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The total and mitochondrial lipidome of *Artemia franciscana* encysted embryos

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ABSTRACT

Encysted embryos (cysts) of the crustacean *Artemia franciscana* exhibit enormous tolerance to adverse conditions encompassing high doses of radiation, years of anoxia, desiccation and extreme salinity. So far, several mechanisms have been proposed to contribute to this extremophilia, however, none were sought in the lipid profile of the cysts. Here in, we used high resolution shotgun lipidomics suited for detailed quantitation and analysis of lipids in uncharacterized biological membranes and samples and assembled the total, mitochondrial and mitoplastic lipidome of *Artemia franciscana* cysts. Overall, we identified and quantitated 1098 lipid species dispersed among 22 different classes and subclasses. Regarding the mitochondrial lipidome, most lipid classes exhibited little differences from those reported in other animals, however, *Artemia* mitochondria harboured much less phosphatidylethanolamine, plasmenylethanolamines and ceramides than mitochondria of other species, some of which by two orders of magnitude. Alternatively, *Artemia* mitochondria exhibited much higher levels of phosphatidylglycerols and phosphatidylserines. The identification and quantitation of the total and mitochondrial lipidome of the cysts may help in the elucidation of actionable extremophilia-affording proteins, such as the 'late embryogenesis abundant' proteins, which are known to interact with lipid membranes.

1. Introduction

Encysted embryos (cysts) exiting females of the species *Artemia franciscana* may enter diapause [46], an extremophilic state during which metabolism is brought into a halt, accompanied by extreme augmentation of stress tolerance [23,51,55,28]. Diapause has been documented in insects [25], rotifers [14], tardigrades [27], crustaceans [45,13], killifish [57] and mammals [61,8]. During this state, *Artemia franciscana* cysts tolerate high doses of UV and ionizing radiation, years of continuous anoxia while hydrated at physiological temperature, thermal extremes, desiccation-hydration cycles, and very high salinity [16,53,15,62]. So far, the extremophilia of these cysts has been attributed to i) elaborate 'metabolic restructuring' [33], ii) high content of the non-reducing disaccharide trehalose [85,19,86] iii) a very large guanine nucleotide pool [80], iv) two heat shock proteins, p26 and artemin [81,10,17,78], and v) expression of 'late embryogenesis abundant' (LEA) proteins [79,34,60]. Other mechanisms that have been reported in *C. elegans* larvae involving polyamine utilization, glyoxalase-dependent detoxification, lipid desaturation, and reactive oxygen species detoxification pathways may also be implicated in desiccation tolerance [24]. Furthermore, it has been discovered that mitochondria obtained from the cysts of *Artemia franciscana* lack the so-called 'permeability transition' [58,50], a non-selective high-conductance channel which leads to cell death by allowing the flux of water and other molecules up to 1500 Da across the inner mitochondrial membrane [3]. In addition, these mitochondria are refractory to bongkrekic acid (BKA), a dual inhibitor of the permeability transition and the adenine nucleotide translocase (ANT) [50]. However, if the *Artemia franciscana* ANT is heterologously expressed in yeasts, there it regains BKA sensitivity [83], a finding that has prompted the postulation that the lipid environment of the ANT may be crucial for the BKA response.

Abbreviations: AC, acylcarnitine; CE, cholesteryl esters; Cer, ceramide; CL, cardiolipin; CoQ, coenzyme Q; DAG, diacylglycerol; PA, phosphatidic acid; LPA, lysophosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LEA, 'late embryogenesis abundant'; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; LPI, lysophosphatidylinositol; PS, phosphatidylserine; LPS, lysophosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol.

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While trehalose is critical to desiccation tolerance in the cysts [19,20,18,64] and it appears to work synergistically with p26 [77,18] and LEA proteins [34], in experiments using liposomes the additive protection by LEA proteins plus trehalose was reported to be dependent on the lipid composition of the target membrane [60]. Accordingly, molecular modeling of the secondary structures of the cytosol-targeted AfrLEA2 and mitochondrially-targeted AfrLEA3m revealed bands of charged amino acids known to interact directly with lipid membranes [60]. Along this line, it has been shown that LEA proteins preferentially stabilize membranes of a particular lipid composition based on the protein's subcellular location [74,75,35].

The lipid compositions of mammalian membranes are well-defined [76,38]. Among different cells and tissues the mitochondrial lipid composition is fairly similar [38], with the exceptions of those isolated from some organs which additionally contain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) plasmalogens [59,1]. Furthermore, the molecular species of cardiolipin, a polyglycerophospholipid found exclusively in the inner mitochondrial membrane [36] exhibit considerable diversity between tissues and among disease states [42,12,32,31,43,11]. On the other hand, lipids from *Artemia franciscana* cysts have been scarcely investigated. So far, it is known that *Artemia franciscana* cysts are unique because they harbour complex fucosyl and neutral glycosphingolipids, not found in other animal species [49,48], and also sphingomyelin (SM) [47], which has been found in species belonging to other invertebrate phyla but not Echinodermata and Lophotrochozoa [47]. Furthermore, it is known that the lipid content of *Artemia* varies considerably during enrichment and starvation periods, implying a dynamic character [63]; this dynamism in lipid profile is also supported by an intricate regiospecific distribution of fatty acids in triacylglycerols of *Artemia franciscana* nauplii enriched with fatty acid ethyl esters [2] or microalgae [9].

Mindful of i) the scarcity of information regarding the lipid profile of *Artemia franciscana* cysts, ii) the potential importance of lipid composition in affording extremophilia and the documented synergism of LEA proteins in doing so as a function of the lipid environment, we investigated the lipidome of the cysts. By using a MS/MS^{ALL} high resolution shotgun lipidomics workflow which is ideally suited for detailed quantitation and analysis of lipids in uncharacterized biological membranes and samples [30] we assembled the total and mitochondrial lipid profile of *Artemia franciscana* cysts. Comparisons of their lipidomes to those obtained from mammalian tissues revealed stark quantitative differences which may help to explain the extremophilia of the cysts, especially in relation to the functions of LEA proteins.

2. Materials and methods

2.1. Hydration and dechorionation of Artemia franciscana cysts

No permits were required for the described study, which complied with all relevant regulations. Dehydrated, encysted gastrulae of *Artemia franciscana* were obtained from Salt Lake, Utah through Artemia International LLC (Fairview, Texas 75069, USA) and stored at 4 °C until used. Embryos (15 g) were hydrated in 0.25 M NaCl at room temperature for 16–18 h during constant aeration. After this developmental incubation, the embryos were dechorionated in modified antiformin solution (1% hypochlorite from bleach, 60 mM $NaCO_3$, and 0.4 M NaOH) for 30 min, followed by a rinse in 1% Na^+-thiosulfate (5 min) and multiple washings in ice-cold 0.25 M NaCl as previously described [52]. For further lipidomic analysis, dechorionated embryos were pelleted by centrifugation for 5 min at 300*g* at 4 °C, snap-frozen with liquid nitrogen and stored in −20 °C, until use.

2.2. Isolation of mitochondria and mitoplasts from Artemia franciscana

Mitochondria from embryos of *Artemia franciscana* were prepared as described elsewhere, with minor modifications [67]. Dechorionated embryos were filtered through filter paper, and ~10 g were homogenized in ice-cold isolation buffer consisting of 0.5 M sucrose, 150 mM KCl, 1 mM EGTA, and 20 mM K^+-HEPES, pH 7.5, using a glass-Teflon homogenizer at 850 rpm for ten passages. The homogenate was centrifuged for 10 min at 3000*g* at 4 °C, the upper fatty layer of the supernatant was aspirated and the remaining supernatant was centrifuged at 11,300*g* for 10 min. The resulting pellet was gently resuspended in the same buffer, avoiding the green core. The green core was discarded, and the resuspended pellet was centrifuged again at 11,300*g* for 10 min. The pellet was resuspended in 0.3 mL of ice-cold isolation buffer consisting of 15% Percoll, 0.5 M sucrose, 150 mM KCl, 1 mM EGTA, and 20 mM K^+-HEPES, pH 7.5 and layered on a preformed Percoll gradient (40 and 23%). After centrifugation at 30,000*g* for 6 min, the fraction between the 15% and 23% Percoll gradient interface and the supernatant above the 15% Percoll layer were discarded, and the mitochondrial fraction located at the interface between the 23% and 40% Percoll layer was removed, diluted with isolation buffer, and centrifuged at 16,600*g* for 10 min. The resulting loose pellet was resuspended in isolation buffer and centrifuged at 6700*g* for 10 min. In pilot experiments where the resulting pellet was resuspended in a 15% Percoll and underwent a second round of a Percoll-gradient centrifugation, no more fractions between the 15% and 23% layers nor above the 15% layer formed, implying that no further purification could be achieved by this methodology. For further mitoplast isolation, the resulting pellet was resuspended in 40 mL of 10 mM K^+-HEPES pH 7.5, and kept under constant stirring at 4 °C for 30 min. Subsequently, this fraction was centrifuged at 6700*g* for 10 min, the supernatant was discarded, and the pellet underwent one more round of centrifugation at 6700*g* for 10 min. The resulting pellet was snap-frozen with liquid nitrogen and stored in −20 °C, until use.

2.3. Western blot analysis

Artemia cysts (dechorionated) homogenates, Percoll-purified mitochondria and mitoplasts were solubilised in 10% sodium dodecyl sulphate, the insoluble pellets were discarded, and the supernatants were frozen at −20 °C for further analysis. These samples were thawed on ice, their protein concentration was determined using the bicinchoninic acid assay as detailed in Section 2.4, loaded at a concentration of 20 μg per well on the gels and separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a methanol-activated polyvinylidene difluoride membrane. Immunoblotting was performed as recommended by the manufacturers of the antibodies. Rabbit polyclonal anti-alpha 1 subunit of Na^+/K^+ ATPase, mouse monoclonal anti-COX IV subunit, and rabbit monoclonal anti-VDAC1 (Abcam, Cambridge, UK), primary antibody were used at titers of 1:1000. Immunoreactivity was detected using the appropriate peroxidase-linked secondary antibody (1:5000, donkey anti-rabbit or donkey anti-mouse, Jackson Immunochemicals Europe Ltd, Cambridgeshire, UK) and enhanced chemiluminescence detection reagent (ECL system; Amersham Biosciences GE Healthcare Europe GmbH, Vienna, Austria). Densitometric analysis of the bands was performed in Fiji [69].

2.4. Protein determination

Protein concentration was determined using the bicinchoninic acid assay, and calibrated using bovine serum standards [73] using a Tecan Infinite® 200 PRO series plate reader (Tecan Deutschland GmbH, Crailsheim, Germany).

2.5. Transmission electron microscopy (TEM)

Mitochondrial and mitoplasts fractions were pelleted by centrifugation and fixed overnight in 4% glutaraldehyde and 175 mM Na^+-cacodylate buffer, pH 7.5, at 4 °C. Subsequently, pellets were