LOCALIZATION OF Na\(^{+}\), K\(^{+}\)-ATP\(_{\text{ase}}\) AND OTHER ENZYMES IN TELEOST PSEUDOBRANCH

II. Morphological Characterization of Intact Pseudobranch, Subcellular Fractions, and Plasma Membrane Substructure

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ABSTRACT

The pseudobranch of the pinfish 
 Lagodon rhomboides 
is an unusually homogeneous and structurally simple tissue, well suited to cell fractionation studies. Its principal cell type, closely related to the chloride cells of teleost gill, is characterized by numerous mitochondria in close association with abundant tubular invaginations of the plasma membrane. Other cytoplasmic organelles are rarely encountered. In broken fresh pseudobranch cells negatively stained with ammonium molybdate, a 40 Å particulate layer was observed on the intracellular surface of the tubular plasma membrane fragments. Nuclear (N), mitochondrial-light mitochondrial (M+L), and microsomal (P) fractions, obtained by differential centrifugation, were characterized by examination of fixed, embedded pellets and unfixed preparations negatively stained with ammonium molybdate and potassium phosphotungstate. Mitochondria, in orthodox configuration and retaining their outer membranes, were observed in M+L and N. Significant amounts of tubular, sheetlike, or vesicular membrane fragments were observed in all three fractions. Many such fragments, when negatively stained, showed the 40 Å particulate surface layer characteristic of plasma membrane invaginations, and in some cases 20-Å projections could be resolved on the opposite (extracellular) surface. Since these morphological observations, together with previously presented biochemical data, suggest a plasma membrane localization of Na\(^{+}\), K\(^{+}\)-ATP\(_{\text{ase}}\), the possible association of the enzyme with membrane projections is discussed.

INTRODUCTION

A great deal of interest has centered on the localization of enzymes and other biomacromolecules in morphologically identifiable components of cells and tissues. Such localization provides links between biochemical and ultrastructural information which greatly facilitate the understanding of cellular events. For example, the detailed localization of sodium- and potassium-activated adenosine triphosphatase (Na\(^{+}\), K\(^{+}\)-ATP\(_{\text{ase}}\)) is important for the elucidation of the mechanism of monovalent cation transport by plasma membranes. This is especially true in the case of
specialized salt-secreting epithelial cells, which are structurally and functionally polarized, and whose plasma membranes would be expected to be biochemically heterogeneous.

This paper is concerned with the morphological characterization of subcellular fractions from the pseudobranch of the teleost Lagodon rhomboides, the biochemical properties of which were discussed in the preceding paper (5). It was hoped that the morphological evidence would help resolve some of the questions regarding the localization of Na⁺, K⁺-ATPase and other enzymes in pseudobranch which were left unanswered by the biochemical evidence.

Particular attention has been paid to the substructure of the plasma membranes of pseudobranch. Minute projections have been seen on the extracellular surface of the tubular invaginations of the plasma membranes of pseudobranch cells and the related chloride cells of gill in situ by Ritch and Philpott (21), and evidence obtained in the present study supports the association of Na⁺, K⁺-ATPase with these tubular extensions of the plasma membrane.

MATERIALS AND METHODS

Intact Tissue

Pinfish, Lagodon rhomboides, were obtained and maintained as described previously (5). Pseudobranchs, either nearly whole or cut into pieces a few millimeters on a side, were fixed in 3% glutaraldehyde in Millonig's phosphate buffer, pH 7.4 (15). For light microscopy, whole pseudobranchs were fixed for 3.5 h at room temperature and then for about 4 days in the cold. After a rinse with Millonig's buffer, the tissue was dehydrated overnight in a 1:1 mixture of methyl Cellosolve and absolute ethanol, followed by 12 h in n-propanol (2 ×), 12 h in n-butanol (2 ×), and 3 days (3 ×) in Ruddell's methacrylate solution A (22). Samples were then placed under pressure (1,380 dyn/cm²) for 20 h in Ruddell's methacrylate solution B (22). The tissue was then immersed in the complete Ruddell mixture at −10°C for 6 h, thawed, and polymerized. All steps were carried out according to the unpublished procedure of Dr. E. Jay Wheeler.¹

For light and electron microscopy, small pieces of tissue were fixed in the above-buffered glutaraldehyde for 90 min, rinsed with buffer, postfixed in buffered 1% OsO₄ for 90 min, rinsed with water, dehydrated with ethanol and propylene oxide, and embedded in Epon (13). Sections approximately 1 μm thick were stained with various common light microscopic stains, including periodic acid-Schiff and Harris' hematoxylin (12). Thin sections, about 500 Å thick, were stained with uranyl acetate (2%, pH 5.0) and lead citrate (20). Preparations were examined with RCA EMU-3 and Philips EM-200 electron microscopes.

Fraction Pellets

Nuclear (N), mitochondrial-light mitochondrial (M+L), and microsomal (P) fractions of pinfish pseudobranch were prepared as described in the preceding paper (5). Thin pellets of fraction material, fixed with phosphate-buffered 1.5% glutaraldehyde, were formed by filtration under pressure on Millipore filters and prepared for electron microscopy (3, 6). The entire thickness of each pellet was included in all sections.

Negative Staining

Unfixed fractions and minced whole fresh pseudobranch were stained with 3% ammonium molybdate, pH 7.0–7.1 (16, 17), or with 2 or 3% potassium phosphotungstate, pH 7.0 (18). The best results were obtained by transferring the sample with a needle to a small container of stain, placing a collodion- and carbon-coated copper specimen grid face down on the resulting surface film, picking the grid up, and draining away excess fluid with filter paper.

RESULTS

Morphology of Pseudobranch Tissue

The pinfish pseudobranch is about 1 cm in length and has the appearance of a comb, with thin gill-like filaments (teeth) extending from a base of vascular and connective tissue. The histology of pseudobranchs from other teleosts has been described (4, 10, 21), and the present observations on pinfish pseudobranch are quite similar.

Fig. 1 is a light micrograph of portions of several filaments and their connection to the vascular base. In the preparation of subcellular fractions this base was always trimmed away before homogenization. Each filament has a thin cartilaginous supporting rod and blood vessels in its core. Around this core are arranged layers of pseudobranch cells and sinusoidal blood spaces, forming leaflets or platelets homologous to the

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Figure 1  Longitudinal section of filaments of pinfish pseudobranch showing tissue homogeneity. FL, fused leaflets; UL, unfused leaflets; C, cartilage; Br, vascular and connective tissue of branchial arch. Stained with periodic acid-Schiff (PAS) and hematoxylin. Scale: 100 µm. X 200.
FIGURE 2  Longitudinal section through parts of three lamellae of a filament of pinfish pseudobranch. 
Ps, pseudobranch cell; S, sinusoidal blood space; Ep, squamous epithelial cells; Pi, pilaster cells. Stained  
with PAS and hematoxylin. Scale: 10 µm. X 700.

FIGURE 3  Section through pinfish pseudobranch leaflet. T, tubular extensions of pseudobranch cell  
plasma membrane; M, mitochondrion; Er, erythrocyte; En, endothelial cell lining blood space (extension  
of pilaster cell); Ep, squamous pavement epithelial cell. Stained with uranyl acetate and lead citrate.  
Scale: 1 µm. X 12,500.
respiratory leaflets of gill filaments. The overall appearance of the filaments is homogeneous, the major components being pseudobranch cells and red blood cells. A small number of mucus cells were observed in certain areas.

Fig. 2 shows a portion of a filament at higher magnification. The large, granular pseudobranch cells, which dominate the epithelium, are covered at the outer edges of leaflets by squamous pavement epithelial cells. The blood space is supported by pilaster cells, whose thin extensions form the endothelial lining of the blood space. This space is continuous with blood vessels in the filament core.

The fine structure of the pseudobranch cells can be seen in Figs. 3 and 4. The most prominent features of the cells are the large number of mitochondria and the tubular membrane arrays in close proximity to them. These tubules have been shown to be extensions of the plasma membrane (4, 21). Aside from nuclei, other organelles such as lysosomes, Golgi complexes, and granular endoplasmic reticulum were observed only rarely.

Abundant plasma membrane extensions and mitochondria have also been found in the cells of a number of epithelial organs which have in common the ability to transport electrolyte solutions across the cell layers against concentration and/or electrical gradients (7, 19). The cells of the pseudobranch are most like the chloride cells of the gills, buccal epithelium, and surface epithelium of teleost fish, but the organization of tubules is much more variable in the pseudobranch (Fig. 4). It is not possible to divide these pseudobranch cells into two distinct types on the basis of tubule form as did Harb and Copeland (10) in their study of flounder pseudobranch. In fact, tubule form often varies considerably within single cells.

Negative staining of minced fresh whole pseudobranch revealed two major components in the tissue, namely mitochondria and long, branch-
ing membrane tubules (Fig. 5). The latter are almost certainly identical with the tubular extensions of the plasma membrane seen in sectioned material. Lollipop-shaped projections from mitochondrial inner membranes were clearly visualized (Fig. 5 a and c), and resemble those first described by Fernández-Morán in beef heart mitochondria (8). The outer mitochondrial membrane has been reported to be free of projections, and this seems to be the case for pseudobranch mitochondria.

The tubular membranes also have a surface structure which was not seen in the fixed, sectioned preparations of pinfish pseudobranch. On the outside of the tubules (intracellular or cytoplasmic side) there appears a layer of particulate matter about 40 Å thick. Its appearance is somewhat variable, probably depending on how well the stain penetrated in different areas of the membrane; structural details are more distinct in samples negatively stained with molybdate than with phosphotungstate. As seen in Fig. 5, this layer frequently appears to consist of rows of individual projections. These projections will be discussed in more detail below. Here it is sufficient to note that this surface structure is characteristic of the plasma membrane and was not observed on other recognizable types of membranous components. In Fig. 5 c it can be seen that the plasma membrane projections (right) are readily distinguishable from the larger, distinctly lollipop-shaped mitochondrial projections (left).

Morphology of Subcellular Fractions from Pseudobranch

Fig. 6 shows sections through a pellet of N fraction material. Large cell and tissue fragments sedimented in this fraction, as did nuclei, whole blood cells, bundles of collagen fibrils, sections of the tubular membrane networks, and long segments of the plasma membranes of adjacent cells, presumably stabilized by the basal lamina between them.

A section through a pellet prepared from a M+L fraction is shown in Fig. 7. The mitochondria of the fraction appeared intact; a few swollen mitochondria were observed but even these apparently retained their outer membranes. Organelles which could be lysosomes were rarely observed in this fraction, a finding consistent with the microscopy of whole pseudobranch.

The fraction did contain a significant nonmitochondrial membranous component, in the form of tubules, sheets, and vesicles. The predominance of tortuous plasma membrane invaginations observed in preparations of whole pseudobranch makes it likely that the major source of this nonmitochondrial component of the M+L fraction was plasma membrane.

The P fraction was the most homogeneous of the fractions in appearance, as seen in Fig. 8. The pellet material consisted primarily of small membrane vesicles, a few clearly tubular fragments, small granules which were probably glycogen, and some amorphous flocculent material. Because of the very large surface area of the plasma membranes of pseudobranch cells, this was probably the major source of these membrane vesicles. Minor contributions could have come from plasma membranes of other cell types, nuclear membranes, and the comparatively rare elements of endoplasmic reticulum and Golgi membranes. The flocculent material could have been cell surface-associated polysaccharide or nucleoprotein from ruptured nuclei.

A different configuration of the P fraction membranes was observed when unfixed samples were negatively stained. As seen in Fig. 9, the membrane fragments were largely tubular. When stained with potassium phosphotungstate (Fig. 9 a), the tubular fragments were rather long. Short tubular fragments and spherical vesicles were seen in molybdate-stained samples (Fig. 9 b and c). Both tubular and spherical fragments showed the surface projections characteristic of the plasma membrane, particularly when stained with molybdate. Smooth-surfaced vesicles were also observed, which could have been fragments of nuclear membranes, mitochondrial outer membranes, etc. There were also some very small mitochondrial fragments displaying the unmistakable large knoblike projections of negatively stained cristae (Fig. 9 b).

Negative staining of M+L fraction material (Fig. 10) demonstrated the same two major components as pellet sections, namely mitochondria and nonmitochondrial membrane material. In this case it was much easier to conclude that the latter were not fragments of broken mitochondria but rather fragments of plasma membrane extensions, since they were distinctly tubular and had the characteristic projecting layer. Distinction between these and the respec-
Figure 5  Negatively stained preparation of disrupted fresh, unfractionated pseudobranch. (a) Mitochondria. × 70,500. (b) Tubular plasma membrane fragment. × 70,500. (c) Comparison of mitochondrial and plasma membrane projections. × 93,000. M, mitochondria; H, headpieces of mitochondrial projections; PI, projections on intracellular surface of plasma membrane. Stained with ammonium molybdate. Scales: 0.1 µm.
Figure 6  Sections of N fraction pellet. (a) General appearance (b, c) Plasma membrane fragments in nuclear fraction. Plasma membranes of adjacent cells, held together by the basal lamina, are shown in c. N, nucleus; Er, erythrocyte; C, collagen; M, mitochondrion. Stained with uranyl acetate and lead citrate. Scales: 1 μm. × 13,000.
**FIGURE 7** Section of M + L fraction pellet. M, mitochondria; T, tubular plasma membrane fragments. Stained with uranyl acetate and lead citrate. Scale: 1 µm × 15,000.

**FIGURE 8** Section of P fraction pellet. Dense small particles are probably glycogen. Stained with uranyl acetate and lead citrate. Scale: 1 µm × 19,000.

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Figure 9  P fraction, showing tubular plasma membrane fragments. PI, PE, projections on intracellular and extracellular surfaces of membranes fragments, respectively; PR, parallel rows of particulate material across thickness of tubule; M, mitochondrial fragments. (a) Negatively stained with potassium phosphotungstate. X 105,000. (b, c) Negatively stained with ammonium molybdate. X 185,000. Scales: 0.1 μm.
Figure 10  Tubular plasma membrane fragments in M+L fraction. PI, PE, projections on intracellular and extracellular surfaces, respectively; PR, parallel rows of particulate material; M, mitochondrial fragment. Stained with ammonium molybdate. × 105,000. Insets × 163,000. Scales: 0.1 µm.
In a number of negatively stained preparations of M+L and P fractions, the interior of membrane tubule fragments was intermittently filled with stain in such a way as to demonstrate another superficial layer on the inside (extracellular side) of the tubules. As seen in Figs. 9 e, 10, and 11, this was similar to, but smaller than, the projecting layer on the intracellular side. Fortuitous interaction between the stain and membranes permitted resolution of the intracellular layer into individual projections about 30–40 Å in diameter having a center-to-center separation of about 50–100 Å, and the extracellular layer into projections about 20 Å in diameter having a center-to-center separation of 40–50 Å. The intracellular projections generally appeared irregular and filamentous, while the extracellular ones were better defined and round. The occasional appearance of regular parallel rows of particulate material in the places where the tubule membranes were seen face-on (Figs. 9 b and 10) suggested regular geometric arrays rather than random placement, but it could not be determined whether these patterns arose from the outside or inside surfaces, or both.

Negative staining has been used to demonstrate membrane projections in fractions from a number of different tissues, but in most cases the side of the membrane on which the projections were located could not be determined since membrane vesicles could conceivably have formed with either side out. In the present case, two different types of projections were observed on long, branched tubular fragments, involution of which appears unlikely. These tubules are certain to be the invaginations of the plasmalemma seen in sections of pseudobranch cells. Thus it can be stated with certainty that the larger projections were observed on the intracellular side of the plasma membrane and the smaller ones on the extracellular side. This conclusion is consistent with the in situ observations of Ritch and Philpott (21) of 20-Å projections along the extracellular sides of plasma membrane tubules in Fundulus gill and pseudobranch.

Fig. 11 apparently demonstrates continuity between the tubular membranes and uninvaginated portions of the plasma membrane. Projections are also seen on both sides of the uninvaginated portions, but in view of the polarity of pseudobranch cells, it is still possible that there...
are regions of uninvaginated plasma membrane lacking projections.

**DISCUSSION**

*Significance of Microscopic Results for Enzyme Localization*

The pseudobranch cells of *Lagodon rhomboides*, like those of other species of teleost fish, have ultrastructural characteristics much like those of a number of other epithelial cells with known or suspected ion transport functions (7, 19). These include cells of teleost gill, elasmobranch rectal gland, mammalian kidney, certain insect organs, and salt glands of reptiles, marine birds, and certain plants. The extensive invaginations of plasma membrane which characterize such cells presumably provide the surface area and enzymatic pumps required for massive ion transport, and the abundant mitochondria near these invaginations are believed to supply the necessary energy in the form of ATP.

As shown here, pseudobranch stands out as an unusually homogeneous tissue, especially in comparison with such tissues as kidney or gill, the latter being most closely related to pseudobranch. This makes it particularly suitable for studies of enzyme localization by cell fractionation techniques. The bulk of the pseudobranch is contributed by a single cell type with a highly invaginated plasma membrane. (The presence of blood components in the tissue can be ignored in the present study, because the enzyme assays of whole pinfish blood indicated negligible activities (5).) Furthermore, this cell type is fairly simple in construction, containing few organelles besides the mitochondria and tubular plasma membrane extensions. These factors greatly facilitate interpretation of the morphological appearance of cell fractions.

Pinfish pseudobranch has another advantage for localization studies. Fragments of plasma membranes can be readily distinguished from fragments of other membranous organelles, both by their often tubular shape and, more importantly, by the characteristic tiny projections which appear on both sides of the membrane when negatively stained.

It is therefore possible to use the morphological characterization of the fractions to support and extend the conclusions made about enzyme localizations from the results of biochemical investigations (5). Although sections of fixed pellets were not analyzed morphometrically to determine the adequacy of the sample studied, the filtration method of pellet formation produces pellets which are more homogeneous than those obtained by centrifugation (3), and we have found no qualitative evidence of heterogeneity between different regions of the pellets examined in these investigations. Negatively stained samples were more difficult to evaluate, but the observations made on these preparations were entirely consistent with those made on sectioned material.

The results of our investigations have led us to several conclusions concerning enzyme localizations in pseudobranch. First, the mitochondrial localization of cytochrome c oxidase appears also to be valid for this tissue, since the relative abundances of mitochondria in the fractions are consistent with the distribution (5) of this enzyme. Second, the relatively minor contributions of mitochondria and mitochondrial fragments to N and P fractions support the suggestion (5) that monoamine oxidase of pseudobranch is not located primarily in outer mitochondrial membranes. Third, the morphological evidence strongly supports the suggestion that the Na⁺, K⁺-ATPase of pseudobranch is localized in plasma membranes. The presence of both the enzyme and large, easily identifiable fragments of plasma membrane in the N and M+L fractions is consistent with this hypothesis, particularly since a mitochondrial localization was ruled out by rate sedimentation analysis (5). The apparent predominance of plasma membrane fragments in the P fraction, which had the highest specific activity of Na⁺, K⁺-ATPase, is further evidence for such a localization.

*Variable Morphology of Plasma Membranes*

As described above, the membrane fragments of the P fraction from pseudobranch appeared as vesicles or tubules of varying size depending upon the technique used for visualization. This may indicate considerable flexibility in pseudobranch plasma membranes. Such an interpretation would be consistent with the striking variation observed *in situ*. It seems likely that the configuration of the plasma membranes in vivo may be quite variable, the exact form being determined by a number of interdependent physical and chemical parameters which change with time. Another possible explanation for this conformational
heterogeneity could be compositional heterogeneity as implied by biochemical studies (5).

Plasma Membrane Projections

The observation of projections on both sides of the plasma membrane fragments from the pseudobranch cells is one of the principal results of these investigations. It is possible that surface tension or other forces involved in negative staining affected the appearance of these projections, and it would be helpful to study pseudobranch with freeze-cleave or freeze-etch techniques for comparison. However, the fact that the projections on the extracellular side have also been visualized in teleost pseudobranch by the lanthanum fixation method of Ritch and Philpott (21) significantly improves the chances that they are real. Similarly, the observations in situ of intracellular projecting layers in several other tissues whose cells have a structure similar to that of pseudobranch cells (1, 9, 24) support the reality of the intracellular projections on pseudobranch plasma membranes. These other tissues, namely fish gill (24), blowfly rectal papilla (9), and cecropia midgut epithelium (1), are all thought to have electrolyte transport functions. This raises the possibility that such projections have a role in transport, whether they consist of glycoprotein, enzyme assemblies, or other membrane constituents.

Although there is no direct evidence for or against the possibility that either or both kinds of projections on the pseudobranch plasma membranes are associated with the ion pump, presumably Na⁺, K⁺-ATPase, the plausibility of such a proposition can be considered. Na⁺, K⁺-ATPase has been solubilized from a number of membrane preparations (see, e.g., 14, 26), that is to say, obtained in membrane fragments too small to be sedimented by the centrifugal procedures which usually define a soluble fraction. Some fragments of this type have been studied with the electron microscope and found to be particles about the same size as the projections (23). These results are consistent with the localization of Na⁺, K⁺-ATPase in a membrane projection. Of course, such fragments could also come from within the membrane.

Molecular weight determinations of Na⁺, K⁺-ATPase have been attempted by various means on membrane fractions, both solubilized and unsolubilized. A number of recent estimates range from 200,000 to 300,000 daltons (2, 11). If the Na⁺, K⁺-ATPase is a single spherical unit, Kepner and Macey (11) calculated that this molecular weight would correspond to a particle with 85 Å diameter. These results would seem to preclude either the 20 Å or 40 Å projections on pseudobranch plasma membranes from comprising the Na⁺, K⁺-ATPase in its entirety, but certainly either or both types of projections could be parts of a larger nonspherical Na⁺, K⁺-ATPase complex.

From the point of view of theoretical models for ion transport by an enzyme complex, it would be possible for either or both kinds of projections to be part of the ion pump. Although many models have assumed that the ion pump extends across the whole thickness of the membrane, a model proposed by Skou (25) specifically envisions a pump at one side of the membrane, in the protein layer on the cytoplasmic side; he pointed out that the pump could also consist of two different proteins on opposite sides of the membrane.

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