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The impact of the thermal sensitivity of cytochrome c oxidase on the respiration rate of Arctic charr red muscle mitochondria

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Abstract To assess if cytochrome c oxidase could determine the response of mitochondrial respiration to changes in environmental temperature in ectotherms, we performed KCN titration of the respiration rate and cytochrome c oxidase activity in mitochondria from Arctic charr (*Salvelinus fontinalis*) muscle at four different temperatures (1 °C, 6 °C, 12 °C, and 18 °C). Our data showed an excess of cytochrome c oxidase activity over the mitochondrial state 3 respiration rate. - Mitochondrial oxygen consumption rates reached approximately 12% of the cytochrome c oxidase maximal capacity at every temperature. Also, following titration, the mitochondrial respiration rate significantly decreased when KCN reached concentrations that inhibit almost 90% of the cytochrome c oxidase activity. This strongly supports the idea that the thermal sensitivity of the maximal mitochondrial respiration rate cannot be dictated by the effect of temperature on cytochrome c oxidase catalytic capacity. Furthermore, the strong similarity of the Q_{10}s of mitochondrial respiration and cytochrome c oxidase activity suggests a functional or structural link between the two. The functional link could be coevolution of parts of the mitochondrial system to maintain optimal functions in most of the temperature range encountered by organisms.

Key words Temperature · Mitochondria · Respiration · Cytochrome C oxidase · Fish

Abbreviations *BSA* bovine serum albumin · *COX* cytochrome c oxidase · *Hepes* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid · *RCR* respiratory control ratio

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Introduction

Mitochondrial metabolism in ectotherms is highly dependent on environmental temperature. For example, in fish, Q_{10}s of mitochondrial respiration have been shown to vary from approximately 2 to 4.8 depending on the species, the tissues, the substrate, and the temperature range (Ballantyne et al. 1989; Blier and Guderley 1993a, 1993b; Guderley and Johnston 1996; Hardewig et al. 1999; Weinstein and Somero 1998). Thermal sensitivity is also a property of higher-level physiological functions. In fish, sustained swimming capacity increases with temperature to an optimum and then decreases (Fry and Hart 1948; Brett 1967). Blier (1992) has proposed that the decrease in mitochondrial catalytic capacity, or impairments of mitochondrial regulatory properties could be the causes of the diminution of aerobic swimming capacity. This suggestion is brought forth following the observation that an increase in the abundance of mitochondria (Egginton and Sidell 1989; Johnston and Maitland 1980; Sänger 1993) and of the aerobic enzyme activities in locomotor muscles (for reviews see Guderley 1990; Guderley and Blier 1988) are common adaptive responses to the acclimation or acclimatization of fish species at low temperatures. The functional limitation of red muscle metabolism at low temperatures could be due to (1) decrease in maximal catalytic capacity of mitochondria; (2) limitations of metabolites or oxygen diffusion transfer between the myofibrillar and the mitochondrial compartment, or (3) a loss of regulator sensitivity (Blier 1992; Blier and Guderley 1993a, 1993b). Given the impact of mitochondrial sensitivity on fish swimming capacity and on animal energetics, we suspect that strong selective pressures have lead to optimization of functional properties of mitochondria at different temperature regimes. Some relevant studies have focused on potential adaptations by comparing the respiration

rates of mitochondria from fish that experience different thermal habitats (Johnston et al. 1994, 1998). These studies failed to reveal any clear compensation patterns, in terms of catalytic capacity, at low temperatures. For example, Johnston et al. (1998) found no significant up-regulation of the maximum rate of oxygen uptake per milligram of mitochondrial proteins in Antarctic fishes when compared to sub-Antarctic and Mediterranean fish species. Since mitochondria are a quite complex metabolic system, it is difficult to speculate on mechanistic explanations of thermal sensitivity, and therefore, to identify functional constraining factors. The latter is a problem that we face when we want to identify specific adaptations of mitochondria to different temperature regimes. Furthermore, the experimental conditions typically used differ from in vivo conditions. Most studies focused on state 3 of mitochondrial respiration, while in real conditions mitochondria are probably solicited at lower levels (somewhere between state 3 and state 4). Finally, we do not know which of the following functional properties (catalytic capacity or regulatory mechanisms) are most severely and significantly affected by temperature changes. As a result, we do not yet understand how the functional properties of this system are affected by temperature changes and which components are responsible for impairment at low and high temperatures.

Since the formulation of the theory of the control of metabolism in the seventies (Heinrich and Rapoport 1974; Kacser and Burns 1973), pioneer studies on rat liver mitochondria (Groen et al. 1982; Tager et al. 1983) found that complex IV (cytochrome c oxidase, COX), the dicarboxylate carrier (a metabolite transporter), and the adenine nucleotide carrier (the carrier of ADP and ATP) share most of the control of mitochondrial respiration. These studies suggest that control of mitochondrial respiration is unevenly distributed along the pathways, with some enzyme complexes contributing to a significantly higher proportion of the total control. Some more recent studies confirmed the importance of these enzymatic complexes, particularly the complex IV (COX), in the control of mitochondrial respiration in different organisms. COX has been identified as a potential site of allosteric regulation based on its far from equilibrium reaction and its multiple tissue specific subunits in mammals (Kadenbach 1986). It also has been shown to have a significant control over mitochondrial respiration rates (Davey and Clark 1996) and, according to recent studies on human cells (Villani et al. 1998), its maximal activity measured in vivo could be close to the rate of electron transfer reached during the state 3 of respiration. Considering the potential importance of COX in the regulation and in the establishment of the catalytic capacities of mitochondria, the thermal sensitivity of this enzyme could, therefore, be of major significance in the impairment of the functional properties of mitochondria during temperature changes in ectotherms.

The aim of this study is to evaluate how the thermal sensitivity of COX can affect the respiration rate of Arctic charr (*Salvelinus alpinus*) red muscle mitochondria. This was achieved by the titration of COX activity and mitochondrial respiration with KCN at different temperatures. The Arctic charr is a stenothermal fish from cold habitats and is strictly adapted to a narrow range of temperatures. According to Baroudy and Elliott (1994) Arctic charr are amongst the least resistant of salmonids to high temperatures. The mitochondrial metabolism of this species should, therefore, be strongly affected by temperature changes. In a recent study (Glémet 1997), we have shown that the functional properties of Arctic charr red muscle mitochondria differ from those of a more eurythermal related species (*S. fontinalis*) with higher level of COX when expressed per unit of mitochondrial proteins. This suggests some functional adaptations of *S. alpinus* red muscle mitochondria that could be modulated partly by COX activity. Consequently, Arctic charr appears to be a good animal model to study the functional determinants of the thermal sensitivity of mitochondrial metabolism.

Materials and methods

Experimental animals

Arctic charr (*S. alpinus*) ranging from 35 cm to 42 cm were obtained from SODEQ fish culture station (Nouvelle, Québec) where they were kept at a temperature of 3–4 °C. They were transported to the Université du Québec à Rimouski 10–15 days before physiological measurements. During this period, fish were kept in 150-l tanks at a temperature of 9 °C and were not fed. This temperature was chosen because it represents the median of measurement temperatures (1 °C, 6 °C, 12 °C, and 18 °C) which closely match the tolerance range for the species (Baroudy and Elliott 1994). However, to make sure that acclimation time did not affect our data, the experimental measurement temperatures were selected randomly and each fish was acclimated at least 10 days and at most 15 days before measurements.

Isolation of mitochondria

Fish were killed by a blow to the head and the superficial lateral red muscle was rapidly removed and minced with a razor blade. All the manipulations were carried out on ice except for the centrifugations, which were performed at 4 °C. The tissue was homogenized in six volumes of 250 mmol l^{-1} sucrose, 1 mmol l^{-1} EGTA, 30 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 1% bovine serum albumin (BSA) (Chamberlin et al. 1991) adjusted to pH 7.4 at 20 °C. The homogenization was performed by one 5-s pass in a homogenizer (Tekmar company, USA) and three passes in a Potter-Elvejhem grinder with a tightly fitting pestle. The homogenate was centrifuged at 750*g* for 10 min. The supernatant was collected without removing the upper lipid layer and centrifuged at 10,000*g* for 5 min. The pellet was resuspended in 20 ml of the isolation medium and then centrifuged for 5 min at 10,000*g*. The resulting mitochondrial pellet was resuspended in the isolation medium (1.20 ml) and kept on ice until used. For COX oxygen consumption measurements, we sampled 500 µl of the mitochondrial suspension and added Triton X-100 to a final concentration of 0.09%. The COX suspension was homogenized two times for 10 s with a Tissue Tearor (Biospec Products) and stored at −80 °C until used.

Table 1 Body mass, mitochondrial (malate, pyruvate) and cytochrome c oxidase (COX) oxygen consumption rate, repiratory control ratio (RCR), and mitochondrial state 3 versus COX respiration rate (V_{max}) ratio at 1 °C, 6 °C, 12 °C, and 18 °C for red muscle of *Salvelinus alpinus*. All values are expressed as mean ± SD with $n = 5$, except for COX respiration rate at 1 °C where $n = 4$

Temperatures	Body mass (g)	State 3 (nmol O_2 min^{-1} mg $protein^{-1}$)	State 4	COX respiration rate	RCR	Mitochondrial/COX respiration rate (%)
1 °C	792.3 ± 52.4	27.3 ± 5.8[a]	6.5 ± 1.1[a]	241.3 ± 53.7[a]	4.4 ± 1.7	11.4 ± 3.5
6 °C	798.7 ± 97.8	46.5 ± 7.7[b]	11.4 ± 1.9[b]	433.2 ± 147.5[a]	4.1 ± 0.6	11.7 ± 3.6
12 °C	873.5 ± 48.8	79.6 ± 14.3[c]	17.0 ± 1.7[c]	677.2 ± 96.1[b]	4.7 ± 0.9	12.2 ± 3.8
18 °C	854.9 ± 111.8	95.2 ± 10.4[c]	23.7 ± 2.0[d]	862.6 ± 166.6[b]	4.0 ± 0.5	11.4 ± 2.7

[a,b,c,d] Significant differences within columns are indicated by different subscript letters ($\alpha < 0.05$)

Mitochondrial and COX oxygen consumption and titration

Mitochondrial and COX oxygen uptake were measured with a Clark-type oxygen electrode (YSI). The respiration chamber was maintained at the incubation temperature (1 °C, 6 °C, 12 °C or 18 °C) and the final volume was 1.22 ml. The system was calibrated with distilled water saturated with oxygen at the incubation temperature.

For mitochondrial respiration rate, the reactive medium contained 130 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 11 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} glucose, 10 mmol l^{-1} KH_2PO_4 and 0.5% BSA (essentially fatty-acid free; Blier and Guderley 1993a). The pH was adjusted to 7.4 at 15 °C. The substrates used were malate and pyruvate at a final concentration of 5 mmol l^{-1} each. State 3 of mitochondrial respiration was induced by addition of ADP at a final concentration of 0.5 mmol l^{-1}. The respiratory control ratio (RCR) was calculated according to Estabrook (1967). We used the terms state 3 and state 4 respiration as defined by Chance and Williams (1956). The titration of mitochondrial respiration by KCN was performed by the addition of different final concentrations of KCN (1, 2, 3, 5, 8, 10, 15, 20 mmol l^{-1}) added to the chamber 2 min before the addition of ADP. For each measurement, 1.8 (1 °C) or 1.3 mg (6 °C, 12 °C, and 18 °C) of mitochondrial protein were added to 1.13 (1 °C) or 1.14 ml (6 °C, 12 °C, and 18 °C) of the medium.

For COX activity measurements, the reactive medium was the same as for mitochondrial respiration rate but with 10 mmol l^{-1} ascorbic acid (Villani and Attardi 1997). To measure the maximal rate of activity, we added cytochrome c at a final concentration of 50 μmol l^{-1}. The titration of COX activity was performed by addition of KCN (5 μl) to reach different final concentrations (0.05, 0.1, 0.5, 1, 2, 3, 5, 8, 10, 15, 20 mmol l^{-1}) in the cells 2 min before starting the reaction (addition of cytochrome c). For each measurement, 143 μg (1 °C), 104 μg (6 °C) or 78 μg (12 °C or 18 °C) of mitochondrial protein with Triton X-100 was added to 1.18 (1 °C), 1.19 (6 °C), or 2.00 (12 °C or 18 °C) of reactive medium.

Q_{10} values for mitochondrial and COX respiration were calculated according to the following formula: $Q_{10} = (\mathrm{Rate}^{t2}/\mathrm{Rate}^{t1}) \exp 10/(t_2 - t_1)$.

Protein assays

Total protein content of each suspension was determined using the bicinchoninic acid method (Smith et al. 1985). Mitochondrial protein was determined by subtracting the protein concentration of the isolation medium from that of the corresponding mitochondrial suspension.

Chemicals

Potassium cyanide (KCN) was obtained from Aldrich Chemical Company (Oakville, Ont., Canada). All others chemicals were obtained from Sigma Chemical (St. Louis, Miss., USA).

Statistical analyses

Statistica software was used for all statistical analyses. *F*-max tests were used to test homogeneity of variances among the four temperatures groups. One-way ANOVAs were used to test differences among temperatures and Tuckey tests were used for a posteriori pairwise comparisons. Significance was assessed at the 0.05 level for all tests.

Results

The mean RCRs of mitochondrial preparation were between 4.0 ± 0.5 (18 °C) and 4.7 ± 0.9 (12 °C). Temperature had no effect (Table 1). The RCR values for muscle tissue suggest that mitochondrial preparations were of good quality. States 3 and 4 of mitochondrial respiration showed significant increases with temperature. State 3 was 27.3 ± 5.8 nmol O_2 min^{-1} mg $protein^{-1}$ at 1 °C and went to 95.2 ± 10.4 at 18 °C while state 4 went from 6.5 ± 1.1 to 23.7 ± 2.0 in the same temperature range. The COX activity showed the same pattern (Table 1). The ratio of mitochondrial respiration to COX remained similar at each temperature (11.4% at 1 °C and 18 °C, 11.7% at 6 °C, and 12.2% at 12 °C). This reflects the similar Q_{10} of mitochondrial respiration and COX activity, and therefore, the similar pattern of Q_{10} changes with temperature range. For state 3 and state 4 of mitochondrial respiration and COX activity, the Q_{10} values were the highest at the lower temperature range (1–6 °C) and decreased with increasing temperature (Table 2).

The titration of mitochondrial respiration and COX activity showed similar curves at the four temperatures (Fig. 1). COX was strongly inhibited at much lower concentration of KCN than the whole mitochondrial respiration. Fifty percent inhibition of COX activity was reached at less than 10 μmol l^{-1} of KCN in the medium. The same level of inhibition of mitochondrial respiration

Table 2 Q_{10} values for mitochondrial and COX respiration rate of Arctic charr (*Salvelinus alpinus*)

Temperature range	State 3	State 4	COX
1–6 °C	2.90	3.08	3.22
6–12 °C	2.45	1.95	2.11
12–18 °C	1.35	1.74	1.50

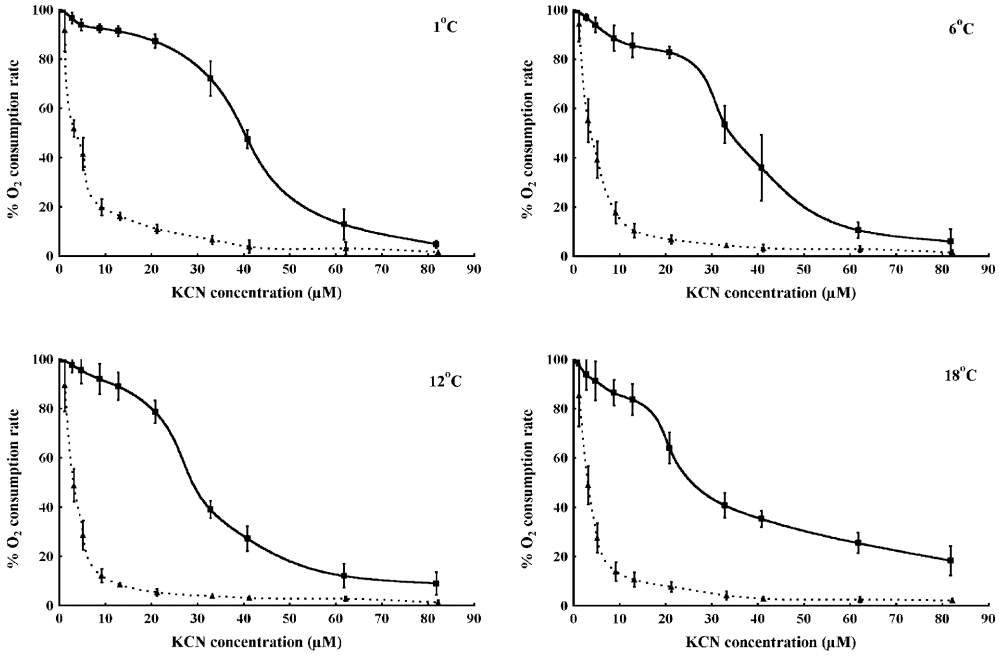

Fig. 1 KCN inhibition of mitochondrial (squares) and COX (triangles) oxygen consumption rate at 1 °C, 6 °C, 12 °C and 18 °C in red muscle of Arctic charr (*Salvelinus alpinus*)

was not reached until KCN concentration reached 30 μmol l^{-1}. The relation between the inhibition of COX activity and the inhibition of mitochondrial respiration was similar under each temperature (Fig. 2). The respiration rate of mitochondria slightly decreased with increasing KCN concentration and remained close to 90% of the maximal value until approximately 90% of the V_{max} of COX had been inhibited. Mitochondrial respiration rate sharply decreased with further increases in KCN concentrations. When 50% of COX V_{max} was inhibited, mitochondrial respiration remained at 91.2% (1 °C) and 96.3% (12 °C) of its maximal values. When the COX inhibition reached 90% of V_{max}, the mitochondrial respiration rates were still between 83.2% (1 °C) and 90.0% (12 °C).

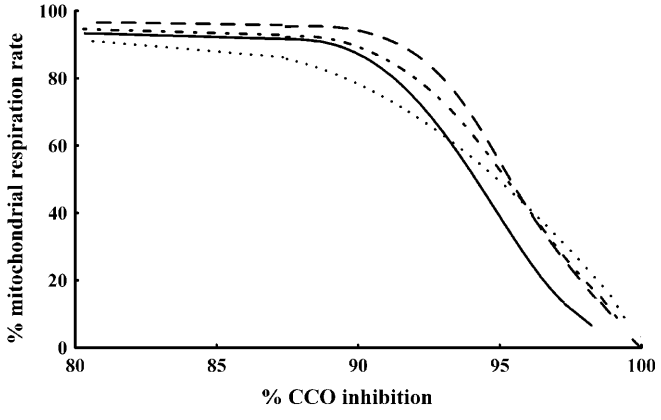

Fig. 2 Percentage of mitochondrial respiration rate as a function of percentage of COX inhibition in red muscle of Arctic charr (*Salvelinus alpinus*) at 1 °C (*solid line*), 6 °C (*dot-dash line*), 12 °C (*dash line*) and 18 °C (*dotted line*)

Discussion

The maximal rates of mitochondrial respiration measured in the present study are similar to most of the values obtained for fish muscle mitochondria when measured in the same temperature range (Moyes et al. 1988, 1992; Johnston et al. 1994, 1998) while they are somewhat higher than values obtained for rainbow trout (*Oncorhynchus mykiss*) red muscle mitochondria (Blier and Guderley 1993b). This could reflect either a higher purity of the mitochondrial preparation, utilization of a respiratory medium that allowed the mitochondria to reach a higher proportion of its maximal capacity, or a higher catalytic capacity of mitochondria from Arctic charr. In the latter case, it would indicate some compensatory mechanism of Arctic charr mitochondrial metabolism to low temperatures. In a recent study (Glémet 1997), the comparison of mitochondria from two wild populations of brook charr (*S. fontinalis*), one pure and one which has been completely introgressed with the mitochondrial genome of Arctic charr (*S. alpinus*), has revealed differences in the respiration rates and in the activity of COX at different temperatures. This clearly suggests that functional adaptation of mitochondria could be associated with mitochondrial genotypes and that mitochondria from different species could have different functional properties according to their ecology and their habitat. The Q_{10}s for pyruvate and malate oxidation are also similar to those obtained from rainbow trout red muscle (Blier and Guderley 1993b; Guderley et al. 1997) and follow the same pattern, which is a decrease at higher temperatures. This decrease of mitochondrial thermal sensitivity at higher temperature is somewhat challenging to explain since

Arctic charr is a strictly low-temperature stenothermic fish and we would intuitively expect a lower thermal sensitivity at low temperature for a cold-water fish. One could speculate that the differences observed in Q_{10} are related to uncoupling of oxidative phosphorylation at some temperature through the modification of membrane structure and concomitant changes in proton leak. However, in the present study, this situation is unlikely since temperature has no effect on RCR (Table 1). In lake charr (*S. namaycush*), the same increase in Q_{10} for pyruvate oxidation by liver mitochondria functioning at low temperature was assumed to reflect phase change in membrane phospholipids (Ballantyne et al. 1989).

Another challenging observation is the similarities of the Q_{10}s of mitochondrial respiration (state 3 and state 4) with those of COX activity (Table 2). Again the thermal sensitivity of COX activity decreases with increases in temperature. It is likely, since COX is an enzymatic complex located within the inner membrane of mitochondria, that the increase of Q_{10} at lower temperatures is related to modification of the structural and physical properties of membrane phospholipids which are in close contact with COX. It has already been established that the physical and chemical properties of the lipids surrounding an enzyme can affect its functional properties (Dahlhoff and Somero 1993). The similarity in Q_{10} of mitochondrial respiration and COX maximal activity could, therefore, result from the modulation of both by a common parameter, which could be the structural and functional properties of membrane phospholipids.

Another interpretation would be that both are functionally linked (the thermal sensitivity of mitochondrial respiration is induced by COX activity). However, if we look at the ratio of the mitochondrial respiration over the COX activity, we realize first that COX activity is highly in excess and that this excess is maintained independently of the temperature changes (the ratio of mitochondrial respiration over COX activity is between 11.4% and 12.2% at the four temperatures). There is, therefore, little chance that the COX activity could set limitations over mitochondrial respiration. This interpretation is concordant with the titration data (Figs. 1, 2). At each temperature, the COX maximal activity should reach a significantly high level of inhibition before impairment of mitochondrial respiration. For example, mitochondria still reached 83–90% of its maximal respiration rate at KCN concentration that inhibited 90% of the COX activity (Table 3, Fig 2). These data clearly match with the ratio of maximal respiration rate to maximal COX activity. The ratios (Table 1) indicate that in our in vitro measurements of maximal respiration rates, mitochondria utilize approximately between 11.4% and 12.2% of the COX activity available. Furthermore, the relationships between COX activity and mitochondrial respiration do not seem to be temperature dependent.

Table 3 Percentage of mitochondrial oxygen consumption rate at 50% and 90% COX inhibition in red muscle of *Salvelinus alpinus*

	COX_{50} (%)	COX_{90} (%)
1 °C	94.2	89.2
6 °C	95.5	89.9
12 °C	95.5	91.5
18 °C	95.0	81.8

Excess of COX activity (in the order of three to four times) has also been reported in mammalian mitochondria (Rossignol et al. 1999). The excess of the catalytic capacity of COX in mitochondria questions the importance of available COX catalytic capacity in the determination of mitochondrial respiration rate. Moreover, this excess is quite constant in the whole temperature range experienced by *S. alpinus*. This suggests that the surplus of catalytic capacity in COX could be required for the adequate functioning of mitochondria and that COX could have evolved to closely match the requirements of mitochondria at any temperature. One can, therefore, ask why so much COX activity in a pathway that operates at 12% of the V_{max} of the enzyme? Enzyme excess in metabolic pathways has been known for a long time. For example, most of the reactions in glycolysis are catalyzed by enzymes whose V_{max} values may exceed pathway flux rates by up to three orders of magnitude (Gitomer and Veech 1988). As pointed out by Suarez et al. (1997), a higher catalytic capacity (k_{cat}) could result from a decline in the affinity of the enzyme for its substrate (Km > [substrate]) in such a way that in vivo reaction can be regulated by changes in the enzyme affinity for the substrate (Atkinson 1977; Ferscht 1977). In recent contributions, Kadenbach and his colleagues have shown that the hyperbolic kinetics of COX in presence of ADP was changed into sigmoidal kinetics in presence of ATP (Arnold and Kadenbach 1997, 1999) due to the exchange of ADP by ATP at the matrix domain of subunit IV (Napiwotzki and Kadenbach 1998). Under in vivo conditions, therefore, the high catalytic capacity of COX would ensure that changes in reduced cytochrome c concentration could lead to adjustments of COX activity, and the affinity of COX to reduced cytochrome c could be modulated by allosteric effectors (ADP and ATP). In studies on human uncoupled and permeabilized cells, Villani and Attardi (1997) and Villani et al. (1998) have found a COX capacity nearly limiting (7–22% excess) for ADP + glutamate/malate-dependent respiration. These data on cellular preparations could reflect in vivo situations where the conditions for the regulation of COX (for example the presence of cellular metabolites in physiological concentrations) are operating in opposition to the in vitro conditions. The allosteric inhibition of COX by ATP is sensitive to various compounds (conditions) and could be uncoupled in most studies performed on isolated mitochondria or COX preparations (see Kadenbach and Arnold 1999). COX activity in physiological conditions (substrate and effectors concentrations, and physical and chemical state of the microenvironment) should, consequently, match the flux that can be reached by mitochondria. Another good

reason to maintain a high catalytic capacity of COX would be to maintain the electron transport chain mainly in an oxidized state to insure a sharp thermodynamic gradient in the electron-transfer system (ETS) under most conditions, and then maintain free access of electrons to the ETS. Finally, an alternative explanation came from Gnaiger et al. (1998) who suggested that the "apparent excess capacity of COX compared to the aerobic mitochondrial capacity is a basis for the high oxygen affinity of the respiration chain" which is required in a low-oxygen environment cell. This explanation could be particularly relevant for fish muscle mitochondria since the oxygen availability is relatively low for aquatic organisms (Withers 1992) and that (at least in mammals) maximal muscle activity and body oxygen consumption is limited by cardiac output and oxygen supply to the muscle (Brown 1992).

Mitochondria are complex and highly integrated enzymatic systems. Each mitochondrial enzyme likely possesses its own thermal sensitivity. Temperature change, consequently, will first affect the integration of the different parts of the system and lead not only to changes in catalytic capacity, but also to changes in the sensitivity of the regulatory process. For example, in a study of the effects of temperature on rat liver mitochondria, Dufour et al. (1996) have shown that at decreasing temperatures the control over the rate of substrate oxidation, at low respiration rate, switched from a shared control by the substrate oxidation system and the phosphorylation system to a control mostly exerted by the substrate oxidation system. While in state 3 of respiration, the same mitochondria switched from a system controlled mainly by substrate oxidation to a system controlled by the substrate oxidation and the phosphorylation. This clearly illustrates that temperature not only affects the catalytic capacity but also the mechanisms of regulation of mitochondria. Furthermore, this effect of temperature depends on the respiration state. The effect of temperature on regulatory mechanism could be highly significant since mitochondria could fail to achieve the appropriate ADP phosphorylation rate, according to the cell requirement, even at moderate respiration rate. This could therefore impose recruitment of anaerobic glycolysis.

Our data indicate that the maximal catalytic capacity of COX cannot limit the state 3 of mitochondrial respiration under all temperatures experienced by *S. alpinus*. The excess measured in the present study suggests that when mitochondria are close to state 3, any adjustment of COX will have only a small impact on mitochondrial respiration rate. However, the identical ratios of maximal COX activity to the state 3 of mitochondrial respiration, as well as the similar threshold of COX inhibition of the titration curves obtained at four different temperatures, suggest that mitochondria and COX could be adapted to maintain a given excess under most of the temperatures encountered by the species. The significance of these adaptations could be dictated by the regulatory function of COX in situ. Further comparative studies among endotherms and ectotherms on the kinetics of COX should reveal the importance of this enzyme on the efficient regulation of aerobic metabolism, as well as its evolutionary plasticity.

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