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Bioenergetic peculiarity of heart mitochondria from the hemoglobin- and myoglobin-free antarctic icefish

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Heart mitochondria of the hemoglobin and myoglobin-free antarctic icefish are specifically noncoupled with respect to succinate oxidation but not during NADH-dependent substrate oxidation. This observation suggests the coexistence of a noncoupled respiratory chain specialized in succinate oxidation and of the energy-conserving respiratory pathway. The alternative pathway of succinate would lead to the dissipation of the redox energy as heat and this thermogenic process could favour recovery after hypoxic stress periods, during which O_2 supply is severely impaired by the lack of both heme pigments.

Antartic fish of the family Channichthyidae, commonly named icefish, are unique among vertebrates, since the genes coding for hemoglobin and myoglobin are not expressed [1,2]. The lack of hemoglobin, lowering the oxygen-carrying capacity of the blood, is partly compensated by remarkable adaptations of the cardiovascular system involving heart hypertrophy, increased cardiac output, higher blood volume and large bore vessels and capillaries [3–5].

Most of the adaptive features designed to compensate for the absence of myoglobin are encountered in cardiac function. Diffusion distances between blood and cardiac cells are substantially reduced with a concomitant increase of the exchange area by an extensive microvascularisation of the spongious myocardium [6,7]. On the other hand, the unusually high lactate dehydrogenase activity reported in myocardium, in fact one of the highest values found in heart, reflects the involvement of anaerobic glycolysis in the energetics of icefish cardiac muscle [8].

Electron micrographs have revealed an unusual complex organisation of cardiac mitochondrial cristae, having a dense and vesiculated aspect strictly different from the classical morphology found in other vertebrates heart mitochondria [7,9]. This observation prompted us to analyze the respiration of icefish cardiac mitochondria. We report here the existence of a noncoupled succinate oxidation and its possible implication in icefish cardiac bioenergetics.

Specimens of *Channichthys rhinoceratus* and of the red-blooded *Notothenia rossii* were caught in the Kerguelen archipelago (49°30′ S, 70° E). Fish were immediately used or kept alive for short time in running sea water. Isolation of cardiac ventricle mitochondria was carried out according to Tyler and Gonze [10], except that exposition to lower concentration of bacterial Nagarse (1 mg/g fresh weight) was reduced to 15 s and that 0.1% bovine serum albumin was added to the extraction buffer for the first dilution of the homogenate. Optimal centrifugation forces were 600 and $10\,000 \times g$.

Oxygen consumptions were recorded polarographically in a 2 ml reaction mixture containing 57.5 mM Tris, 15 mM KCl, 2 mM EDTA, 2.5 mM KH_2PO_4 (pH 7.4). Substrate-supported respiration rates were measured in the presence of 2.5 mM ADP and the indicated concentrations of substrates and inhibitors. Cytochrome oxidase activity was recorded in the above reaction mixture plus 2 mM ascorbate, 0.1 mM TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine), 5 μM rotenone and 5 μM antimycin A. Respiratory control and ADP/O were recorded by pulses of 150–300 nmol ADP and were calculated by the usual procedure [11] with a starting O_2 concentration in the polarographic

Abbreviations: 2-OG, 2-oxoglutarate; CAT, carboxyatractyloside; FCCP, carbonylcaynide-*p*-trifluoromethoxyphenylhydrazone.

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TABLE I

Oxygen consumptions, respiratory controls (RC) and ADP/O ratios of isolated cardiac ventricle mitochondria from two antarctic fish species

Substrate-supported respiration rates, expressed as nmol O_2 consumed per mg protein per min at 10 °C, were measured in the presence of 2.5 mM ADP and the indicated concentrations of substrates and inhibitors. RC and ADP/O are range values.

Species	2-Oxoglutarate (5 mM)			Succinate (5 mM) rotenone (5 μM)			Palmitoyl CoA (40 μM) carnitine (2 mM)			3-Glycerophosphate (5 mM)	Cytochrome oxidase
	O_2 uptake	RC	ADP/O	O_2 uptake	RC	ADP/O	O_2 uptake	RC	ADP/O	O_2 uptake	O_2 uptake
C. rhinoceratus	45±2 (4)	5.5–10	2.9–3.2	22±2 (6)	1	0	53±6 (3)	1.5–3.7	1.5–1.9	0 (2)	45±3 (3)
N. rossii	51±3 (4)	4.5– 6	2.8–3.0	44±3 (4)	1.9–2	1.8–2.0	33±3 (3)	4.2–4.3	2.4–2.5	0 (2)	69±4 (3)

vessels of 260 μM. Protein concentrations were determined by the Biuret method [12]. Determination of cytochrome concentrations from frozen mitochondrial pellets was carried out according to Ref. 13.

Polarographic measurements of oxygen uptake and oxidative phosphorylation parameters of cardiac mitochondria from the icefish *C. rhinoceratus* and a common red-blooded antarctic species, *N. rossii*, are given in Table I. Icefish cardiac mitochondria differ from those of the red-blooded species by lower cytochrome concentrations (Table II) and lower activities of 2-oxoglutarate (2-OG) oxidase, cytochrome oxidase and mainly of succinoxidase (Table I). A significantly higher respiration rate is, however, recorded when a fatty acyl-CoA is used as a substrate. Phosphorylation capacities have been assayed in phosphate-acceptor limiting conditions (Fig. 1A, Table I). Using a NADH-dehydrogenase-linked substrate (2-OG), tightly coupled cardiac mitochondria from both fishes maintain ADP/O ratios of 3 even after 6–8 ADP pulses, and respiratory control greater than 5 even when the oxygen concentration is lower than 12 μM. In the case of *N. rossii*, reducing equivalents arising from succinate oxidation reach the respiratory chain via complex II with a concomitant ADP/O ratio around 2 (Table I). Surprisingly, under the same conditions, succinate oxidation by icefish cardiac mitochondria is totally independent of ADP (Fig. 1B), leading to a lack of respiratory control (Table I). This observation strongly suggests that unlike 2-OG oxidase activity, succinate-supported respiration is not coupled to oxidative phosphorylation in these mitochondria (ADP/O = 0). In order to confirm polarographically such an unusual property of the icefish cardiac respiratory chain, we inhibited mitochondrial respiration by carboxyatractyloside (CAT), inhibitor of the adenylate translocator, and oligomycin, inhibitor of the ATP synthase. Whereas 2-OG-supported respiration is severely inhibited by these substances (Table III), illustrating the control of the respiration by ADP levels inside the mitochondria, oxidation of succinate is unaffected by CAT and oligomycin. The succinate-supported respiration of icefish cardiac mitochondria is not therefore under the control of the phosphate potential and would not be linked to ATP synthesis. Furthermore, addition of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) does not affect succinate oxidation (Table III). By contrast, 2-OG oxidation is stimulated by FCCP, even in the presence of CAT and oligomycin, showing the classical effect of the uncoupler. Succinate oxidation in these mitochondria is thus independent of proton conductance, indicating that the electrochemical proton gradient ($\Delta\mu_{H^+}$) would not be generated during this process.

To investigate the organization of this peculiar respiratory chain, we have tested the effects of specific inhibitors on the four complexes. Table III describes the action of inhibitors on 2-OG- and succinate-supported respiration. One can observe that the noncoupled succinate oxidation is blocked by malonate, a competitive inhibitor of succinate dehydrogenase and that all inhibitors show their usual effects on both respirations. This

TABLE II

Cytochrome concentrations (in nmol per mg proteins) in cardiac mitochondria from two antarctic fish species

Species	aa_3	b
C. rhinoceratus	0.92±0.18 (3)	0.09±0.006 (3)
N. rossii	1.27±0.12 (3)	0.09−0.11 (2)

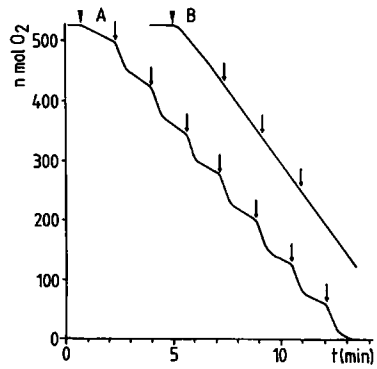

Fig. 1. Electrode tracings of oxidative phosphorylation capacities in heart ventricle mitochondria of *C. rhinoceratus* with 5 mM 2-OG (A) or 5 mM sucinate+5 μM rotenone (B) as substrate. Arrowheads and arrows indicate addition of mitochondria and 300 nmol ADP pulses, respectively. Mitochondrial protein final concentration: 1.8 mg. The same mitochondrial preparation is used in A and B.

TABLE III

Effect of various inhibitors, an uncoupler (FCCP) and guanosine 5'-diphosphate on the cardiac mitochondrial respiration of the icefish C. rhinoceratus

Oxygen consumptions are expressed as nmol O_2 consumed per mg proteins and per min at 10 °C. Final concentrations of substrates and added substances are indicated. Before experiment, pooled mitochondria were tested for their respiratory control: only those with respiratory control higher than 5 were used. n.d., not determined.

Additions	O_2 uptake in presence of	
	2-OG (5 mM)	succinate (5 mM) rotenone (5 μM)
None (State III)	45	20
Carboxyatractyloside (5 μM)	7	20
Oligomycin (20 μM)	11	20
FCCP (1 μM)	51	20
Rotenone (5 μM)	0	
Antimycin A (5 μM)	< 0.5	< 0.5
Malonate (1.5 mM)	n.d.	0
KCN (125 μM)	0	0
SHAM (2 mM)	45	20
GDP (1.25 mM)	n.d.	20

indicates that the modifications of the respiratory chain properties allowing the occurrence in these mitochondria of coupled and noncoupled respirations are not translated in terms of respiratory complexes sensitivity to inhibitors and general organization.

In view of the low succinoxidase activity of icefish cardiac mitochondria it is conceivable that the passive proton backflow could counteract a weak protonmotive force associated with succinate oxidation, leading to the absence of detectable respiratory control. This was however ruled out by inhibiting with malonate the succinoxidase activity of rat mitochondria to level similar to that recorded in icefish. Table IV shows that these mitochondria still display significant ADP stimulation. On the other hand, succinoxidase activity of icefish cardiac mitochondria has been assayed at 25 °C, thus thermally increasing the activity to 30 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$. Despite the one-third enhancement of the succinoxidase activity no ADP stimulation was seen.

TABLE IV

Respiratory rates (State III) and respiratory control of isolated rat liver mitochondria before and after addition of malonate

Oxygen consumptions are expressed as nmol O_2 consumed per mg proteins and per min at 25 °C.

Substrate	2-OG (5 mM)	Succinate (5 mM) rotenone (5 μM)	Succinate (5 mM) rotenone (5 μM) malonate (1 mM)
O_2 consumption	42	78	23
Respiratory control	6.6	2.9	2.1

A totally noncoupled succinate-oxidation process in mitochondria though perfectly able to drive ATP synthesis from NADH oxidation cannot be directly explained by the well-accepted model of the respiratory chain [14]. For example, it is not conceivable that, depending on the origin of reducing equivalents, either from complex I (NADH → complex I → ubiquinone → complex III → complex IV → O_2), or complex II (succinate → succinate dehydrogenase (II) → ubiquinone → complex III → complex IV → O_2) the protonmotive force generation would be switched on or off. This would require a ubiquinone able first to recognize the origin of reducing equivalents and second to control the proton-translocation properties of complexes III and IV.

A plausible hypothesis would be the coexistence of two different populations of cardiac mitochondria: one having very high succinoxidase activity, very low NADH dehydrogenase activity and membranes highly permeable to hydrogen ions and the other having opposite properties. However, one would then expect that succinate, formed in the Krebs cycle of the population oxidizing NADH-linked substrates, could be transported to the other population in order to be further oxidized. An alternative model may assume that the NADH oxidation pathway and the non-proton translocating pathway of succinate oxidation are located within the same mitochondrion in morphologically separated domains of the inner membrane, e.g., the crystal part and the boundary region, respectively. This could be tentatively related to the peculiar mitochondrial morphology reported previously [7]. Nonproton translocating alternative pathways are not unusual and have already been described in plant mitochondria [15]. These pathways are characterized by their insensitivity to cyanide ions and their sensitivity to hydroxamate ions. However, there is no analogy between these pathways and icefish cardiac respiratory chains as demonstrated by the lack of inhibitory effects of salicylhydroxamate and the blocking effect of KCN (Table III). It should be pointed out that no bypass exists between the two proposed respiratory chains if one considers on the one hand the perfect coupling of icefish cardiac mitochondria where no NADH-linked reducing equivalents are lost in the noncoupled pathway, and on the other hand, the total lack of respiratory control of succinate oxidation, indicating that succinate-linked reducing equivalents are not introduced in the H^+-translocating chain.

Beside mechanistic aspects, noncoupling of succinate oxidation has profound implication in icefish heart energetics. Indeed, at each turn of the Krebs cycle, the ATP yield of substrate oxidation is lowered by 2 units when succinate dehydrogenase oxidizes succinate into fumarate. If energy conservation mechanisms are lacking, the redox energy will be dissipated with a concom-

itant heat production. On the basis of the succinoxidase activity (Table I) and a yield of 6.6 mg of mitochondrial proteins per gram of heart tissue, one can calculate a theoretical thermogenic capacity of 0.72 W/kg, a value close to that of man, which liberates 1 W/kg body weight at rest [16]. The lack of effect of GDP on the succinate oxidation (Table III) indicates that a GDP-dependent proton channel, namely thermogenin, is not involved in the possible heat production in icefish heart.

This thermogenic process in the hemoglobin and myoglobin-free antarctic fish would provide a new adaptative feature to the lack of both heme pigments. Indeed, the heart is thought to become hypoxic as a consequence of the low oxygen supply during burst swimming or prey catching situations and would therefore rely on its powerful anaerobic capacity, with a subsequent lactate gradient built up [8]. Following activity, aerobic conditions are restored at rest and heat production associated with oxidative processes would improve oxygen diffusion through the cardiac plasma membrane and would also contribute to the lactate washout from sarcoplasm in the blood stream. Such a function of heat accumulation has also been proposed in the case of tuna skeletal oxidative muscles [17].

Succinate is also an end-product of a powerful ATP-generating anaerobic pathway of many hypoxia tolerant species where complex II is working as fumarate reductase [18]. If that anaerobic pathway is involved in icefish heart bioenergetics, succinate would be quickly metabolized during periods of rest if its oxidation is not coupled to ATP synthesis; otherwise the high phosphate potential produced and the low passive permeability of cell membrane to dicarboxylates would limit its elimination.

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