Role of the posterior vacuole in Spraguea lophii (Microsporidia) spore hatching

Ann M. Findley¹, Earl H. Weidner², Kevin R. Carman², Zhimin Xu³ and J. Samuel Godbar³

¹Department of Biology, University of Louisiana at Monroe, Monroe, Louisiana 71209, USA; ²Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803, USA; ³Department of Food Sciences & Human Ecology, Louisiana State University, Baton Rouge, Louisiana 70803, USA

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Abstract. Microsporidia constitute a large group of obligate intracellular protozoan parasites that inject themselves into host cells via the extrusion apparatus of the infective spore stage. Although the injection process is poorly understood, its energy source is thought to reside in the posterior vacuole that swells significantly during spore firing. Here we report the presence and localisation of the key peroxisomal enzymes catalase and acyl-CoA oxidase (ACOX) within the posterior vacuole of Spraguea lophii (Doflein, 1898) spores. Western blot analyses show that these enzymes discharge out of the spore and end up in the medium external to the extruded sporoplasm. The presence of a catalase enzyme system in the Microsporidia was first made evident by the detection of significant levels of molecular oxygen in the medium containing discharging spores in the presence of hydrogen peroxide. Catalase was visualised in inactive, activated, and discharged spores using alkaline diamino-benzidine (DAB) on glutaraldehyde-fixed cells. The position of these enzymes within the extrusion apparatus before and during spore discharge support the Lom and Vávra model that postulates discharge occurs by an eversion process. In addition to these enzymes, spores of S. lophii contain another characteristic peroxisomal component, the very long chain fatty acid (VLCFA) nervonic acid. A sizeable decrease in nervonic acid levels occurs during and after spore discharge. These data indicate that nervonic acid is discharged from the spore into the external medium during firing along with the catalase and ACOX enzymes.

Peroxisomes are found in virtually all mitochondria-bearing eukaryotes that have long-term associations with oxygen, hydrogen peroxide detoxification, and oxidation of very long chain fatty acids (Mullen and Trelease 1996). We report a peroxisomal-like organelle in a primitively prescribed amitochondriate microsporidian, Spraguea lophii (Doflein, 1898). Although best known as intracellular parasites of fish and arthropods, microsporidians are eukaryotes that have achieved a wide distribution in nature (Canning and Lom 1986). Microsporidians are noteworthy because of their invasive spore stage that functionally resembles a missile cell that is uniquely adapted for injecting its infective contents into a target host cell. The firing mechanism includes a coiled protein filament surrounded by membrane pleats and a posterior vacuole (Weidner et al. 1995). During spore activation, the posterior vacuole swells significantly followed by an explosive evertive discharge of the tube through a spore aperture with micosecond velocity. It is through the invasion tube that the spore contents (sporoplasm) are ejected into a newly-formed membranous sac at the tube tip. After discharge, the everted contents of the extrusion apparatus end up exterior to the sporoplasm cells. We report major molecular markers for peroxisomes within the microsporidian spore extrusion apparatus: very long chain fatty acids (nervonic acid), acyl-CoA oxidase (ACOX), catalase, and the most interactive of the peroxisomal proteins (peroxin) Pex19.

MATERIALS AND METHODS

Source of S. lophii spores from Lophius americanus. Microsporidian cysts containing S. lophii spores were recovered from the infected neurons of the anglerfish, Lophius americanus (Valenciennes, 1837). All of the infected animals used in this study were provided by the Marine Biological Laboratory Aquatics Facilities at Woods Hole, MA. The cysts were dissociated with a glass homogenizer and the spores were purified through repetitive centrifugation/wash cycles. Purified spores were stored in distilled water (with a trace of calcium) at 4°C. Spores were prepared for activation and hatching as described previously (Weidner et al. 1995). It should be noted that the classification scheme for the group of microsporidians that infect the anglerfish is currently under revision and that the species designation of the organism used in this study may soon be changed (Freeman et al. 2004).

Oxygen generation as a measure of catalase. Oxygen saturation levels were monitored continuously in the medium bearing discharging S. lophii spores (in Hepes buffer, pH 7) and were measured with a Clarkstyle microelectrode (Model

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In the control treatment, no H2O2 was added to the medium conditions eliminate any resident peroxidase activity (Angersson apparatus during spore germination, spores were activated and fixed quickly before they fired. This procedure enabled the capturing of images in which the vacuole continues to swell up to the point of spore firing. Standard electron microscopy revealed no detectable oxygen and spore hatching was greatly reduced. The enzyme ACOX is always found in association with peroxisomes (Van Veldhoven et al. 1991, Wanders et al. 2001). Colorimetric determination of ACOX levels in hatching spores of S. lophii revealed significant amounts of enzymatic activity present with the amount of activity dependent upon spore sample concentration, i.e., a tenfold increase in ACOX activity was observed with a tenfold increase in spore sample material (Fig. 1b, M1 vs. M2). Nervonic acid (C24:1) was the only detectable very long chain fatty acid (VLCFA) discharged along with the catalase and ACOX enzymes into the medium external to the discharged sporoplasm cells. To determine whether there was β-oxidation of nervonic acid in the medium, aliquots were examined 5, 30 and 60 min after spore discharge. The results showed a marked decrease in the nervonic acid profile of the medium during the first thirty minutes of incubation (Fig. 1c). The data presented in Fig. 1 provide a representative series of several experiments performed for each parameter measured (O2 generation, ACOX levels, and nervonic acid concentrations). Statistical treatment of these data was deemed impracticable due to large variations in percent spore firing between each experiment and the difficulty associated with counting spores that clump together upon discharge.

Western blot analyses were used to identify microsporidian ACOX, catalase, and Pex19. Antibodies directed against ACOX were tested on mouse liver peroxisomes and discharged S. lophii sporoplasm cells. To determine whether there was β-oxidation of nervonic acid in the medium, aliquots were examined 5, 30 and 60 min after spore discharge. The results showed a marked decrease in the nervonic acid profile of the medium during the first thirty minutes of incubation (Fig. 1c). The data presented in Fig. 1 provide a representative series of several experiments performed for each parameter measured (O2 generation, ACOX levels, and nervonic acid concentrations). Statistical treatment of these data was deemed impracticable due to large variations in percent spore firing between each experiment and the difficulty associated with counting spores that clump together upon discharge.

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RESULTS

Catalase is the centrepiece enzyme of the eukaryotic peroxisome (Purdue and Lazarow 1996, 2001). Its presence in S. lophii was first indicated by the production of significant levels of molecular oxygen in medium containing discharging spores (Fig. 1a). The oxygen surge resulted when hydrogen peroxide was added either slightly before or during spore discharge. The amount of oxygen generated was proportional to the level of spore firing and the amount of H2O2 substrate added to the medium containing primed spore material. In the absence of hydrogen peroxide, activated spores yielded no detectable oxygen and spore hatching was greatly reduced. The enzyme ACOX is always found in association with peroxisomes (Van Veldhoven et al. 1991, Wanders et al. 2001). Colorimetric determination of ACOX levels in hatching spores of S. lophii revealed significant amounts of enzymatic activity present with the amount of activity dependent upon spore sample concentration, i.e., a tenfold increase in ACOX activity was observed with a tenfold increase in spore sample material (Fig. 1b, M1 vs. M2). Nervonic acid (C24:1) was the only detectable very long chain fatty acid (VLCFA) discharged along with the catalase and ACOX enzymes into the medium external to the discharged sporoplasm cells. To determine whether there was β-oxidation of nervonic acid in the medium, aliquots were examined 5, 30 and 60 min after spore discharge. The results showed a marked decrease in the nervonic acid profile of the medium during the first thirty minutes of incubation (Fig. 1c). The data presented in Fig. 1 provide a representative series of several experiments performed for each parameter measured (O2 generation, ACOX levels, and nervonic acid concentrations). Statistical treatment of these data was deemed impracticable due to large variations in percent spore firing between each experiment and the difficulty associated with counting spores that clump together upon discharge.

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Fig. 1. Peroxisomal activity in *Spraguea lophii*. a – Oxygen generation with addition of 0.01% H$_2$O$_2$ to medium containing $10^6$ hatching spores (closed circles); without H$_2$O$_2$ substrate (open circles), no O$_2$ detection was recorded. b – Colorimetric measurement of ACOX. S0 was a standard recorded without ACOX in medium containing hatching spores; S1 and S2 are external standards with 10 and 20 units of ACOX present in medium. M1 and M2 are experimental treatments with the only ACOX present coming from the spore material. M2 represents a 10× increase in spore sample. Note that the ACOX colorimetric measurement indicates a concomitant 10× increase in enzyme activity with increased spore concentration. c – Nervonic acid (C24:1) is a very long chain fatty acid found in the medium with discharged *S. lophii* spores. In a time course experiment following spore germination, the results show a sharp decline (between 0 and 30 min) in nervonic acid indicating β-oxidation of this fatty acid.

Fig. 2. Western blots of peroxisome enzymes. a – ACOX. Lane a1 shows a 72-kDa ACOX band in the supernatant of discharging spore. Lane a2 shows that the *Spraguea lophii* sporoplasm pellet is free of ACOX. Lane a3 contains the ACOX band from mouse liver peroxisomes for comparison. b – Catalase. Lane b1 shows 72-kDa CATA and 60-kDa CATB bands from *S. lophii*. Lanes b2 and b3 show known catalase bands from *Neurospora crassa* (72 kDa and 60 kDa) and mouse liver peroxisomes (60 kDa), respectively. c – Peroxin 19. Antibody is directed against peroxin (Pex19). In c1, mouse liver peroxisomal Pex19 gives a single 40-kDa band. C2 demonstrates a clear band against Pex19 from *S. lophii*, however, the peptide is believed to be between 50–55 kDa.

Catalase was selectively visualised with diaminobenzidine (Angermuller and Fahimi 1981, Yokota et al. 1987). In the presence of catalase, DAB reacts with H$_2$O$_2$, to form a precipitate that was confined to the microsporidian spore posterior vacuole (Figs. 3b, c). As the invasion tube emerges from the firing spore, the DAB reaction is associated first with the inner surface and then subsequently along the outer surface of the discharged tube (Weidner and Findley 2002, 2003). After spore discharge, no detectable DAB activity was found in either the spore ghosts or the extruded sporoplasms. Control spores that were primed for hatching but for which the hydrogen peroxide substrate was not provided were negative for the DAB reaction product; however, the posterior vacuole was clearly increased in size when compared to unactivated spores (Fig. 3a).

Before activation, *S. lophii* spores have an extrusion apparatus that bears a posterior vacuole that is much reduced in size. This vacuole is osmiophilic and does
Fig. 3. Catalase visualised by diaminobenzidine. a – Spraguea lophii spores do not react to DAB in the absence of H₂O₂ in the medium, pH 10.5. b, c – The reaction of spores with DAB in the presence of H₂O₂. Spore cells were prefixed with glutaraldehyde to eliminate any resident peroxidase activity. Arrows show that all catalase activity is confined to the extrusion apparatus posterior vacuole. Scale bars = 0.5 µm.

react with the lipid probe, Nile Red (not shown). During spore activation, the posterior vacuole grows significantly and rapidly until it occupies over half the spore volume (Figs. 4a, b). Ultimately, the spore aperture collapses and the extrusion apparatus everts and the sporoplasm is sent out of the spore with millisecond velocity (Fig. 4c). As the sporoplasm passes out of the spore, the spore compartment collapses and the completely discharged spore appears flattened.

Based upon the identification and localisation of key enzyme systems (catalase, ACOX) and metabolites (O₂, VLCFA) before, during, and following spore discharge, we propose a composite model for spore germination (as illustrated in Fig. 5). The microsporidian spore extrusion apparatus consists of the spore aperture, polar filament protein coil, polaroplast membrane system, and posterior swelling vacuole. Previous studies have shown that when the extrusion apparatus discharges, the polar filament coil (shown in blue) emerges through the spore aperture instantaneously along with the polaroplast membrane (shown in red) and that these form the invasion tube by an eversion process (Lom and Vávra 1963, Weidner et al. 1995). In the present model of spore hatching, the extrusion apparatus membrane is exteriorized and becomes the outer envelope of the discharged sporoplasm cell. As the extrusion apparatus completely everts, the contents of the posterior vacuole (catalase, ACOX, VLCFA; represented by black stars) are discharged into the external medium. The sporoplasm (nucleus and cytoplasm; labeled c and represented in yellow) enters into the compartment formed from the everted polaroplast membrane. The original plasma membrane is left behind on the spore wall.

DISCUSSION

Despite the importance of the Microsporidia as the etiological agents of disease in a wide variety of invertebrate and vertebrate hosts, the specifics of the spore hatching process are, as of yet, poorly understood. Although the sudden swelling of the posterior vacuole has been implicated in playing a pivotal role in the force generation necessary for spore germination (Lom and Vávra 1963, Weidner et al. 1995), the details of the mechanism by which this might occur have remained undetermined. Interest in the study of posterior vacuole dynamics during spore discharge stems from the demonstrated efficacy of small amounts of H₂O₂ in in vitro hatching protocols of spore smears and the observation of O₂ generated concomitant with this H₂O₂ addition (Weidner and Findley 2002, 2003). Oxygen evolution was shown to be proportional to spore firing efficiency and the amount of H₂O₂ added to spores primed for hatching. In as much as H₂O₂ serves as the substrate for the antioxidant enzyme catalase, we sought to demonstrate the presence of this enzyme and to localise its position within the microsporidian spore prior to and during spore germination. The DAB reaction product indicates the presence of catalase is initially confined within the posterior vacuole, subsequently moves to the extruding polar tube during the hatching process, appears on the outside of the discharged tube, and finally...
Fig. 4. Electron micrographs of activated *Spraguea lophii* spores. **a, b** – A significant increase is seen in the volume of the posterior vacuole following spore activation. **c** – Additional increase in vacuole size results as the extrusion apparatus is discharged. It is presumably within the extrusion apparatus where the catalase, nervonic acid, and other components of the peroxisome are located. The volumetric shift within the vacuole is likely due to β-oxidation within the activated spore peroxisome and accompanying water production. Scale bars = 0.5 µm.

Although the specifics of the mechanism by which catalase works in the posterior vacuole remain unclear, it may be associated with the β-oxidation of very long chain fatty acids. VLCFA have been reported in significant amounts within *Spraguea lophii* spores (El Alaqui et al. 2001). Nervonic acid is a characteristic peroxisomal component (Sandhir et al. 1998), and the data indicate that it is discharged from the spore upon hatching. The decrease in nervonic acid levels observed following spore hatching and the presence of the catalase and ACOX enzymes in the external medium surrounding discharged spores are consistent with β-oxidation activity associated with the posterior vacuole contents and its probable identification as a peroxisomal-like organelle. Acyl-CoA oxidase is an essential enzyme of lipid catabolism and its action produces hydrogen peroxide. The co-location of catalase and ACOX within the same cytoplasmic compartment, namely the posterior vacuole, provides an efficient means for the conversion of the H$_2$O$_2$ produced during β-oxidation of VLCFA to H$_2$O and O$_2$. Consequently, the oxidation of long chain fatty acids and its resultant production of significant amounts of water and molecular oxygen may lead to the observed rapid swelling of the posterior vacuole. This volumetric increase in the posterior vacuole ultimately causes the spore aperture to be breached and begins the evertive discharge of the spore.

In the amitochondriate Microsporidia, the peroxisome becomes the only logical candidate for VLCFA assembly and β-oxidation. The data presented are consistent with the identification of the posterior vacuole of the microsporidian spore as a presumptive (primitive or extremely specialised) peroxisomal organelle. Western blots of external media samples collected from hatching spores have been probed with antiserum raised against mammalian peroxisomal matrix proteins and have successfully identified a number of peptides with peroxisomal-leading sequences (Fransen and Weidner, unpublished data).

Peroxisomes are extremely adaptable organelles that exhibit considerable functional plasticity performing disparate roles in different organisms (Titorenko and Rachubinski 2001, 2004). Although best known for their role in lipid catabolism and H$_2$O$_2$ detoxification, recent evidence also suggests a prominent role for this organelle in the intracellular signaling processes operative during development. In this context, peroxisomes may function as an organizing and coordination centre providing critical information that affects differentiation and morphogenesis events within cells (Titorenko and Rachubinski 2004). Derived from the endoplasmic reticulum of cells, the peroxisome has been shown to be structurally diverse as well. For example, in yeast the overexpression of certain Pex genes that code for the peroxin proteins needed for peroxisome assembly can result in a small number of greatly enlarged peroxi-
Fig. 5. Model of microsporidian spore discharge. Probable sequence of firing events during the eversion of the polar filament which represents a modification of the Lom and Vávra (1963) model. C indicates the cytoplasmic contents of the posterior vacuole that are discharged into the surrounding medium. Blue – polar filament protein; red – membrane of extrusion apparatus; yellow – spore cell cytoplasm; *** – contents of posterior vacuole, the peroxisomal proteins, that are expelled during spore hatching.

The body of data presented herein point to the posterior vacuole of the microsporidian spore as a peroxisome-like organelle. Biochemical, cytochemical, and western blot analyses have identified and localised several key peroxisomal components, namely catalase, ACOX, Pex19, and nervonic acid oxidation, as being associated with the posterior vacuole of *S. lophii* spores. Following spore hatching, all of these components, and O₂ generation, are found in association with the external medium that bathes the sporoplasm cells. These results support the Lom and Vávra (1963) model of spore discharge. In this model, the posterior vacuole swells upon spore activation, the extrusion apparatus everts, and a membranous sac forms at the tip of the discharged tube to accommodate the nascent spore cell, or sporoplasm. Additionally, this model implies that the membrane of the extruded sporoplasm may be derived from the extrusion apparatus and may therefore be reversed. Indeed, preliminary studies with cytoplasmic protein probes indicate that in discharged sporoplasts, the membrane orientation is reversed as proposed (De Giorgis and Weidner, unpublished data). Comparative studies of catalase activity and posterior vacuole dynamics in other microsporidian species are currently underway to adjudicate whether the spore firing mechanism proposed here is in widespread use throughout the Microsporidia. Additionally, whether catalase activity and posterior vacuole dynamics are solely responsible for spore firing in *S. lophii* has yet to be established. The role of increased osmotic potential within germinating spores as a result of trehalose conversion to glucose has also been implicated as a possible spore firing mechanism in aquatic microsporidian species (Undeen and Vander Meer 1999). Further studies are warranted to discern the relative importance of these mechanisms in the hatching process.

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REFERENCES


