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Respiratory and Calcium-Accumulating Properties of Muscle Mitochondria of Some Aquatic Arthropods

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Abstract

Mitochondria were isolated from the muscles of *Streptocephalus dichotomus, Caridina nilotica, Paratelphusa hydrodromous, Sphaerodema rusticum,* and *Enithares rogersi,* all of which belong to the phylum arthropoda, and their properties studied. All mitochondria showed qualitatively similar respiratory and Ca^{2+} uptake capacities but there was much quantitative variation. There emerges an apparent correlation between locomotor activity of the organism and the respiratory activity of the mitochondria. Similar correlation is found between the calcium need of the animal and the Ca^{2+} uptake capacity of the mitochondria. The results also suggest an inverse correlation between respiration and Ca^{2+} uptake.

Introduction

That the mitochondrial preparations obtained from most of the organisms studied so far have the capacity to actively accumulate calcium ions has led to the general contention that this property is intrinsic to the organelle [1]. The physiological implications of such a process, however, have not been clearly understood. Most research work in this area of calcium uptake of mitochondria is built around the hypothesis that this organelle might be the intracellular ion regulator [2].

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During the past few years, research work in our laboratory has centered around the fundamental aspect of the problem, namely the universality of the mitochondrial calcium-uptake process. In other words, we are asking the question: will this phenomenon be exhibited by mitochondria isolated from organisms at different phylogenetic levels? A positive answer seems to arise from our preliminary survey of more than 15 organisms.

Also arising from the data are some interesting correlations between the mobility of the organism and respiratory capacity of muscle mitochondria as well as between the calcium uptake capacity of muscle mitochondria and their calcium need. In this communication we deal with the comparative analysis of the properties of mitochondria isolated from the muscles of 5 arthropods. The selection of this group assumes significance since the arthropods parallel no other invertebrate group in their dominance and diversification of animal life.

Materials and Methods

The five arthropods chosen were: (a) *Streptocephalus dichotomus,* the fairy shrimp (Branchiopoda, Crustacea); (b) *Caridina nilotica,* a small prawn (Decapoda, Crustacea); (c) *Paratelphusa hydrodromous,* the field crab (Decapoda, Crustacea); (d) *Sphaerodema rusticum,* a water bug (Belostomatidae, Hemiptera, Insecta); (e) *Enithares rogersi,* the back-swimmer (Notonectidae, Hemiptera, Insecta).

S. dichotomus and *E. rogersi* were reared in their original pond water with frequent replenishment to avoid depletion of natural food. Herbivorous *C. nilotica* and *S. rusticum* were reared in tap-water aquaria with rich Hydrilla vegetation. *P. hydrodromous,* usually collected from the mud holes of paddy fields, were kept in shallow water tanks with a substratum of gravel, sand, and pebbles and fed *ad libitum* on meat.

The habitat, mode of locomotor activity, size, and the number used per experiment are given in Table I.

Isolation of Muscle Mitochondria

The particular muscle tissue chosen in each animal (Table I) was excised out as carefully and quickly as possible, keeping the animals on ice blocks during excision. The excised muscle blocks were either minced thoroughly or gently pounded in a chilled mortar with a pestle and then suspended in 5 volumes of the isolation medium (see below). The brew was strained through cheese-cloth to remove exoskeletal bits. The filtrate was then homogenized using a Teflon homogenizer (A.H. Thomas Co., Philadelphia) for 30–60 sec. The resulting homogenate was diluted twice

TABLE I. Some relevant details about the species chosen, mitochondrial source, and yield

Species	Habitat	Locomotor activity	Size in cm	No. used/ experi- ment	Muscle type	Mito- chondrial yield (mg protein/g wet tissue)
S. dichotomus	Astatic pools	Slow, steady swimmer	3	40–50	Thoraco- abdominal	2–3
C. nilotica	Streams	Active swimmer	4	60–80	Abdominal	4–4.5
P. hydrodromous	Semi- aquatic mudhole dweller	Slow walking movement	4-5	5–6	Cephalo- thoracic	3–4
S. rusticum	Large tanks	Fast swimmer	2	30–50	Thoracic	4–5
E. rogersi	Astatic pools	Ever-active backswimmer	1	100	Thoracic	4–5

(to give approximately a 10% homogenate). The nuclei and debris were removed by centrifuging at 600*g* for 5 min and the mitochondrial pellet obtained from the supernatant at 10,000*g* for 10 min. The pellet obtained was washed twice with EDTA-free isolation medium and suspended in reaction media (see below). The mitochondria were suspended at a concentration of 15 mg protein/ml in the medium appropriate to the subsequent experiments. All centrifugations were carried out in a refrigerated centrifuge (Janetzki-K.24) at 0–4° C.

In a few experiments, the homogenate was centrifuged at 10,000*g* for 10 min to start with and the resulting pellet was resuspended and fractioned for nuclei and mitochondria as before. Since basically there were no major quantitative differences observed between the two methods, the former procedure was used routinely. The mitochondrial protein was estimated by the biuret method [3].

Isolation Media

Optimal and reproducible results were obtained for the crustacean-muscle mitochondria when the Chappel-Perry medium [4] containing 0.1 M KCl, 0.05 M Tris-HCl, pH 7.4, 5 mM $MgSO_4$, and 1 mM EDTA was used. For the insect thoracic muscle mitochondria, the best medium was found to be 0.154 M KCl solution containing 1 mM EDTA and pH adjusted to 7.4 with KOH at 0° C [5].

Reaction Medium

Several different media were tried for measurement of mitochondrial respiration. Media containing sucrose (0.25 M), sorbitol (0.3 M), mannitol (0.2 M), or KCl (0.15 M) with phosphate buffer pH 7.4 (0.05 or 0.1 M) and magnesium salt (0.01–0.004 M) as common ingredients were tried. Consistent results were obtained with a medium of the following composition: KCl (0.15 M), potassium phosphate buffer, pH 7.4 (0.004 M), Tris-sulphate buffer, pH 7.4 (0.005 M), and $MgSO_4$ (0.004 M). This was routinely used unless otherwise mentioned. Addition of bovine serum albumin improved the activity only marginally in all cases.

Assay of Succinic Dehydrogenase Activity

Succinic dehydrogenase was measured in the mitochondrial preparation according to the procedure described earlier [10] in presence of azide.

Measurement of Oxygen Uptake

The respiration was measured polarographically using a Clark-type oxygen electrode (Yellow Springs, Ohio) at 32° C in 3.5 ml total volume.

Measurement of Energy-Independent Binding of Ca^{2+}

The procedure described by Reynafarje and Lehninger [6], was followed with modifications suggested by Carafoli [7]. For these experiments, the pelletized mitochondria were suspended during isolation in a medium containing 0.25 M sucrose, 2 mM Tris-chloride pH 7.4, at a concentration of about 1 mg protein/ml. Various 1-ml aliquots were taken and to each was added rotenone (120 μM) and antimycin A (110 μM). Radioactive $^{45}CaCl_2$ was added to the tubes at increasing concentrations from 1–60 nmole. After 30 sec of incubation with shaking the contents of each tube was rapidly filtered through a Millipore filter (0.45 μm diameter pore size) and washed with cold reaction medium. The filters dried were glued onto planchets and radioactivity determined in a gas-flow counter (efficiency 8%).

Measurement of Energy-Dependent Matrix-Loading of Ca^{2+}

The general procedure adopted was as described by Loyter et al. [8]. The reaction mixture used was the same as for respiratory studies. To each ml of mitochondrial suspension (2–3 mg protein), succinate (5 mM) and ATP (7.5 mM) were added and the mixture equilibrated to room temperature (30° C) for 1 min. At the end of this period, 120 nmole of $^{45}CaCl_2$ were added and incubation continued for 15 min (time having

been determined by preliminary experiments). At the end of this period, 0.1 ml of 0.4 M $MgSO_4$ was added, mixture shaken well to release unbound calcium and the mitochondria filtered quickly through a millipore filter. The radioactivity in the filters was determined as mentioned above. Omissions or additions of chemicals to this basic reaction mixture are mentioned wherever appropriate.

Chemicals

Radioactive calcium ($^{45}CaCl_2$) specific activity 18.5mCi/g Ca^{2+} was obtained from the Isotopes Division, Bhabha Atomic Research Centre, Trombay, India. Antimycin A was the generous gift of Professor E. Carafoli, Zürich. ADP was obtained from Sigma Chemicals, U.S.A., and rotenone was purchased from E. Merck, Germany. The other chemicals used were of Analar grade.

Results

Mitochondrial Yield and Stability

The yield of mitochondrial protein from each muscle type used is given in Table I. The figures given are the average of a number of experiments. In the experiments described below, the results are also the mean of at least three independent experiments.

Since it has been reported earlier [9, 10] that mitochondria from invertebrates are unstable, the stability of mitochondria from these 5 organisms was tested using succinic dehydrogenase assay as a criterion. It was indeed found that storage of the mitochondrial preparations for 1 h even at 0° C resulted in 90% of the succinic dehydrogenase activity being lost, whereas under similar conditions, rat-liver mitochondrial preparations were stable. All subsequent experiments, therefore, were carried out within 15 min of isolation of mitochondria (also see *Discussion*).

Respiratory Capacity

The respiratory control index and ADP/O ratio of the preparations, calculated as per Estabrook [11] are given in Table II.

The following facts become obvious from the data presented:

(a) The mitochondria isolated from all these organisms respire with succinate as substrate. Several other substrates like pyruvate + malate, α-ketoglutarate, proline, and arginine were tried, but the activity was low with these added substrates. Similarly, the effect of various inhibitors such as oligomycin, antimycin A, rotenone, and azide were also studied

TABLE II. Respiratory properties of the muscle mitochondria of various species (Data on rat-liver mitochondria worked out in our laboratory is given as a standard for comparison of results)[a]

Species	Succinate oxidation nmole of O_2/mg protein/min	Respiratory control index	ADP/O ratio
S. dichotomus	10.5	3.3	1.5
C. nilotica	12.8	3.4	1.7
P. hydrodromous	7.1	2.4	1.1
S. rusticum	14.4	3.0	1.5
E. rogersi	27.0	2.0	1.8
Rat liver	65.0	20.0	1.5

[a] The assay mixture (3.5 ml) contained 2.5 mg mitochondrial protein, Succinate 10 mM to start with. ADP (0.05–0.1 mM) was added after about 2 min.

and the results were similar to that of rat-liver mitochondria (data not given).

(b) The respiratory rate is 3–9 times lower than rat-liver mitochondria.

(c) The ADP/O ratios are in a reasonably acceptable range for succinate, thereby assuring us of the phosphorylating efficiency of the particles isolated.

(d) The respiratory control index (R.C.) between 2 and 3 obtained would indicate that, at least to a certain extent, the mitochondria are functionally intact. In all experiments where Ca^{2+} uptake was studied, only those mitochondria which gave at least this value of R.C. were used.

(e) Comparison of data given in Tables I and II reveals an interesting correlation between the locomotor activity and the respiratory activity

TABLE III. The number and *Kd* values of high and low affinity binding sites in the energy-independent muscle mitochondrial membrane binding of calcium of the various species

	Number of binding sites (nmole of Ca^{2+}/mg mito protein)		Affinity constants (*Kd* in μM)	
Species	High affinity	Low affinity	High affinity	Low affinity
S. dichotomus	—	8.0	—	14.5
C. nilotica	0.83	5.5	1.4	42.0
P. hydrodromous	—	11.0	—	711.0
S. rusticum	0.33	—	1.3	—
E. rogersi	—	4.0	—	67.0

of muscle mitochondria. There is a gradual increase in respiratory rate from the muscle mitochondria of the slow walking field crab (*P. hydrodromous*) to the ever-active swimmer (*E. rogersi*).

Energy-Independent $^{45}Ca^{2+}$ *Uptake*

The data of energy-independent $^{45}Ca^{2+}$ binding by mitochondria have been plotted according to the convention of Scatchard and the derivation of high and low binding sites, done from the extrapolated intercepts (Table III). Calculations of affinity constants were done according to Reynafarje and Lehninger [6]. The plots are shown in Fig. 1.

The following point emerges for discussion. The shape of the curve for each organism is different. Four of the organisms gave monophasic curves, but the slope varies from the very steep curve for *S. rusticum* to the other extreme of *P. hydrodromous* where the curve is more horizontal. *C. nilotica* differs from the others in giving a biphasic curve, indicative of two types of binding.

Energy-Dependent Matrix-Loading of $^{45}Ca^{2+}$

As shown in Fig. 2, the muscle mitochondria of all 5 organisms show energy-dependent matrix-loading. Omission of substrate or ATP or

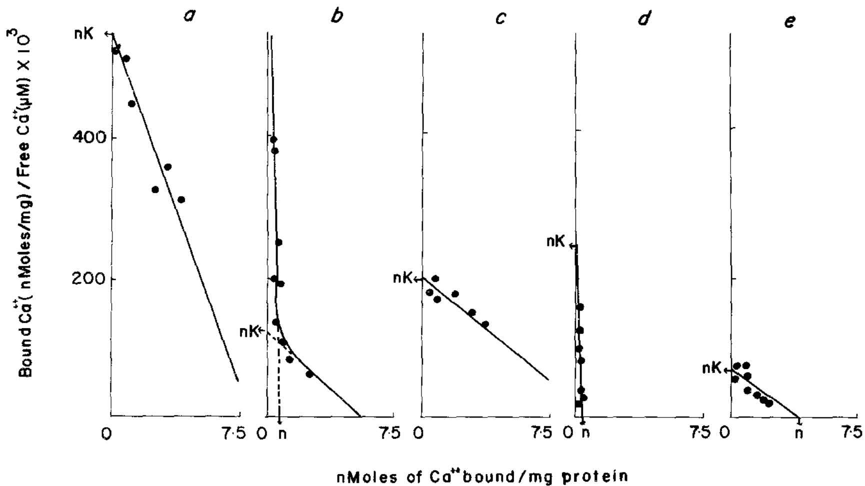

Figure 1. Scatchard plots of the energy-independent membrane binding of calcium in the muscle mitochondria of the five species. (a) *Streptochephalus dichotomus*, (b) *Caridina nilotica*, (c) *Paratelphusa hydrodromous*, (d) *Sphaerodema rusticum*, and (e) *Enithares rogersi*. From the *nK* on the ordinate and *n* on the abscissa, the number of binding sites and affinity constants are derived [6] (Table III).

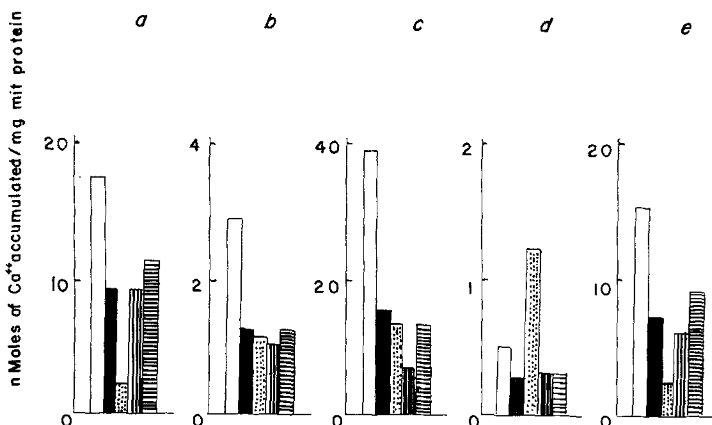

Figure 2. Histograms showing the energy-linked calcium accumulation in the muscle mitochondria of the five species studied. (a) *Streptocephalus dichotomus*, (b) *Caridina nilotica*, (c) *Paratelphusa hydrodromous*, (d) *Sphaerodema rusticum*, and (e) *Enithares rogersi*. Open bars: uptake of calcium with all necessary components in the medium. Closed bars: in the absence of substrate, succinate. Dotted bars: in the absence of ATP. Vertical barred bars: with uncoupler, 2,4-Dinitrophenol (30 μM). Cross-barred bars: with respiratory inhibitors, azide (20 μM). Experimental conditions are described in *Materials and Methods*.

addition of uncoupler or respiratory inhibitor reduces the uptake significantly. An anomalous behavior is found however in the case of *S. rusticum* thoracic-muscle mitochondria, where the uptake is doubled in the absence of ATP. Elimination of phosphate in the total medium decreases the Ca^{2+} accumulation by 50% (not shown in Fig. 2).

Quantitatively, even the highest amount of Ca^{2+} taken up by *P. hydrodromous* muscle mitochondria (38 nmole/mg protein) is less than one-tenth of the "limited-loading" demonstrable in rat-liver or kidney mitochondria [12]. Moreover, in all the five cases, added Ca^{2+} failed to stimulate state of 4 respiration to state 3 (unpublished data)' which can also be related to the low level of accumulation.

Discussion

Choice of Tissue

In any comparative study of widely varying organisms, the choice of proper tissues to be compared is of vital importance and one which is likely to face maximal criticism. Proper muscle function needs a regulated supply of Ca^{2+} ions as well as ATP as the energy source. Since

our main interest has been on these very two functions of mitochondria, the choice of muscle tissue for isolation of the organelle became almost a necessity for the project.

Stability of Mitochondria

The mitochondria isolated from these organisms, reported here as well as by others (unpublished results), seem to be extremely unstable. A variety of explanations, such as the intrinsic nature or the release of lysosomal components into the supernatant during homogenization, could be proposed and some of these are already being tested, but what needs stressing is the fact that various experiments have been carried out taking as many precautions as possible to avoid such damage–the data presented in Table II indicate that we have been fairly successful.

Correlation between Respiratory and Locomotor Activity

If the organisms used in this study were to be arranged in decreasing order of locomotor activity, then the order would be *E. rogersi, S. rusticum, C. nilotica, S. dichotomus,* and *P. hydrodromous.* Interestingly this is the same order of respiratory activity (Table II).

Calcium Uptake Capacity

(a) Calcium has a basic role in the activation of many enzymes and also in the contraction-relaxation cycle of muscle [7, 13]. Therefore it is not surprising that muscle mitochondria of the animals studied show qualitatively the capacity to actively accumulate calcium.

(b) Attempts to interpret the quantitative variations should take into account: (i) the requirement of calcium by the organism for its exoskeleton and (ii) the role of calcium in altering the permeability of the cell membrane [13, 15, 16], thereby playing an important role in osmotic regulation.

P. hydrodromous needs the maximum Ca^{2+} (among the organisms studied) and this is reflected in its muscle mitochondria having the maximum number of sites. The "low affinity" of these sites might reflect the slow and steady demand for calcium.

At the end of the spectrum are *S. rusticum* and *E. rogersi,* whose calcium need is low [14, 17] and results presented in Table III correlate this with the mitochondrial capacity.

Although the organisms *S. rusticum* and *S. dichotomus* lack a calcified exoskeleton, they are osmotically active. Hence a clear-cut correlation between the calcium need and mitochondrial capacity becomes rather difficult.

General Conclusion

The results presented here, albeit with many limitations such as nonuniformity of the physiological status of the organisms, and lack of quantitative data on calcium need and osmotic load, clearly show an underlying pattern. Whereas there appears to be a direct correlation between respiratory activity and locomotor activity on the one hand, and between calcium need and calcium uptake capacity on the other, these two parameters are inversely related to each other. Whether this pattern gives a clue to the mode of energy dissipation by mitochondria is currently under investigation.

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