Investigation of the Ca^{2+}-inducible mitochondrial permeability transition pore and Bongkrekate sensitivity in distantly related animal species

Ph.D Thesis

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Introduction

The mitochondrion is a major player in regulating and shaping cellular Ca^{2+} levels. Mitochondrial Ca^{2+} uptake is driven by the high electrical potential across the inner mitochondrial membrane and is carried out by the calcium uniporter. Sequestered Ca^{2+} forms precipitates allowing matrix $[Ca^{2+}]_{total}$ to reach values as high as 1 M. Ca^{2+} release is also a membrane potential driven event that involves two antiporters: the mitochondrial Na^{+}/Ca^{2+} exchanger and the Ca^{2+}/H^{+} exchanger, both of which reside in the inner mitochondrial membrane.

Ca^{2+} uptake capacity can be limited by the regulated permeabilization of the inner mitochondrial membrane, on which most processes of a mitochondrion relies. The entity responsible for the phenomenon was termed the mitochondrial permeability transition pore (PTP). During permeability transition, as the mitochondrial matrix is hyperosmotic compared to the cytosol, water content of the matrix increases, which is accompanied by swelling. The inner mitochondrial membrane being folded in mitochondria to form cristae has a high surface, and can withstand the expansion of the matrix, while the outer mitochondrial membrane ruptures. This allows proteins residing in the intermembrane space to be released to the cytosol, of which some are pro-apoptotic factors, e.g. cytochrome c, Smac/Diablo, Endonuclease G, or AIF. When a large fraction of mitochondria simultaneously opens the PTP in response to stress, the resulting ATP depletion makes it impossible to coordinate the apoptotic machinery, leading to necrosis induction.

A large body of evidence highlights the relevance of permeability transition in a variety of untreatable conditions. The involvement of PTP in neurodegenerative diseases such as Alzheimer's, Parkinson's Huntington's disease and amyotropic lateral sclerosis is widely accepted. Furthermore, permeability transition is an important event in the pathology of ischaemia/reperfusion injury. It is becoming evident from transgenic mouse models in which the induction permeability transition is inhibited, that pharmacological intervention targeting the PTP would be of great potential in the mentioned diseases.

A number of chemicals and changes in different mitochondrial parameters are known to affect the probability of PTP opening, most of which were documented when the phenomenon was first described. The tremendous efforts put into the identification of the structure of the PTP are yet to yield protein candidates that are indispensible components of the PTP.

Mitochondria from various species have been described to exhibit pore forming properties. At the beginning of our investigation the only animal species described to lack the phenomenon of Ca^{2+} inducible PTP was the brine shrimp, *Artemia franciscana*.

Artemia are extremophiles inhabiting harsh environments, with remarkable resilience to different kinds of physical and chemical stress. The absence of the PTP in these animals was originally investigated to provide an explanation for this resilience. Based on the fact that distinctly related eukaryotes such as fungi plants and all other animals that had been examined by the time exhibited the PTP, the original authors assumed this was a unique trait of *Artemia* embryos.

Objectives

The primary objective of the ongoing study described in the thesis is the identification of the proteins comprising the PTP. This goal is highly ambitious and is well beyond the scope of a single PhD project. The work I participated in aims at laying down the fundamentals to achieve this goal.

Initially we were focused on the thorough biochemical characterization of the brine shrimp, *Artemia franciscana*, in order to find hints about the molecular basis of their unique trait. Based on our findings in *Artemia* we hypothesized that the unique isoform of adenine nucleotide translocase expressed in *Artemia* could be connected to the absence of PT in this species.

To elaborate, we sought for a model animal exhibiting PTP and phylogenetically close to *Artemia*, and analyzed species that belong to the crustacean subphylum. Surprisingly, our results (which have been published: [1, 2]) suggest that crustaceans generally lack the specific machinery for orchestrating the Ca^{2+}-induced permeability transition pore. We became interested

if the loss of the PTP was a unique trait of crustaceans, and at what point in their evolution did it happen.

Because the analysis of crustaceans instead of identifying good models for comparison, yielded further examples that lack the PTP, we began studying species belonging to a number of different phyla in which the presence of the PTP was unknown. Our goal at this point was not only to find proper models for comparison, but to possibly identify other phyla lacking the PTP.

We found that the adenine nucleotide translocatorin mitochondria isolated from the embryos of *Artemia* was refractory to one of its known inhibitors: Bongkrekik acid. A parallel goal to investigating the PTP was to find the BKA binding site of the ANT which is a potential target for PTP inhibition.

We believe that there is a chance to identify key proteins of the PTP, by data from state of the art techniques of proteomics and genome sequencing subjected to bioinformatics methods. The aim of the project now is identify the structural components of the permeability transition pore by gene mapping and phylogenetic analysis of organisms that do not exhibit the Ca^{2+} inducible permeability transition versus those that do.

Methods

Mitochondrial isolation

Artemia franciscana

Dehydrated, encysted gastrulae of *A. franciscana* were stored at 4 °C until use. Embryos (15 g) were hydrated in 0.25 M NaCl at room temperature for at least 24 h. Subsequently they were dechorionatedin modified antiformin solution for 30 min, and this was followed by a rinse in 1% sodum thiosulfate (5 min) and multiple washings in ice-cold 0.25 M NaCl. ~ 10 g of embryos were homogenized in ice-cold isolation buffer consisting of 0.5 M sucrose, 150 mM KCl, 1 mM EGTA, 0.5% (w/v) fatty acid-free BSA, and 20 mM K^+-Hepes(pH 7.5) with a glass–Teflon homogenizer. The homogenate was centrifuged for 10 min at 300 g and 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 gentlyresuspended in the same buffer. The resuspendedpellet was centrifuged again at 11 300 g for 10 min. The final pellet was resuspended in 0.4 ml of ice-cold isolation buffer consisting of 0.5 M sucrose, 150 mMKCl, 0.025 mM EGTA, 0.5% (w/v) fatty acid-free BSA, and 20 mM K^+-Hepes(pH 7.5), and contained ~ 80 mg protein·ml−1 (wet weight). Experiments were performed at 27 °C.

Vertebrates (*Xenopuslaevis*, mouse and rat)

Mitochondria from the livers of *Xenopus* were isolated in a similar manner as for rat and C57Bl/6N mouse liver mitochondria, as described in. All animal procedures were performed according to the local animal care and use committee (EgyetemiAllatkiserletiBizottsag) guidelines. The incubation media used for experimentation contained 8 mM KC, 110 mM K-gluconate, 10 mMNaCl, 10mM Hepes, 10 mM KH_2PO_4(where indicated), 0.005 mM EGTA, 10 mMmannitol, 0.5 mM $MgCl_2$(or 1, where indicated), glutamate 1, succinate 5 (substrates where indicated), 0.5 mg/ml bovine serum albumin (fatty acid-free), pH 7.25. Experiments were performed at 30 °C for *Xenopus* and 37 °C for the rodents.

Drosophila melanogaster* and *Caenorhabditis elegans

Wild type *D. melanogaster* were supplied by Viktor András Billes from the Department of Genetics, Eötvös Loránd University. 500-600 animals were pooled together and mitochondria were isolated in a similar manner as for the mammals, with minor modifications.

C. elegans was provided by CsabaSőti from the Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University.

Experimental buffer for these species was identical with what was used for the vertebrate species. Experiments were performed at 28 °C.

Freshwater crustaceans (*Cyclops vicinusvicinus* and *Daphnia pulex*)

Daphnia and *Cyclops* can be harvested at local ponds. The plankton samples of both *C. vicinusvicinus* and *D. pulex* harvested were 90-95% pure. Isolation was done in a similar manner as for the mammals, but with modified isolation buffers. The plankton was homogenized in ice-cold buffer containing, in mM: mannitol 225, sucrose 125, Hepes 5, EGTA 1, and 1 mg ml−1 bovine serum albumin (BSA, fatty acid-free), with the pH adjusted to 7.4 using Trizma. The

homogenates were passed through one layer of muslin and centrifuged at 1,250 g for 10 min; the pellets were discarded, and the supernatants were centrifuged at 10,000 g for 10 min; this step was repeated once. At the end of the second centrifugation the pellets were suspended in 0.15 ml of the same buffer with 0.1 mM EGTA. The experimental medium contained 225 mM mannitol, 125 mM sucrose, 5mM Hepes, 0.1 mM EGTA, 10 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM glutamate, 5 mM malate, 5 mM succinate, 0.5 mg/ml bovine serum albumin (fatty acid-free), pH = 7.25. Experiments were performed at 27 °C.

Marine species

The marine species used in this study (*Crangon crangon, Palaemon serratus, Carcinus maenas, Pagurus bernhardus, Asterias rubens, Paracentrotus lividus, Nephtys hombergii, Mytilus edule, Cerastoderma edule, Patella vulgata, Branchiostoma lanceolatum*) were obtained from Service d'Expédition de Modèles Biologiques - CNRS/FR2424 - Station Biologique de Roscoff, France. Animals were kept in aquariums filled with artificial sea water and algae at 6–8°C on 12 hours light/dark illumination cycles, until use. No ethical permissions are required for handling invertebrates for our research purposes. The harvesting of tissue for mitochondrial preparation differed for the species: For *C. crangon* and *P. serratus* 10-15 animals were pooled for each preparation. The cephalothorax of each animal was removed and then the abdominal muscle was de-shelled. For *C. maenas* the hepatopancreas of 4-5 animals was pooled. For *P. bernhardus* the cephalothorax of each animal was removed and then the abdomen was used. For *A. rubens* and *P. lividus* the gastrointestinal tracts of 3-4 animals were pooled. For *N. hombergii and B. lanceolatum* 10-15 of animals were used. For *M. edule, C. edule* and *P. vulgata* 9-12 animals were de-shelled and pooled. The tissue harvested was chopped and homogenized in ice-cold buffer and processed in the same manner as *C. vicinusvicinus* and *D. pulex*. Experimental buffer used for the investigation of these species was identical to that described for freshwater crustaceans. Experiments were performed at 27 °C.

ΔΨm determination

ΔΨm was estimated by fluorescence quenching of the cationic dye Safranin O owing to its accumulation inside energized mitochondria. Mitochondria (amounts can be found in the figure legends) were added to 2 ml of the incubation medium (described for the specific species under

mitochondrial isolation) plus 5 μM Safranin O. Fluorescence was recorded in a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK) at a 2-Hz acquisition rate, with 495- and 585-nm excitation and emission wavelengths, respectively. Pilot experiments with various substrates showed that the combination of glutamate, malate and succinate (all at 5 mM) yielded the most reproducible and most negative $\Delta\Psi m$ values.

Extramitochondrial [Ca^{2+}] determination by Ca-Gr 5N fluorescence

Mitochondria (amounts can be found in the figure legends) were added to 2 ml of incubation medium (described for the specific species under mitochondrial isolation) plus 1 μM CaGr-5N. Fluorescence was recorded in a Hitachi F-4500 spectrofluorimeter at a 2-Hz acquisition rate, with 506- and 530-nm excitation and emission wavelengths, respectively.

Mitochondrial swelling

Swelling of isolated mitochondria was assessed by measuring light scatter at 660 nm in a Hitachi F-4500 fluorescence spectrophotometer. Mitochondria (amounts described in the figure legends) were added to 2 ml of experimental medium. At the end of each experiment, the nonselective pore-forming peptide alamethicin was added as a calibration standard to cause maximal swelling.

Determination of ADP–ATP exchange rate

The ADP–ATP exchange rate was estimated with the method recently described by our team, exploiting the differential affinity of ADP and ATP for Mg^{2+}. The rate of ATP appearing in the medium following addition of ADP to energized mitochondria (or vice versa in the case of de-energized mitochondria) is calculated from the measured rate of change in $[Mg^{2+}]_f$ with the use of standard binding equations. Mitochondria (amounts described in the figure legends) were added to 2 ml of an incubation medium plus 2 μM Magnesium Green pentapotassium salt and 50 μM A_p5A. Fluorescence was recorded in a Hitachi F-4500 spectrofluorimeter at a 2-Hz acquisition rate, with 506- and 530-nm excitation and emission wavelengths, respectively. For the calculation of [ATP] or [ADP] from free [Mg^{2+}], the constants for K_{ADP}, and K_{ATP} were estimated for the respective buffer and temperature conditions.

Transmission electron microscopy (TEM)

Isolated mitochondria were pelleted by centrifugation (10 000 g for 10 min) and fixed overnight in 4% gluteraldehyde and 175 mM sodium cacodylate buffer (pH 7.5) at 4 °C. Subsequently, pellets were postfixed with 1% osmium tetroxide for 100 min, dehydrated with alcohol and propylene oxide, and embedded in Durcupan. Series of ultrathin sections (76 nm) were prepared with an ultramicrotome, mounted on single-slot copper grids, contrasted with 6% uranyl acetate (20 min) and lead citrate (5 min), and observed with a JEOL 1200 EMX (Peabody, MA, USA) electron microscope. The volume fraction of intramitochondrial Ca^{2+}–P_i precipitates was determined by adaptive thresholding performed in image analyst MKII (Image Analyst Software, Novato, CA, USA).

Mitochondrial respiration

Oxygen consumption was performed polarographically using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). 0.5 mg of mitochondria were suspended in 2 ml incubation medium. Oxygen concentration and oxygen flux (pmol/(s*mg); negative time derivative of oxygen concentration, divided by mitochondrial mass per volume) were recorded using DatLab software (Oroboros Instruments).

Mitochondrial matrix pH (pH_i) determination of mouse liver and Artemia cyst mitochondria

Mouse liver mitochondria (20 mg) were suspended in 2 ml of medium containing (in mM: 225 mannitol, 75 sucrose, 5 Hepes, and 0.1 EGTA, pH 7.4 using Trizma) and incubated with 50 µM BCECF-AM (Invitrogen, Carlsbad, CA, USA) at 30°C. After 20 min, mitochondria were centrifuged at 10,600 g for 3 min (at 4°C), washed once and re-centrifuged. The final pellet was suspended in 0.2 ml of the same medium and kept on ice until further manipulation. A similar procedure was used for *Artemia* cysts with the exception that the medium consisted of 500 mM sucrose, 150 mM KCl, 1 mM EGTA, 0.5% (w/v) fatty acid-free BSA, and 20 mM K^+-Hepes(pH 7.5), and the temperature was 27°C. Fluorescence of hydrolyzed BCECF trapped in the matrix was measured in a Hitachi F-4500 spectrofluorimeter in a ratiometric mode at a 2 Hz acquisition rate, using excitation and emission wavelengths of 450/490 nm and 531 nm, respectively.

Results and Discussion

Our experiments on *Artemia* revealed a potent Ca^{2+} uptake machinery, that mechanistically resembled that of the mammalian consensus, but was different from it in some aspects. The Ca^{2+} uptake was sensitive to Ru 360, indicating the process is primarily executed by the Ca^{2+}uniporter. P_i was the necessary counter ion for Ca^{2+} uptake, which fact was also confirmed by EELS microscopy.

In contrast to mammals, ADP and ATP both decreased Ca^{2+} uptake capacity in *Artemia*. For mammals the well-established explanation is the inhibitory effect of these nucleotides on the PTP. The inhibitory effect of ADP in *Artemia* could be abolished by blockage of ADP/ATP exchange either directly by inhibition of the ANT by cATR or indirectly, by inhibition of the F_oF_1 ATP-ase by oligomycin. Therefore ADP needs to enter the matrix in order to mediate its effect. Matrix ADP could cause this by two different mechanisms: I) by decreasing membrane potential or II) by binding to a specific matrix binding site. The two inhibitors, cATR and oligomycin have different effects on the matrix ADP pool: at sufficiently high membrane potential (both ANT and the F_oF_1 ATP-ase in forward mode), when cATR inhibits the ANT the ADP concentration slightly decreased (it is normally low even in phosphorylating mitochondria due to the higher flux control coefficient of the F_oF_1 ATP-ase compared to the ANT), as matrix ATP cannot be exchanged for ADP, however in the case of oligomycin, the matrix ADP level is increased, because ADP cannot be converted into ATP by oxidative phosphorylation (or substrate-level phosphorylation in the matrix by succinyl-thiokinase, as we used succinate in our substrate combination, which disfavors the reaction). Therefore we conclude that Ca^{2+} uptake in *Artemia* is unaffected by matrix ADP concentration, and it is more likely that ADP inhibits Ca^{2+} uptake in *Artemia* mitochondria due to reducing membrane potential and thereby decreasing the driving force for Ca^{2+} uptake, rather than regulating Ca^{2+} dynamics by binding to a specific binding site inside the matrix.

ATP on the other hand does not need to enter the matrix and likely binds on the outer surface of mitochondria to affect Ca^{2+} uptake. This finding provides another possible explanation for the inhibitory effect of ADP: the inhibition is indirect and is in fact caused by the ATP produced by mitochondria, which can be abolished by the inhibition of ATP production by either cATR or

oligomycin. These findings open up several new interesting questions regarding Ca^{2+} uptake in *Artemia*, however answering these questions was not our main objective.

We investigated mitochondrial morphology of *Artemia* with transmission electron microscopy. *Artemia* mitochondria have a highly similar appearance to mammalian mitochondria regarding size and christae structure. However when loaded with high amounts of Ca^{2+}, needle like electron-dense formations appear in the matrix of *Artemia* mitochondria, whereas in mammals, Ca^{2+} sequestration results in ring-shaped or dotted structures. Electron energy loss spectroscopy was used to confirm the electron-dense structures to be rich in Ca and P. Similar morphology can be observed in mammals only when Mg^{2+} and ADP concentrations are low. In the presence of ADP Artemia mitochondria also showed dot like precipitates. We have not investigated whether the morphological change is due to the decreased Ca^{2+} uptake capacity caused by ADP or the direct effect of ADP on the precipitates, however the possibility that the needle-like structure of the precipitates is a contributor to the high Ca^{2+} uptake capacity in *Artemia* cannot be ruled out.

Based on the findings above, we conclude that the Ca^{2+} uptake machinery in the primitive arthropod *Artemia franciscana* is similar in its basic principles of mechanism to the mammalian consensus. However we confirm that uptake capacity is higher and PT cannot be evoked in this species, furthermore we described unique traits of the Ca^{2+} uptake machinery and matrix Ca^{2+} precipitates in *Artemia*. We believed these traits of mitochondria from *Artemia* embryos are less relevant to the unavailability of the PTP in this species, and decided to focus our attention on other results.

During our investigation we found that other classical PTP inhibitors that effectively increase Ca^{2+} uptake capacity on mammalian mitochondria had no effect on Ca^{2+} uptake in *Artemia*. A well-described inhibitor of the ANT, BKA is also such inhibitor of the PTP. We found *Artemia* to be insensitive to the ANT inhibitory effects of BKA: it failed to reverse the depolarizing effect of ADP on $\Delta\Psi m$ experiments and in contrast to cATR, it did not affect ATP production. Inhibition by BKA on *Artemia* mitochondria could only be achieved by substantial decrease of the pH and the increase of BKA concentration. This finding is relevant, as the ANT is a known regulator of the PTP, but it is dispensable to the process. The possibility that these characteristics

of *Artemia* mitochondria are connected to the absence of the PTP could not be overlooked, and we wished to test this hypothesis.

We realized that more closely related organisms to *Artemia* were needed for a reliable comparison. Surprisingly, when we tested other non-extremophile, salt- and freshwater inhabiting crustaceans, we found that without exception they all lacked the PTP. Furthermore we observed no resistance to BKA in any of the newly investigated species, which falsified our early hypothesis regarding a connection between these two characteristics. Our new assumption was that the PTP was lost at some point in the evolution of the crustacean subphylum. We believed, that narrowing down at which point the mechanism of PT was lost in these animals would lead to clues about the molecular entity of the PTP. We therefore characterized species that branched off from crustaceans at different points during evolution. We were also interested if we would find species in other phyla that lacked PT.

In this thesis six novel species that do not exhibit the permeability transition pore are described. Furthermore we showed that PT can be provoked in six other species, which belong to taxa, in which presence of PTP had not been studied previously. Our investigation was thorough, utilizing variable methods to test the functionality of the PTP in each of the organisms. Based on our results, we postulate that the PTP is a universal trait in the animal kingdom, except for the crustacean subphylum.

Tough the BKA resistance of *Artemia* is unrelated to the absence of PTP, we believed that further investigation could lead to the identification of the bongkrekate binding site of the ANT. The BKA binding site is an unknown potential drug target for PTP inhibition and we sought to identify it by comparison of the *Artemia* ANT to multiple other sensitive ANT sequences. So far, our success in sequencing the full *Artemia* sequence, and the partial sequences of *Crangon crangon* and, *Palaemon serratus* highlights an amino acid sequence possibly responsible for the BKA resistance of *Artemia*.

With our collaborators, we were unsuccessful in demonstrating BKA insensitivity in yeast expressing the cloned *Artemia* ANT. Though these results are disappointing, some conclusions can be made: the insensitivity of *Artemia* to BKA might be due to some other protein entity either absent in mitochondria of the *Artemia* that is otherwise necessary for conferring sensitivity

to BKA, or present that is masking the BKA binding site. Insensitivity could also be caused by unique physical properties of these mitochondria, such as differences in membrane characteristics.

Publications related to the thesis

1.) Konrad, C., G. Kiss, B. Torocsik, V. Adam-Vizi, and C. Chinopoulos, *Absence of Ca2+-induced mitochondrial permeability transition but presence of bongkrekate-sensitive nucleotide exchange in C. crangon and P. serratus.* PloS one, 2012. **7**(6): p. e39839.

2.) Konrad, C., G. Kiss, B. Torocsik, J.L. Labar, A.A. Gerencser, M. Mandi, V. Adam-Vizi, and C. Chinopoulos, *A distinct sequence in the adenine nucleotide translocase from Artemia franciscana embryos is associated with insensitivity to bongkrekate and atypical effects of adenine nucleotides on Ca2+ uptake and sequestration.* The FEBS journal, 2011. **278**(5): p. 822-36.

Publications independent from the thesis

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