclude the possibility of contamination. Such contamination could only result from a soluble tubulin form, since the difference in sedimentation of these two fractions is substantial. More likely it is a membrane protein. The protein at 47,000 daltons is in the range of molecular weights of actins.

When Chlamydomonas flagellar membranes were analyzed by electrophoresis on sodium dodecyl sulfate-urea acrylamide gels, one major protein band was observed. It had an apparent molecular weight considerably greater than 170,000 (19). The electrophoretic conditions (especially the percentage of acrylamide used in the gel) were sufficiently different in those experiments to allow a closer examination of a different molecular weight range. The presence of a large protein band at the origin of our gels is consistent with the Chlamydomonas result.
Fig. 7. Purified mastigonemes solubilized in 1% sodium dodecyl sulfate/1% mercaptoethanol/0.01 M sodium phosphate buffer (pH 7.0) and subjected to electrophoresis on sodium dodecyl sulfate/10% acrylamide gels for 4½ hours. The corresponding densitometric scans of the gels at 600 nm are shown. A (O—O), mastigonemes stained with periodic acid-Schiff procedure; B (x—x), mastigonemes stained with Coomassie blue.

Fig. 8. Whole flagella (WF, x—x) and axonemes (AX, O—O) solubilized in 1% sodium dodecyl sulfate/1% mercaptoethanol/0.01 M sodium phosphate buffer (pH 7.0) and subjected to electrophoresis on sodium dodecyl sulfate/10% acrylamide gels for 4½ hours. Both gels were stained with Coomassie blue. The corresponding densitometric scans of gels at 600 nm are also shown.

Mastigonemes—The sodium dodecyl sulfate-acrylamide gel electrophoresis analysis of the mastigonemes displayed three major protein bands. Since all mastigoneme proteins are glycoproteins, (including the 54,000 band) the molecular weight should be accepted only tentatively, because some glycoproteins do not migrate in sodium dodecyl sulfate-acrylamide gels at rates proportional to their true molecular weights (22). The band at 54,000 apparent molecular weight is probably due to intact flagella contamination, since the tubulin is not a glycoprotein.

The sensitivity of the periodic acid-Schiff stain is much lower than that of Coomassie blue. Mastigoneme glycoproteins, however, gave strong reactions with the periodic acid-Schiff stain, indicating a high carbohydrate content. The protein component was identified by both the Coomassie blue stain and Lowry assay (23).

Tubular mastigonemes have been previously isolated by Bouck (14), solubilized in urea/Tris-EDTA, and subjected to electrophoresis on urea-containing 7.5% acrylamide gels, yielding four Coomassie blue-stained bands. All these bands stained with periodic acid-Schiff as well. Because he did not use sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it is hard to compare his results with the sodium dodecyl sulfate-polyacrylamide gel pattern found in this laboratory. It is clear from the work in both laboratories (14) that there are at least four mastigoneme glycoproteins. Chlamydomonas mastigonemes contain a single glycoprotein of about 170,000 daltons (19). This organism, however, has a single type mastigoneme without extramastigoneme filaments.

In nonregenerating Ochromonas danica, presumptive mastigoneme-like structures, lacking lateral filaments, were found within the perinuclear continuum (14). However, in the regenerating cells these structures could also be localized within the cisternae of the Golgi complex and within vesicles free in the cytoplasm. There they possessed all the morphological characteristics of mature mastigoneme including extramastigoneme filaments. The flagellum itself possesses two rows of mastigonemes from the first appearance of the flagellum stub, which indicates that mastigonemes are attached concomitantly with flagella growth in O. danica (14). The extramastigoneme filaments are added to the mastigoneme shaft in the Golgi complex. It was postulated by Bouck (14) that these extramastigoneme filaments are wholly or in part carbohydrate.

Axonemes—The axonemes that were isolated using these procedures contained a small amount of intact flagella contamination. Evidence for this statement is derived from observations in the electron micrographs of the axoneme preparation, although the sodium dodecyl sulfate-acrylamide gels did not show a membrane protein pattern in the axoneme preparation probably because of the large amount of a single band. The one
major protein band was found at 54,000 daltons. These results should be compared to those of Olmsted, et al. (24), who examined the microtubules of neuroblastoma cells, brain, and Chlamydomonas. These investigators found that two tubulin proteins dominated the sodium dodecyl sulfate-acrylamide gel pattern. The proteins had apparent molecular weights of 58,000 and 56,000. The identification of a single tubulin band in O. danica may be due to two tubulins having the same molecular weight, or to the occurrence of only one tubulin in O. danica flagella. Judging from the gels, however, it is just conceivable that two tubulins might differ by 1 or 2 thousand daltons and overlap, although repeated runs never showed a double band.

The dyneins of flagella first described by Gibbons (20, 25) were found by Linck (26) on sodium dodecyl sulfate electrophoresis to have a subunit molecular weight of 500,000. We presume the dynein proteins would remain at the top of the gel in the system herein described.

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