*J. exp. Biol. 157, 543–549 (1991)*
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## SHORT COMMUNICATION

# OXYGEN CONSUMPTION, CARBON DIOXIDE PRODUCTION AND ENZYME ACTIVITIES OF ISOLATED WORKING *OCTOPUS* HEART

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*Accepted 17 December 1990*

The heart of *Octopus vulgaris* (Lam.) presents an ideal tissue for *in vitro* studies. Its performance, when perfused with haemocyanin solutions or blood, closely matches that *in vivo* in terms of power output (Agnisola and Houlihan, 1991) and the heart tissue is self-perfused so that with each contraction a proportion of the fluid in the lumen passes through the heart wall in a system of blood vessels to produce coronary flow (Houlihan *et al.* 1987). Comparison of *in vitro* activity levels of key enzymes from energy-generating pathways (Driedzic *et al.* 1990) with rates of oxygen consumption of perfused hearts, working at subphysiological levels (Houlihan *et al.* 1987) suggested that the tissue is fuelled primarily by a glucose-based metabolism. In this experiment, the analysis is refined with the simultaneous measurement of oxygen consumption, carbon dioxide production and enzyme activity levels on the same tissues. Hexokinase activity was determined since it is a good indicator of maximal aerobic glucose use in a variety of muscles (Crabtree and Newsholme, 1972), citrate synthase and cytochrome oxidase activities were measured as markers of aerobic capacity, and 3-hydroxyacyl CoA dehydrogenase activity was assayed as a qualitative index of the capacity to oxidize fatty-acid-derived fuels.

Low respiratory quotient's (RQs) in some preparations suggested that either amino acids or fatty acids were utilized as a metabolic fuel in addition to glucose. The efficacy of selected amino acids and fatty acid derivatives to support oxygen consumption of isolated mitochondria was assessed. Finally, we tested the hypothesis that mitochondria from *Octopus* heart can oxidize fatty acids directly, without a carnitine-facilitated transport process, as occurs in some insects (Stevenson, 1968).

*Octopus vulgaris* were collected in May 1989 and used within 2–9 days of

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Key words: *Octopus vulgaris*, heart, oxygen consumption, energy metabolism.

capture. Animals were kept, without food, in flowing sea water at 20°C. The isolated heart was prepared as described previously (Foti *et al.* 1985; Houlihan *et al.* 1987) with some modifications (Agnisola and Houlihan, 1991). Perfusion occurred through the two cannulated atria and the aortic output was collected from the cannulated aorta. The heart was mounted in a chamber and the coronary flow was collected on the chamber's floor. The height of the input reservoir was set to obtain the maximal cardiac output and varied among preparations. The output reservoir was always 20 cm higher than the input reservoir. Hearts were perfused with either oxygenated sea water or haemocyanin solutions containing 2.8 mmol $l^{-1}$ glucose, or whole blood (Agnisola and Houlihan, 1991), at a temperature of 18–22°C. If necessary, the pH of the haemocyanin solution was adjusted to 7.5 at 20°C. During the course of parallel studies it became apparent that hearts perfused with whole blood achieved higher levels of oxygen consumption than under other conditions. However, regression equations of the relationship between oxygen consumption and power output were similar, regardless of the composition of the perfusate (Agnisola and Houlihan, 1991).

Intraventricular pressure, aortic pressure, aortic and coronary flow rates, heart rates, input and output $P_{O_2}$, total oxygen content and total carbon dioxide content were measured over 10 min or until they had stabilised. If this had not occurred within 15 min the heart was discarded. Mineral oil was then added to the chamber so that the coronary output collected at the bottom of the bath was out of contact with air. Pressure development, flow rates and perfusate gases were determined for a further 10 min. Aortic and coronary outputs were collected over 1 min intervals and weighed and the values were corrected for temperature and density and expressed as a volume measurement. Pressures were referenced to the level of the input cannula. Power output of the heart was calculated from the sum of the aortic and coronary power outputs and is expressed as mW $g^{-1}$ (Agnisola and Houlihan, 1991). Perfusate samples were taken anaerobically by syringe for measurement of gases and analyzed within 3 min. Oxygen concentrations were determined by the modification of the Tucker (1967) method described by Bridges *et al.* (1979). Total $CO_2$ concentrations were determined by the method of Cameron (1971) using 50 $\mu$l samples, unknowns being bracketed by sodium bicarbonate standards. $O_2$ consumption and $CO_2$ production were assessed from changes in gas content in fluid passing both through the ventricular walls (coronary flow) and through the lumen (aortic output), as previously detailed (Houlihan *et al.* 1987; Agnisola and Houlihan, 1991). Repeated sampling of a haemocynanin solution covered with oil at an initial $P_{O_2}$ of 0.93 kPa revealed that the $P_{O_2}$ did not change significantly for up to 60 min.

Enzyme activities were determined on hearts utilized in the perfusion studies. Following perfusion, hearts were weighed and bisected. One portion was used immediately for hexokinase (HK; EC 2.7.1.1) assays; the second portion was frozen and used later for citrate synthase (CS; EC 4.1.3.7), 3-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35) and cytochrome *c* oxidase (CO; EC 1.9.3.1) assays. The latter three enzymes were freeze stable. Measurements of

enzyme activity were performed at 20°C on crude homogenates. Hearts were homogenized in nine volumes of 75 mmol $l^{-1}$ Tris, 1 mmol $l^{-1}$ EDTA, pH 7.4, with an Ultra-turrax homogenizer. Assays were conducted at optimal concentrations of substrates and cofactors, and at the pH (within 0.1 unit) necessary to achieve maximal enzyme activites. Methods and data analysis are described in detail elsewhere (Sidell *et al.* 1987; Driedzic *et al.* 1990). Absorbance was monitored by using a Beckman DU-65 spectophotometer interfaced with a Beckman data capture system.

Additonal animals were used in the mitochondria experiments. Approximately 2 g of systemic heart was taken up in 30 ml of medium containing 500 mmol $l^{-1}$ sucrose, 150 mmol $l^{-1}$ KCl, 2 mmol $l^{-1}$ EDTA and 25 mmol $l^{-1}$ Hepes, pH 7.4, at 22°C (Mommsen and Hochachka, 1981). Because of the ruggedness of the tissue, it was necessary to homogenize for three 10-s bursts with an Ultra-turrax to obtain a workable yield of mitochondria. The suspension was centrifuged at 700 *g* for 10 min, the supernatant was recentrifuged at 9000 *g* for 20 min, and the pellet was resuspended in 2–3 ml of assay medium and used directly. The assay medium contained 50 mmol $l^{-1}$ NaCl, 25 mmol $l^{-1}$ $KH_2PO_4$, 30 mmol $l^{-1}$ Hepes and 200 mmol $l^{-1}$ KCl, pH 7.4, at 22°C. In initial studies, the KCl concentration was varied in steps of 50 mmol $l^{-1}$ from 50 to 400 mmol $l^{-1}$ final concentration. Medium containing 200 mmol $l^{-1}$ KCl provided the highest respiratory control values. The addition of glycine at 100–400 mmol $l^{-1}$ had no effect on oxygen consumption. Respiration was routinely assessed in the presence of pyruvate, malate, glutamate, aspartate and proline, all at 5 mmol $l^{-1}$. Some experiments were also conducted with octanoate (0.1 mmol $l^{-1}$), oleate (0.1 mmol $l^{-1}$), palmitate (0.1 mmol $l^{-1}$), acetyl CoA (0.5 mmol $l^{-1}$), palmitoyl CoA (0.5 mmol $l^{-1}$) and L-carnitine (5 mmol $l^{-1}$). State 3 respiration was initiated with 0.13 mmol $l^{-1}$ ADP. In studies with palmitate and octanoate, mitochondrial suspension was added to the assay medium containing the substrate; in all others experiments, substrates were made up in assay medium and delivered directly to the respiratory cell in small volumes. Reactions were followed at 22°C, in water-jacketed glass cells, with Clark-type electrodes interfaced to a YSI model 16582 oxygen sensor. The final reaction volumes were 1.9 ml and contained mitochondrial suspension equivalent to 1.2–2.5 mg of protein. Protein was assayed following freeze-thawing and sonication according to Bradford (1976) against BSA standards. Respiratory control ratios were calculated according to Estabrook (1967) and respiratory states are defined according to Chance and Williams (1956).

Maximal power output of the perfused, isolated hearts ranged from 0.95 to 3.39 mW $g^{-1}$ (Table 1). $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ both increased linearly as a function of power output. The quantitative relationships between $\dot{M}_{O_2}$ and power output and between $\dot{M}_{CO_2}$ and power output were consistent with the regression equations previously established (Agnisola and Houlihan, 1991). RQ values for perfused hearts were as follows: 0.82 and 0.99 with oxygenated sea water, 0.82 and 1.0 with haemocyanin solutions, 0.60 and 0.76 with blood.

Under resting conditions the *Octopus* heart has a power output of approxi-

Table 1. *Power output, oxygen consumption, carbon dioxide production and enzyme activity levels in* Octopus vulgaris *systemic heart*

| | |
|---|---|
| Power output ($mW\,g^{-1}$) | 1.68±0.36 |
| $\dot{M}_{O_2}$ ($\mu mol\,g^{-1}\,min^{-1}$) | 2.43±0.64 |
| $\dot{M}_{CO_2}$ ($\mu mol\,g^{-1}\,min^{-1}$) | 2.08±0.53 |
| Hexokinase ($\mu mol\,g^{-1}\,min^{-1}$) | 3.17±0.51 |
| Citrate synthase ($\mu mol\,g^{-1}\,min^{-1}$) | 34.36±1.91 |
| 3-Hydroxyacyl CoA dehydrogenase ($\mu mol\,g^{-1}\,min^{-1}$) | 2.66±0.19 |
| Cytochrome oxidase ($\mu mol\,g^{-1}\,min^{-1}$) | 17.02±1.26 |

Power output, $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were obtained from perfused, isolated hearts at 18–22°C. Enzyme activity levels were determined at 20°C on the same tissues.

Values are presented as mean±S.E.M. $N=7$ for power output and $\dot{M}_{O_2}$, $N=6$ for $\dot{M}_{CO_2}$ and all enzymes except cytochrome oxidase, where $N=5$.

mately $2\,mW\,g^{-1}$ and during activity this may increase to about $6\,mW\,g^{-1}$. These estimates are based upon cardiac outputs calculated from the Fick equation and measured with flow probes combined with measurements of aortic and venous pressures (Wells *et al.* 1987). However, it is likely that these performances are not substainable and part of the energy may be derived anaerobically. The average $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ values presented in Table 1 are most certainly underestimates of maximal respiration by the tissues and, based upon the regression equation reported in Agnisola and Houlihan (1991), they probably represent about half of the maximum value.

Maximal *in vitro* activities of selected enzymes were assessed in hearts that had been perfused. Pairwise correlations between enzymes and $\dot{M}_{O_2}$ or $\dot{M}_{CO_2}$ failed to reveal any significant relationships, probably because the hearts had attained variable levels of their maximal power output. Thus, the data were converted to the mean values. The activities reported here for cytochrome oxidase, hexokinase and citrate synthase are similar to previous findings, once the difference in assay temperature is accounted for (Driedzic *et al.* 1990). However, the present data allow a refinement in analysis of the relationship between metabolic flux rates, based upon *in vitro* enzyme activity levels, and $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$, since all the information is obtained from the same specimens. In the cytochrome oxidase assay the conversion of 4 $\mu$mol of reduced to oxidised cytochrome *c* requires 1 $\mu$mol of $O_2$. Maximal *in vitro* activities of this enzyme correspond to an $\dot{M}_{O_2}$ of about $4.25\,\mu mol\,O_2\,consumed\,g^{-1}\,min^{-1}$, which is a very close match to the maximal rate of 4.8 (i.e. two times $2.4\,\mu mol\,O_2\,consumed\,g^{-1}\,min^{-1}$) extrapolated from the perfusion studies. If it is assumed that all glucose that flows through hexokinase enters the citric acid cycle, this could yield maximal $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ values of $19\,\mu mol\,O_2$ consumed or $CO_2$ produced $g^{-1}\,min^{-1}$, which are well in excess of the observed levels. This finding is in contrast to a previous interpretation, based on a number of assumptions, of a close match between oxygen consumption and hexokinase activity (Driedzic *et al.* 1990). Our current view is that, *in vivo*,

Table 2. *Oxygen consumption rate of mitochondria isolated from systemic heart of* Octopus vulgaris

| Substrate | *N* | Respiratory control ratio | State 3 rate |
|---|---|---|---|
| Malate | 6 | 2.9 | 47.6±21.6 |
| Malate plus pyruvate | 4 | 2.2 | 45.2±5.4 |
| Proline | 5 | 1.3 | 25.8±9.8 |
| Proline plus pyruvate | 5 | 2.0 | 33.2±18.4 |
| Glutamate | 3 | 1.7 | 14.6±6.0 |
| Glutamate plus pyruvate | 4 | 2.9 | 31.0±9.0 |
| Aspartate | 3 | – | 2.4±1.8 |
| Aspartate plus pyruvate | 3 | 2.5 | 11.4±4.6 |

Oxygen consumption is expressed in natoms O $mg^{-1}$ protein $min^{-1}$ at 22 °C.
Concentration of all substrates was 5 mmol $l^{-1}$.
Data represent the mean±S.E.M. of three separate isolations with 1–3 runs per preparation.
*N* refers to the total number of determinations.

hexokinase activity in *Octopus* heart, as in vertebrate hearts (Sidell *et al.* 1987), is inhibited to a large extent under conditions of aerobic metabolism. It is possible that some of the glycolytic potential is utilized to support an anaerobic metabolism partially dependent upon blood-borne glucose. The assay-temperature-adjusted activity of HOAD was about 2.5-fold higher than in a population of *Octopus* previously sampled (Driedzic *et al.* 1990), placing it in the same range as activities found in hearts of teleost fish, which are considered to have a fatty-acid-based metabolism (Sidell *et al.* 1987). Citrate synthase (CS) activity was close to 35 $\mu$mol $g^{-1}$ $min^{-1}$. In vertebrate hearts, the maximal *in vitro* activity of CS is linearly related to power output (Driedzic *et al.* 1987). Substituting the value for *Octopus* into the equation for vertebrate heart yields a resting power output of 3 mW $g^{-1}$, a level very close to the measured power output of *Octopus* heart, implying a fundamental relationship between these two variables which transcends phyletic organisation.

Mitochondria isolated from *Octopus* systemic heart were able to oxidize malate, proline and glutamate (Table 2). In the absence of ADP, oxygen consumption with aspartate as the only exogenous fuel was negligible. ADP stimulated respiration by as much as threefold with some substrates. Relatively low respiratory control ratios (RCRs) suggest that the isolated mitochondria were partially uncoupled. However, this does not diminish the qualitative insights into the nature of the fuels that support respiration. Pyruvate alone did not support oxygen consumption. Oxygen consumption was higher in media containing glutamate or aspartate plus pyruvate than in those containing glutamate or aspartate alone, indicating that these amino acids could spark carbohydrate metabolism. Mitochondria from *Octopus* systemic ventricle are similar to mitochondria from squid ventricle in their ability to oxidize malate and proline

independently, and in the effectiveness of the combination of glutamate plus pyruvate to support oxygen consumption (Ballantyne *et al.* 1981; Mommsen and Hochachka, 1981). Free amino acid levels in *Octopus* heart and plasma are not known, but it is reasonable to anticipate high concentrations of glutamate and proline in common with *Octopus* mantle (Florkin and Bricteux-Gregoire, 1972), *Loligo opalescens* heart (Ballantyne *et al.* 1981) and *Sepia officinalis* blood (Hochachka *et al.* 1983). The current finding reveals that *Octopus* mitochondria have the capacity to oxidize four-carbon intermediates in the absence of input from carbohydrates. Thus, amino acids could serve as a metabolic fuel under some conditions and could contribute to RQ values lower than one.

Although the cephalopod heart is generally considered to be unable to oxidize fatty acids, the low RQ values exhibited by heart preparations perfused with blood and the high HOAD activity levels compelled us to re-examine the question of the potential for fatty acid oxidation. Neither acetyl CoA nor palmitoyl CoA, even in the presence of carnitine and ADP, supported oxygen consumption. State 3 respiration in medium containing acetyl CoA, carnitine and malate was no greater than in medium containing malate alone as a metabolic fuel (43.2 *vs* 45.2 natoms $O\,mg^{-1}$ protein $min^{-1}$, respectively; average of two trials from two separate preparations). Octanoate, palmitate or oleate alone could not be oxidized even in the presence of ADP. State 3 respiration in medium containing palmitate plus malate ($37.8\pm2.8$ natoms $O\,mg^{-1}$ protein $min^{-1}$, $N=5$) was no greater than in medium containing malate alone ($43.6\pm10.2$ natoms $O\,mg^{-1}$ protein $min^{-1}$, $N=3$). The same results were found with octanoate and oleate. The similarity of rates of malate-fuelled respiration with and without free fatty acids or CoA derivatives indicates that the high concentrations of these compounds did not disrupt mitochondrial function. The present findings confirm that CoA derivatives are not a suitable metabolic fuel for cephalopod mitochondria, as has been shown for squid (Ballantyne *et al.* 1981; Mommsen and Hochachka, 1981), and that a carnitine-independent mechanism for transport of fatty acids across the mitochondrial membrane, as reported for some insects (Stevenson, 1968) but not previously assessed in cephalopods, does not exist. These studies lend support to the concept that fatty acids are poorly oxidized by mitochondria from cephalopod heart and thus could not contribute to the low RQ values.

In conclusion, in *Octopus* heart the maximal *in vitro* activity of hexokinase overestimates the maximal aerobic use of glucose, but the maximal *in vitro* activity of cytochrome oxidase is closely matched to the maximal *in vivo* oxygen consumption. Low RQ values for heart perfused with blood, in concert with mitochondrial studies, suggest that amino acids may be catabolized on a sustained basis to support energy metabolism in *Octopus* heart.

We are very grateful to the staff of the Zoological Station and particularly to Professor B. Tota for facilitating these experiments. A portion of this work was supported by NSERC by grants to W.R.D. and J.M.S.

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