ANAEROBIC METABOLISM OF RED SKELETAL MUSCLE OF GOLDFISH, *CARASSIUS AURATUS* (L.)

Mitochondrial produced acetaldehyde as anaerobic electron acceptor

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1. Introduction

Among vertebrates, goldfish (*Carassius auratus*) and other cyprinidae have a remarkable tolerance to long periods of anoxia [1–3]. The anaerobic survival of goldfish is 16 h at 20°C [3] and several days at 4°C [4]. During these periods of long-term anoxia another terminal electron acceptor than oxygen should be operative to maintain redox balance and energy supply. The biochemical basis of this capability is not well understood. Exposure to 12 h anoxia at 20°C markedly decreases glycogen storage of skeletal muscle and liver indicating the activation of a glycolytic process [5]. Accumulation of lactate, the most important anaerobic endproduct of vertebrates, however is low compared to the depletion of glycogen storage [4–6]. Other metabolic endproducts of anaerobic metabolism like alanine, succinate, acetate and propionate are of minor importance [7,8]. Estimating the energy yield from different sources during anoxia only ~30% of the total energy need could be explained [7]. On this basis it was supposed that an unknown electron acceptor, coupled to energy production should be involved in anaerobic metabolism of goldfish. An indication for the operation of another metabolic pathway is found in the continuing excretion of metabolically produced carbon dioxide by anaerobic goldfish [3,9,10]. In [11] ethanol excretion was reported.

Here, the anaerobic metabolism of red skeletal muscle of goldfish is investigated. It is shown that anaerobic mitochondria convert pyruvate into acetaldehyde. The acetaldehyde produced, after transport to the cytoplasma, may serve as electron acceptor since the presence of a high activity of alcohol dehydrogenase allows the formation of ethanol. The anaerobic metabolism of red skeletal muscle will be discussed in relation to regulatory consequences and survival of long-term anoxia.

2. Materials and methods

2.1. *Animals*

Healthy 3-year-old goldfish (±80 g) were acclimated to 20°C for at least 2 months.

2.2. *Preparation of red muscle mitochondria*

The fish were anaesthetized with 100 ppm tricaine-methane sulfonate and killed by decapitation. Superficial red muscle was rapidly excised and cooled to 4°C. Mitochondria were isolated as in [3]. A slightly modified mannitol–sucrose-buffered medium was used: 210 mM mannitol, 120 mM sucrose, 50 mM Tris–HCl, 20 mM HEPES, 2 mM EGTA and 0.3% bovine serum albumin (pH 7.4). Pellet weight of the mitochondrial fraction, obtained by centrifugation at 10 000 × *g*, was used as parameter for mitochondrial activity.

2.3. *Incubation procedure*

The freshly prepared mitochondrial suspension (200 mg/ml) was diluted to 6 mg/ml in 3.5 ml reaction medium composed of 210 mM mannitol, 120 mM sucrose, 50 mM Tris–HCl, 20 mM HEPES, 2 mM EGTA, 10 mM KH_2PO_4 and different substrates. During incubation the reaction medium was continuously stirred. Reaction vessels were thermostatted at 20°C and closed by a Clark-type oxygen electrode

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(Yellow Springs Instr.) to record the oxygen tension during incubation. Anaerobic vessels were closed after complete expulsion of oxygen from the reaction medium by gassing with pure nitrogen for ⩾15 min. Reactions were initiated by injection of ADP or mitochondrial suspension into the closed vessels.

2.4. *Assay of metabolites*

During incubation 1 ml samples were taken with a syringe. Reaction medium and mitochondria were rapidly separated by means of ultrafiltration (Millipore, 0.45 μm pore size). Filtrates were analyzed for pyruvate, malate and acetaldehyde by means of standard enzymatic methods [12–14]. Determination of acetaldehyde is based on the reaction with aldehyde dehydrogenase. Since this enzyme is only group-specific the reaction product was analyzed by isotachophoresis. Formation of acetate was demonstrated, so the filtrate contained acetaldehyde. Ethanol was enzymatically determined with a test combination (Boehringer).

2.5. *Enzyme kinetics*

Properties of alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) were determined in tissue homogenates. Homogenates were prepared in a buffer, consisting of 250 mM sucrose, 20 mM Tris–HCl, 10 mM HEPES, 1 mM EGTA (pH 7.4) by a Potter-Elvehjem-type homogenizer with a Teflon pestle and centrifuged for 30 min at 30 000 × *g*. Oxidative properties of alcohol dehydrogenase (ADH) were determined in a continuous assay according to [15] with a modification of the acetaldehyde trapping reaction [14] by means of aldehyde dehydrogenase. Reduction of acetaldehyde by ADH was measured as in [16].

3. Results

Biochemical intactness of red muscle mitochondria was controlled by determining coupled aerobic properties as indicated by ADP:O and respiratory control (RC) ratio [17]. In the standard reaction medium with 2.5 mM pyruvate and 1 mM malate the mean ADP:O ratio was 3.60 ± 0.56 and RC ratio was 5.79 ± 1.43. These data provide evidence that isolated red muscle mitochondria are biochemically intact.

As a result of oxidative phosphorylation, the rates of aerobic pyruvate and malate uptake are markedly stimulated by ADP (table 1). In contrast, the low rate of aerobic acetaldehyde formation is not significantly influenced by ADP. The ratio of aerobic, ADP stimulated, pyruvate uptake and oxygen consumption is 0.72 ± 0.20. In absence of ADP the rate of pyruvate uptake exceeds oxygen uptake (ratio 1.77 ± 0.47)

Table 1
Aerobic and anaerobic metabolism of red muscle mitochondria in presence of pyruvate and malate (nmol . min^{-1} . 10 mg mitochondria wet w^{-1})

Substrates	Oxygen uptake	Pyruvate uptake	Malate uptake	Acetaldehyde production
Aerobic				
Pyruvate + malate	7.94 ± 2.14 (13)	14.04 ± 4.66 (10)	0.97 (2)	2.10 ± 1.14 (6)
Pyruvate + malate + ADP	44.42 ± 11.29 (13)	32.66 ± 9.64 (10)	9.70 (2)	1.91 ± 0.33 (3)
Anaerobic				
Pyruvate + malate	–	15.88 ± 4.87 (8)	0.00 (2)	4.33 ± 1.63 (7)
Pyruvate + malate + ADP	–	22.88 ± 7.29 (10)	0.00 (2)	6.72 ± 2.45 (9)
Pyruvate + ADP	–	24.68 (1)	–	7.78 (2)
Malate + ADP	–	–	n.d.[a]	0.00 (2)

[a] Not determined

Substrate concentrations of the reaction medium were 2.5 mM pyruvate, 1 mM malate and 2.5 mM ADP. 10 mM oxamate was added to inhibit low activity of lactate dehydrogenase, which is enclosed in the mitochondrial pellet. Mean values and standard deviations (where appropriate) from independent determinations (between brackets) are given

Table 2
Properties of alcohol dehydrogenase (EC 1.1.1.1) in goldfish tissues

	Ethanol (pH 9.05)		Acetaldehyde (pH 7.42)	
	K_m[a]	V_m[b]	K_m[a]	V_m[b]
Red muscle	3.32 ± 1.15	16.50 ± 1.63	0.22 ± 0.10	42.05 ± 1.85
White muscle	2.98 ± 1.19	4.04 ± 0.36	0.26 ± 0.03	13.75 ± 3.05
Heart muscle	–	–	n.d.[c]	0.00
Liver	0.19 ± 0.12	0.20 ± 0.06	0.27 ± 0.11	2.28 ± 0.22
Brain	–	–	n.d.[c]	0.00

[a] K_m-values expressed as mM
[b] V_m-values expressed as μmol . min^{-1} . g wet w^{-1} at 25°C
[c] Not detectable

indicating an anaerobic contribution to pyruvate metabolism. In absence of oxygen, ADP-mediated uptake of pyruvate is lowered but the stimulatory effect of ADP remains (table 1). In contrast to pyruvate uptake, the rate of malate uptake is completely inhibited by anoxia. Acetaldehyde formation is markedly stimulated to 30% of the anaerobic pyruvate uptake (table 1).

Investigating the intracellular localization of acetaldehyde formation, it appears that pyruvate decarboxylase activity is exclusively located in the mitochondria since no acetaldehyde formation could be demonstrated in the cytoplasma of red muscle. Alcohol dehydrogenase (ADH) of red skeletal muscle is located in the cytoplasma for ADH activity of mitochondria is very low (0.020 μmol . min^{-1} . g red muscle wet w^{-1}) compared to total activity (table 2). The low activity of ADH in mitochondria may be due to enclosure of the enzyme in the mitochondrial pellet. This assumption is confirmed by the observation that no ethanol formation of mitochondrial suspensions could be detected.

Concerning the tissue distribution of ADH it is evident that high activities are present in red and white skeletal muscle (table 2). At physiological pH the enzyme has a particular preference for reduction of acetaldehyde into ethanol. Red muscle ADH was found to be insensitive to iodoacetic acid, similar to mammalian liver ADH [18].

No ADH activity could be detected in heart muscle or brain. Liver ADH has a low activity and appears to be of a different type with a higher affinity to ethanol (table 2).

4. Discussion

We show that anaerobic red muscle mitochondria of goldfish convert pyruvate partially into acetaldehyde. The rate of acetaldehyde formation is ~30% the anaerobic pyruvate uptake. Aerobic acetaldehyde production is low and may originate from an anaerobic contribution to aerobic pyruvate metabolism. The oxygen-independent part of aerobic pyruvate conversion in vitro is possibly based on a similar mechanism as anaerobic metabolism of pyruvate. Intramitochondrial acetaldehyde may be produced from pyruvate in the first step of the pyruvate dehydrogenase complex, similar to single step decarboxylation in yeast. Acetaldehyde will be released from the decarboxylating part of the pyruvate dehydrogenase complex (thiamine–pyrophosphate-bound pyruvate dehydrogenase) as a result of the reduced (or absent) ability of the dehydrogenating part of the complex to oxidize the enzyme bound hydroxyethyl derivative into acetyl-CoA. This suggests an enzyme complex with loosely coupled properties that are different from mammalian pyruvate dehydrogenase. After the acetaldehyde has diffused into the cytoplasma it serves as electron acceptor to produce ethanol as the anaerobic end-product of skeletal muscle metabolism.

The high activity of ADH and the preference of the enzyme to reduce acetaldehyde at physiological pH indicate that skeletal muscle is well equipped to maintain anaerobic redox balance. Although fermentation of glycogen to ethanol produces only 2 ATP/glucose units, this pathway may be advantageous to animals living in a hypo-osmotic environment [19].

The anaerobic, neutral endproduct, ethanol, will easily penetrate membranes, diffuse into the surrounding water and hence cause no acidosis of the tissue [11].

In contrast to skeletal muscle, heart muscle does not possess any activity of ADH. Thus during exposure to anoxia this tissue should produce energy by another metabolic pathway, possibly by production of lactate. This may be transported via the blood-stream to skeletal muscle and be metabolized into ethanol [10].

Taking alcohol dehydrogenase activity as a measure for slow and long term anaerobic metabolism, and lactate dehydrogenase activity for fast and short term metabolism, it is clear that there is no unequivocal anaerobic pathway in different tissues of goldfish. Skeletal muscle and especially red skeletal muscle is well provided with metabolic properties to survive long periods of anoxia. The high density of mitochondria [3] and the activity of ADH in combination with a high content of glycogen [5] provide evidence for a special role of red skeletal muscle during anoxia. The physiological significance of the high anaerobic capacity of red skeletal muscle may be clear since this slow muscle is used to equilibrate the fish.

In conclusion the slow anaerobic metabolism of goldfish red muscle is based on cooperation of cytoplasmic and mitochondrial activities. The switch to slow anaerobic metabolism is made possible by the separate formation of the electron acceptor acetaldehyde in the mitochondria and the reduced endproduct, ethanol, in the cytoplasma. In addition to the slow anaerobic mechanism, red skeletal muscle possesses a high activity of lactate dehydrogenase [3] for fast production of anaerobic energy. Therefore it may be expected that a strict regulation of anaerobic pyruvate metabolism exists in order to discriminate between slow, ethanol producing, and fast, lactate producing, metabolism.

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