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Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach

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Abstract *Lucina pectinata* is a large tropical clam living deeply burrowed in the black, reducing mud of mangrove swamps. It is known to possess hemoglobin in the cyttoplasmic areas of its bacteriocytes, which harbor sulfide-oxidizing bacteria. The bacteriocytes also possess lysosome-like microbodies containing either membrane whorls or electron-dense granules in which free heme compounds have been identified. The cytochemical detection of acid phosphatase and arylsulphatase through EDX (energy-dispersive X-ray) microanalysis strongly suggests that the bacteriocytes of *L. pectinata* contain, in fact, two different types of microbodies. Some of these (devoid of dense granules) possess a variable amount of lysosomal enzymes and occasionally a limited quantity of iron, which may result from a recycling process of hemoglobin. Their main function seems to be the digestion of a limited proportion of symbiotic bacteria. They represent genuine secondary lysosomes with a functionally acidic pH. The second type of microbodies is characterized by dense granules containing sulfur and iron hemes but no lysosomal enzymes. Their sulfide-oxidizing activity was substantiated by benzyl viologen assay, with Na₂S as a substrate. These microbodies appear to be similar to the sulfide-oxidizing bodies (SOBs) described in the bacteriocytes of other bivalve species with symbiotic thioautotrophic bacteria; however, their sulfide-oxidizing activity appears to be non-enzymatic. They are discrete organelles, characterized by a functionally basic pH and pseudoperoxidasic activity, and have been termed SOBs. Therefore, the bacteriocytes of *L. pectinata* possess at the same time functional lysosomes and functional SOBs.

Introduction

*Lucina pectinata* is a large lucinid clam living deeply burrowed in the black, reducing mud of mangrove swamps, ranging from the Caribbean area (Warmke and Abbott 1962) to Brazil (Rios 1985). Its red gills were noted by Read (1962), who demonstrated that this color is due to a large supply of hemoglobin. As in other lucinid clams, *L. pectinata* harbors endosymbiotic sulfide-oxidizing bacteria, but, of the various species of Lucinidae studied, *L. pectinata* contains one of the highest concentrations of hemoglobin in gill tissue (Kraus 1995). However, this hemoglobin is not located in the yellow-brownish, dense granular inclusions as was concluded by Read (1962), but in patchy, dark cytoplasmic areas (Frenkiel et al. 1996).

Ultrastructural studies revealed that, in *L. pectinata*, bacteriocytes contain lysosome-like inclusions as large as the nucleus in their basal part. These inclusions appear to be crowded with membrane whorls and/or electron-dense granules in which heme compounds have been identified by using the diaminobenzidin (DAB) reaction; however, the inclusions do not contain hemoglobin but free hemes (Frenkiel et al. 1996). Non-protein-bound hemes present in the electron-dense granules of *L. pectinata* microbodies may, therefore, correspond to hematin, an active free heme compound (defined as an oxidized heme compound without associated protein) that appears to catalyse the oxidation of hydrogen sulfide in specific brown granules of *Urechis caupo* coelomocytes and *Calyplogena magnifica* gill cells (Powell and Arp 1989). Similar specific organelles, initially identified in the gill cells of the gutless clam *Solemya reidi*, in which a sulfide oxidase enzyme system can rapidly oxidize sulfide entering the cells into non-toxic forms of sulfur, were named sulfide-oxidizing bodies (SOBs) (Powell and Somero 1985). The initial metabolism of sulfide, in the symbiont-containing gills of this species, appears to occur not in the bacterial symbionts, but rather in the SOBs, which therefore represent a key structure of the bacterioocyte.
In addition to the internal sites of sulfide detoxification, a “peripheral defense” strategy against sulfide has been described in the superficial cell layers of symbiont-free tissues, such as the foot tissue of *S. reidi* (Powell and Somero 1985). However, this detoxification activity has not been attributed to a specific cell type.

On the other hand, cytoenzymological studies have demonstrated that active lysosomal resorption of the symbionts occurs in chemoheterotrophic symbioses, and may be a strategy for organic molecule transfer (Fiala-Médioni et al. 1989; Streams et al. 1997). Lysosomal digestion of symbionts could also be considered as a way to control the abundance of symbionts by elimination of senescent bacteria (Fiala-Médioni et al. 1989).

Previous identification of distinct lysosome-like microbodies, with or without electron-dense granules, in the bacteriocytes of *L. pectinata* (Frenkel et al. 1996) has raised new questions. Thus, the present study was designed to characterize the large microbodies observed in the bacteriocytes of *L. pectinata* and to determine whether they are either secondary lysosomes, which may be involved in symbiont resorption or hemoglobin breakdown, or functional sulfide-oxidizing bodies, probably involved in sulfide detoxification.

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**Materials and methods**

Adult specimens of *Lucina pectinata* (30–60 mm shell length) were collected by hand in the black mud of mangrove swamps in Guadeloupe (2 m average depth of water).

Histology and histochemistry

Cryosectioning of unfixed gill and foot tissues frozen at –30 °C in Tissue-Tek II O.C.T. compound in a cryostat was used for sulfide-oxidizing activity detection and related controls. Paraaffin histology was used to compare information on mucosubstances with results obtained by cytoenzymological techniques. Fixation and staining methods were all described in a previous paper on *L. pectinata* gill structure (Frenkel et al. 1996). Alcian blue staining, generally performed on paraaffin sections, was adapted to cryosections in order to compare the exact location of mucocytes with the location of sulfide-oxidizing activities. Good results were obtained by fixation of the cryosections with 5% formalin in seawater and staining with Alcian blue at pH 3 and 0.5 for 2 h.

TEM preparation

Small pieces, dissected from various locations in the gills of freshly collected individuals, were fixed for TEM, for 2 h at 8 °C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 7.2 and to 1000 mOsM with NaCl and 2 mM CaCl₂; they were post-fixed for 1 h in 1% osmium tetroxide in the same buffer at the same temperature, and dehydrated through ascending ethanol and propylene oxide. After embedding in epoxy (Epon-Araldite) or in acrylic (LR White) resins, sections were cut on an Ultratome E ultramicrotome. Semi-thin sections (0.5 µm thick) were stained with 0.5% Toluidine blue in 1% borax; thin sections (60 nm thick) mounted on 100-mesh copper grids with pyroxulin film were contrasted with aqueous uranyl acetate and lead citrate before being observed in a Hitachi TEM H-8000 at an acceleration voltage of 100 kV.

For EDX (energy-dispersive X-ray) analysis, fixtures were performed in a mixture of 1% paraformaldehyde and 2% glutaraldehyde in the same buffer, but pieces were dehydrated and embedded in Epon-Araldite without post-fixation in osmium tetroxide and without use of propylene oxide. The contrast of those un-omissiated sections in TEM was improved by addition of picric acid (0.2%) to the fixative. The thickness of sections was tested, and 200 nm was selected to obtain the best signal. The sections were collected on 200-mesh copper grids, without supporting film, and observed, without additional treatment, within 2 days.

Purification of small vesicle fraction and microbody fraction

To obtain the small vesicle fraction, small pieces of delaminated gills, washed in sterile seawater, were crushed before centrifugation at 4000 g for 5 min. The supernatant was centrifuged at 8000 g for 5 min, and the new pellet was suspended and washed twice in sterile distilled water under the same conditions in order to remove salt from the seawater. The last pellet was suspended in 30 µl of sterile distilled water. Five microliters of this enriched fraction of refringent small vesicles, measuring approximately 1 µm, were loaded on copper grids coated with pyroxulin film and air dried before EDX microanalysis in the STEM mode, without delay.

To obtain the fraction containing microbodies, crushed gill tissue suspended in a 50 mM Tris-HCl buffer at pH 8.0 was centrifuged at 4000 g for 5 min to eliminate the cytoplasmic hemoglobin as well as the small vesicles in the supernatant. The pellet was resuspended in the same buffer and filtered on a 40 µm mesh nylon tissue to eliminate coarse debris. The filtrate was centrifuged at 300 g for 3 min. The last pellet, which contained 2–10 µm large, yellow or brown corpuscles, was resuspended in distilled water and loaded on copper grids as described for the small vesicles.

EDX microanalysis

Sections prepared from blocks especially fixed for microanalysis as well as isolated small vesicles and microbodies were analyzed in the STEM mode of the Hitachi H-8000, using a pentafet detector (Oxford Instrument), monitored by a Link Isis system, in the point mode. EDX results obtained from these sections were compared with those obtained from classical glutaraldehyde- and osmium-fixed tissues and from those submitted to cytoenzymological detection of hydrolases. The Temquant software of the Isis system was used for comparison between different structures in the same section, or between similar structures in different individuals. All these quantified comparisons are valid only if the thickness of sections and conditions of acquisition are similar. They were designed only to obtain relative abundance for the various fixation methods used and not to provide true quantification. As a positive control, sections of rat blood cells, obtained under the same conditions of inclusion and thickness, were used. A negative control was constituted by the various cells, devoid of any iron, present in the same sections as the bacteriocytes.

Cytochemical characterization of lysosomal activities

Two frequent hydrolase enzymes, acid phosphatase and arylsulphatase, which have already been detected in various mollusk tissues (Owen 1972; Pipe and Moore 1985) were chosen as lysosomal markers. Acid phosphatase activity was detected by the β-glycerophosphate and lead nitrate method of Barka and Anderson (1962, modified by Pott-Boumendil 1990) in Tris-maleate buffer adjusted to pH 5.2, modified for marine mollusks by addition of NaCl in the incubation mixtures. Arylsulphatase activity was detected by the method of Hopsu-Havu et al. (1967, cited in Lewis and Knight 1992), with P-nitroanilide and barium salt in acetate buffer adjusted to pH 5, also adapted to the osmolarity of marine mollusks. For both procedures, incubation at 37 °C was main-