

## MITOCHONDRIAL ACTIVITY IN RAINBOW TROUT RED MUSCLE: THE EFFECT OF TEMPERATURE ON THE ADP-DEPENDENCE OF ATP SYNTHESIS

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### Summary

By coupling mitochondrial ATP production to an enzymatic NADPH-producing reaction, we monitored the rates of ATP production spectrophotometrically. ATP production was coupled to the oxidation of malate and pyruvate. Interference by adenylate kinase was eliminated by using P<sup>1</sup>,P<sup>5</sup>-di(adenosine-5')pentaphosphate and we found no interference by NADPH oxidase. We studied the kinetics of ATP synthesis by mitochondria from rainbow trout red muscle at three temperatures (8, 15 and 22°C) and under two pH regimes (a constant pH of 7.4 and temperature-dependent pH). The mitochondria oxidized pyruvate and malate. The apparent Michaelis constant ( $K_{m,app}$ ) for ADP as well as the maximal velocity ( $V_{max}$ ) for ADP phosphorylation are markedly affected by temperature but not by pH. The  $K_{m,app}$  for ADP decreases with increasing temperature while the  $V_{max}$  increases. These data suggest that reduced temperatures decrease mitochondrial sensitivity to control by ADP availability.

### Introduction

Temperature has pervasive effects on the swimming capacity of fish. It has been known for at least 40 years that low-temperature acclimation can partly overcome these limitations on locomotor capacity (Fry and Hart, 1948; Brett, 1967). Over this time, many researchers have examined the structural and metabolic responses to cold acclimation in the swimming musculature of fish. The commonly observed responses are increases in the activity of enzymes in oxidative pathways and in the mitochondrial volume density in red and white fibres (for reviews, see Johnston and Dunn, 1987; Guderley and Blier, 1988; Egginton and Sidell, 1989). These responses suggest that the oxidative capacity of muscle fibres becomes limiting at low temperatures. Increases in mitochondrial abundance are commonly assumed to reflect compensation for catalytic or diffusive limitations (for a review, see Guderley, 1990). An alternative interpretation is that an increased abundance of mitochondria enhances the sensitivity of mitochondrial respiration to changes in the levels of cytosolic signals such as ADP (Egginton and Sidell, 1989). This interpretation is supported by the demonstration of Dudley *et al.* (1987) that increases in the oxidative capacity of mammalian muscle lead to larger increases in

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oxygen consumption for a given change in the level of modulators [ADP, ATP/ADP or ATP/(ADP×P<sub>i</sub>)], where P<sub>i</sub> is inorganic phosphate. While the precise nature of mitochondrial control mechanisms is somewhat controversial (for a review, see Brand and Murphy, 1987), the availability of ADP seems to play a key role.

The purpose of this study is to determine the effect of temperature and pH on the sensitivity of mitochondria to control by ADP availability and on the maximal catalytic capacity of mitochondria *in vitro*. We have done this by determining the kinetic variables, apparent Michaelis constant ( $K_{m,app}$ ) and maximal velocity ( $V_{max}$ ) that describe the ADP-dependence of mitochondrial ATP production/ADP utilization. To reproduce the physiological conditions in which mitochondria normally function, we chose pyruvate and malate as substrates. Fish muscle mitochondria have a high capacity to oxidize these substrates (P. U. Blier and J. Ballantyne, unpublished data; Mourik, 1983; Johnston, 1987; Moyes *et al.* 1989, 1992). As fish muscles metabolize amino acids during sustained swimming (Mommensen *et al.* 1980; Van den Thillart, 1986), pyruvate is likely to be an important substrate oxidized by red muscle *in vivo*. Finally, we have determined the kinetics of ATP production/ADP utilization at two pH/temperature regimes to evaluate the impact of the cellular adjustment of pH during body temperature change (Heisler, 1984).

Oxygen polarography has been, and continues to be, a major tool in the study of the mechanisms and the regulation of mitochondrial activity (Rickwood *et al.* 1987). This technique is particularly useful in assessing the fuel preferences of various tissues, the regulation of different pathways and the role of the elements of a pathway. However, because such polarographic measurements require that the oxygen activity in the reaction medium should decrease significantly in a reasonable time, each measurement requires many mitochondria. For example, Lemaster (1984) used 1–4mg of mitochondrial protein per millilitre of reaction medium in a study of the ATP-to-oxygen stoichiometry of oxidative phosphorylation. The large quantity of tissue required for this technique is a major obstacle for repeated determinations in a given preparation. We therefore developed a spectrophotometric method for measuring the rate of mitochondrial ATP synthesis.

## Materials and methods

### *Animals*

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), were purchased from a local fish hatchery (La Pisciculture du Lac William Inc.) and kept in a 1500l tank at 12±1°C for at least 4 weeks before experimentation. The fish (200–300g) were fed weekly and kept at a photoperiod of 10h:14h L:D. The two fish used for each mitochondrial preparation were starved for at least 24h prior to the experiment.

### *Isolation of mitochondria*

The fish were killed by a blow on the head, and the superficial lateral red muscle was immediately removed and minced. All the manipulations were carried out on ice except the centrifugations, which were performed at 4°C. The tissue was homogenized in six

volumes of  $150\text{mmol l}^{-1}$  KCl,  $20\text{mmol l}^{-1}$  Hepes,  $10\text{mmol l}^{-1}$  EDTA,  $5\text{mmol l}^{-1}$   $\text{MgCl}_2$  and 1% bovine serum albumin (BSA, essentially fatty-acid free) adjusted to pH 7.2 at  $22^\circ\text{C}$ . The homogenization was performed in a Potter–Elvehjem grinder by two passes with a loosely fitting pestle and one pass with a tightly fitting pestle. This homogenate was centrifuged at  $500g$  for 10min. The superficial lipid layer was removed and the remaining supernatant was recentrifuged at  $9000g$  for 10min. The resulting pellet was resuspended in  $210\text{mmol l}^{-1}$  mannitol,  $120\text{mmol l}^{-1}$  sucrose,  $20\text{mmol l}^{-1}$  Hepes and 0.5% BSA (essentially fatty-acid free). The pH was adjusted to 7.2 at  $22^\circ\text{C}$ . This suspension was centrifuged at  $500g$  for 10min. The supernatant was collected and centrifuged at  $9000g$  for 10min. The final pellet was then suspended in the same medium at a concentration of 20–25mg of mitochondrial protein per millilitre of preparation. We used the buffer containing  $150\text{mmol l}^{-1}$  KCl during the two first centrifugations to maximize the sedimentation of contractile proteins. The second buffer was used to avoid elution of cytochrome *c* from mitochondrial membranes by KCl and to ensure mitochondrial stability subsequent to the purification (for a review of these isolation procedures, see Sherratt *et al.* 1988).

#### *Measurement of mitochondrial respiration*

Mitochondrial oxygen uptake was measured with a Clark-type oxygen electrode (YSI). The respiration chamber was maintained at  $15^\circ\text{C}$  and the final volume was 1.6ml. The electrode was connected to a home-made transducer and a chart recorder (Baxter Canlab, Montréal Canada). The system was calibrated with the respiration medium saturated with oxygen at  $15^\circ\text{C}$ . The values of oxygen concentration were calculated from the data of Graham (1987) for physiological salines at different temperatures. They were corrected for the atmospheric pressure during calibration. The respiration medium contained  $130\text{mmol l}^{-1}$  KCl,  $30\text{mmol l}^{-1}$  Hepes,  $11\text{mmol l}^{-1}$   $\text{MgCl}_2$ ,  $20\text{mmol l}^{-1}$  glucose,  $10\text{mmol l}^{-1}$   $\text{KH}_2\text{PO}_4$  and 0.5% BSA (essentially fatty-acid free). The pH was adjusted to 7.4 at  $15^\circ\text{C}$ . The substrates used in this study were malate and pyruvate at final concentrations of  $0.5\text{mmol l}^{-1}$  and  $0.2\text{mmol l}^{-1}$  respectively. To measure the maximal rate of respiration we added ADP at a final concentration of  $0.25\text{mmol l}^{-1}$ . The RCR (respiratory control ratio) was calculated according to Estabrook (1967) and we used the terms state 3 and state 4 respiration as defined by Chance and Williams (1956). For each measurement, about 2mg of mitochondrial protein was added to 1.5ml of the medium.

#### *Spectrophotometric measurement of ATP synthesis*

The rate of ATP production was followed spectrophotometrically with a Varian Cary 210 spectrophotometer regulated at  $15^\circ\text{C}$ . The assay medium contained  $130\text{mmol l}^{-1}$  KCl,  $30\text{mmol l}^{-1}$  Hepes,  $11\text{mmol l}^{-1}$   $\text{MgCl}_2$ ,  $20\text{mmol l}^{-1}$  glucose,  $10\text{mmol l}^{-1}$   $\text{KH}_2\text{PO}_4$ ,  $0.4\text{mmol l}^{-1}$  NADP,  $0.14\text{mmol l}^{-1}$   $\text{P}^1, \text{P}^5$ -di(adenosine-5')pentaphosphate ( $\text{Ap}_5\text{A}$ ) and 0.5% BSA (essentially fatty-acid free). The substrates of the reaction were malate and pyruvate at final concentrations of 0.5 and  $0.2\text{mmol l}^{-1}$  respectively. The final volume was 1ml. Hexokinase (HK) was added in excess (1U, i.e. the quantity that converts  $1\ \mu\text{mol}$  of substrate to product per minute at  $25^\circ\text{C}$ ) to utilize ATP, and glucose-6-phosphate dehydrogenase (G-6-PDH) was added in excess (1U at  $25^\circ\text{C}$ ) to utilize

glucose 6-phosphate and to reduce NADP. In studies with rat liver mitochondria, Jacobus *et al.* (1982) established that hexokinase exerted no control over mitochondrial respiration when the ratio milligrams mitochondrial protein to units of hexokinase was less than 3. In our studies, this ratio was consistently less than 0.4, and this with mitochondria from an ectotherm. Furthermore, over the range of experimental temperatures, the  $Q_{10}$  values of our coupling enzymes were close to those of mitochondrial ATP synthesis (1.67 and 1.40 for HK+G-6-PDH between 8 and 15°C and between 15 and 22°C, respectively, compared to 1.60 and 2.00 for ATP synthesis between 8 and 15°C and between 15 and 22°C, respectively). We are therefore confident that in our system all the control of NADP reduction is assumed by mitochondrial activity.

NADP reduction was measured at 340nm and converted into rates of ATP synthesis using a molar extinction coefficient of  $6.23 \times 10^3 \text{ cm}^2 \text{ mol}^{-1}$ . For each measurement we used 0.1–0.2mg of mitochondrial protein. Ap<sub>5</sub>A was used as a specific inhibitor of adenylate kinase (Lüstorf and Schlimme, 1976). To ensure that NADP reduction was coupled to the oxidation of the substrates, we measured the rate without exogenous substrate and then added Ap<sub>5</sub>A to estimate the proportion of this activity which was due to adenylate kinase. Finally, we measured the rate of NADP reduction in the presence of saturating concentrations of substrates and of either sodium azide ( $1 \text{ mmol l}^{-1}$ ) or carboxyatractyloside ( $1 \mu\text{mol l}^{-1}$ ) to block specifically the electron transport system (cytochrome oxidase) or the adenine nucleotide translocase. The ADP/O ratio was measured either by the standard polarographic method (Chance and Williams, 1956) or by dividing the rate of ATP production at the maximal reaction rate measured spectrophotometrically by the maximal respiration rate measured polarographically for the same mitochondrial preparation.

#### *Calculation of kinetic variables*

The kinetic variables were calculated by least squares fitting of the equation:

$$1/v = [(K_m/V) \times (1/A)] + 1/V, \quad (1)$$

where  $v$  is the velocity,  $A$  is the substrate concentration,  $K_m$  is the apparent Michaelis–Menten constant and  $V$  is the maximal velocity. These calculations were made with a program that we adapted for Macintosh (Microsoft BASIC) from an earlier version developed by Cleland (1979) for the statistical analysis of enzyme kinetic data.

For each temperature/pH condition, mitochondrial activity was measured at ten different concentrations of ADP. The useful range of ADP concentrations for the statistical analysis of mitochondrial kinetics was established in a preliminary study. Every set of measurements was repeated on four preparations and the measurements were carried out at each experimental condition for each preparation. Experiments were carried out at three temperatures: 8, 15 and 22°C. The pH of the medium was adjusted to 7.40 and 7.61 at 8°C, to 7.40 at 15°C and to 7.40 and 7.19 at 22°C. pH values were adjusted at the experimental temperature with a pH meter (Fisher, Accumet) calibrated at this temperature. Given that the pH/temperature slope for arterial blood of rainbow trout overlaps that of catfish (*Ictalurus punctatus*; Cameron, 1984) and that no data were

available for the intracellular pH of red muscle of rainbow trout, we chose the pH of catfish red muscle at 15°C as an approximation for that of rainbow trout red muscle. The pH/temperature slope ( $\Delta\text{pH}/\Delta T$ ) that we used ( $-0.03^\circ\text{C}^{-1}$ ) is close to the maximum pH/temperature slope observed for fish red muscle (Cameron, 1984). This maximized the possibility of revealing a pH effect on the kinetic variables while staying within a physiological range. Kinetic variables were calculated using the total concentration of ADP and the free ADP concentration (i.e. not ligated to  $\text{Mg}^{2+}$ ).

#### *Calculation of free ADP concentration*

Given the high concentration of  $\text{MgCl}_2$  ( $11\text{mmol l}^{-1}$ ) and the high affinity of ADP for  $\text{Mg}^{2+}$ , we had to estimate the free concentration of ADP (not ligated to  $\text{Mg}^{2+}$ ) in the medium. We used an apparent stability constant of  $15001\text{mol}^{-1}$  at 25°C and pH8.7 (Taqui Khan and Martell, 1962, cited by O'Sullivan and Smithers, 1979). This constant was corrected for the different pH and temperature values according to O'Sullivan and Smithers (1979). The ADP concentration was calculated using the following general equation for a metal–ligand complex:

$$[\text{L}]_0 = [\text{BL}] / ([\text{B}] \times K'_{\text{BL}}) \quad (2)$$

where  $[\text{L}]_0$  is the sum of forms of L other than BL under the specified conditions, BL is the complexed form of L, B is the total concentration of the metal and  $K'_{\text{BL}}$  is the apparent stability constant.

#### *Proteins*

Protein concentrations were assayed by using the bicinchoninic acid (BCA) reagent method (Pierce, Rockford, Illinois).

#### *Statistics*

To evaluate the effect of pH on the kinetic variables, we used a Wilcoxon test on the data obtained at 8°C and 22°C. Because we found no significant effect of pH, we combined the values obtained at the two different pH values at 8°C and 22°C for each individual to evaluate the effect of temperature on the kinetic variables. Given that every experimental condition was examined for each mitochondrial preparation, the significance of the temperature effect on  $K_{\text{m,app}}$  and  $V_{\text{max}}$  was tested by a randomized block analysis of variance (ANOVA) (Zar, 1984). Values are presented as mean  $\pm$  s.e.

#### *Materials*

All the chemicals were purchased from Sigma Chemical Co. (St Louis, MI, USA) except mannitol, sucrose, KCl and  $\text{MgCl}_2$ , which were purchased from Anachemia (Ville Saint-Pierre, Québec, Canada).

## **Results**

### *Mitochondrial properties*

In the experiments validating the spectrophotometric method, the RCR of the

Table 1. *Effect of different inhibitors on the spectrophotometrically measured rate of ATP synthesis*

	Activity	
	(nmolmin <sup>-1</sup> mg <sup>-1</sup> protein)	(%)
Pyruvate+malate+ADP	198±44	100
ADP	72±30	36.4
ADP Ap <sub>5</sub> A	ND	ND
Pyruvate+malate+ADP Ap <sub>5</sub> A	147±22	74.2
Pyruvate+malate+ADP Ap <sub>5</sub> A Sodium azide	9±3	4.5
Pyruvate+malate+ADP Ap <sub>5</sub> A Carboxyatractyloside	10±3	5.1

P<sup>1</sup>,P<sup>5</sup>-di(adenosine-5)pentaphosphate (Ap<sub>5</sub>A) inhibits adenylate kinase, sodium azide inhibits cytochrome oxidase and carboxyatractyloside inhibits the adenine nucleotide translocase.

Values are means ± s.e., *N*=3 for all determinations.

ND means not detectable.

mitochondria, determined in the presence of MgCl<sub>2</sub>, was 3.6±0.2 (mean ± s.e., *N*=8) when measured at 15°C. The mitochondrial preparations used for the study of the kinetics of ATP synthesis had an RCR of 3.3±0.6 (*N*=4). The RCR of trout red muscle mitochondria was little affected by an increase in temperature from 8 to 15°C, but increased between 15 and 22°C [3.9±0.4 (*N*=6) at 8°C, 4.0±0.2 (*N*=10) at 15°C and 5.2±0.5 (*N*=6) at 22°C]. Because these values are over 3.0 and are obtained with muscle mitochondria respiring at a high concentration of MgCl<sub>2</sub> (11mmol l<sup>-1</sup>), the mitochondrial preparations appear to be of good quality (Thorne and Bygrave, 1973; Sherratt *et al.* 1988). During the preliminary experiments in which we developed our isolation method, RCR values were determined in the absence of MgCl<sub>2</sub> and were consistently over 5.0.

#### *Validation of the spectrophotometric method*

In the absence of exogenous substrates (pyruvate and malate) and at saturating concentrations of ADP, NADP reduction was completely inhibited by Ap<sub>5</sub>A (Table 1). This indicates that the residual reduction of NADP is due to adenylate kinase, which catalyzes the synthesis of ATP and AMP from ADP, and that exogenous substrates are required for the reduction of NADP when Ap<sub>5</sub>A is present.

The addition of sodium azide or carboxyatractyloside blocked 95% of the maximal

rate of NADP reduction. Since sodium azide is an inhibitor of cytochrome oxidase and carboxyatractyloside is a specific inhibitor of the adenine nucleotide translocator, the marked inhibition of NADP reduction clearly indicates that this reduction of NADP is coupled to the reduction of oxygen by cytochrome oxidase and to mitochondrial adenylate transport. Another clear indication of the 1:1 stoichiometry between mitochondrial ATP synthesis and NADP reduction is the similarity between the ADP/O ratio measured polarographically ( $2.82 \pm 0.10$ ,  $N=8$ ) and that deduced from the maximal rate of NADP reduction in presence of Ap<sub>5</sub>A and the maximal respiration rate of mitochondria ( $3.02 \pm 0.24$ ,  $N=8$ ). In other words, the rate of ATP synthesis calculated from the ADP/O ratio measured polarographically and the maximal respiration rates ( $130 \pm 12 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ ;  $N=8$ ) is equivalent to the rate of ATP synthesis measured spectrophotometrically for the same preparation in the presence of Ap<sub>5</sub>A ( $142 \pm 19 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ ;  $N=8$ ). When these values are compared with a Wilcoxon's test, they do not differ ( $P > 0.05$ ). Given these results, we are convinced that the reduction of NADP in our reaction medium is coupled to substrate oxidation and to mitochondrial ADP phosphorylation.

A potential source of error is sedimentation of mitochondria in the cuvette. When mitochondria were maintained in a cuvette in the reaction medium without substrate, ADP or coupling enzymes, the optical density at 340nm did not decline with time, suggesting that no net sedimentation occurred in the cuvettes. This is as expected given the equivalent density of mitochondria and the medium. Another potential source of error is interference by the mitochondrial NAD/NADH pool. However, the stability of absorbance at 340nm in the absence of G-6-PDH is a good indication that the mitochondrial NAD/NADH pool does not interfere with the measurement of ATP synthesis. Finally, a simultaneous oxidation of reduced NADP would underestimate the rate of ATP production and the ADP/O ratio. In our complete reaction medium, lacking only pyruvate and malate, no NADPH oxidase activity could be detected at 56, 112, 224 or 560  $\mu\text{mol l}^{-1}$  NADPH, nor could NADPH oxidation be detected in the basic reaction medium (without coupling enzymes, Ap<sub>5</sub>A, malate or pyruvate), in either the presence or absence of ADP.

#### *ADP control of ATP synthesis*

The rate of ATP synthesis follows a hyperbolic relationship with increases in ADP concentration (Fig. 1). The  $V_{\text{max}}$  calculated from equation 1 at 15°C ( $126 \pm 23 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ ,  $N=4$ ) did not differ from the values measured at saturating concentrations of ADP at 15°C ( $142 \pm 19 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ mitochondrial protein}$ ,  $N=8$ ) ( $P > 0.05$ ). This indicates that the ADP-dependence of mitochondrial ATP synthesis is well described by Michaelis–Menten kinetics. Furthermore, the coefficients of variation of the values of  $K_{\text{m,app}}$  obtained by fitting the data to equation 1 were consistently lower than 25% of the  $K_{\text{m,app}}$  values, suggesting that the variables  $K_{\text{m,app}}$  and  $V_{\text{max}}$  are good descriptors of the kinetics of the reaction (Cleland, 1979).

Although the  $K_{\text{m,app}}$  and  $V_{\text{max}}$  values measured at 8°C and 22°C were not significantly modified by the experimental pH ( $P > 0.05$ ; Table 2), at 8°C  $K_{\text{m,app}}$  tended to decrease slightly with an increase in pH. The effect of temperature on both variables is significant

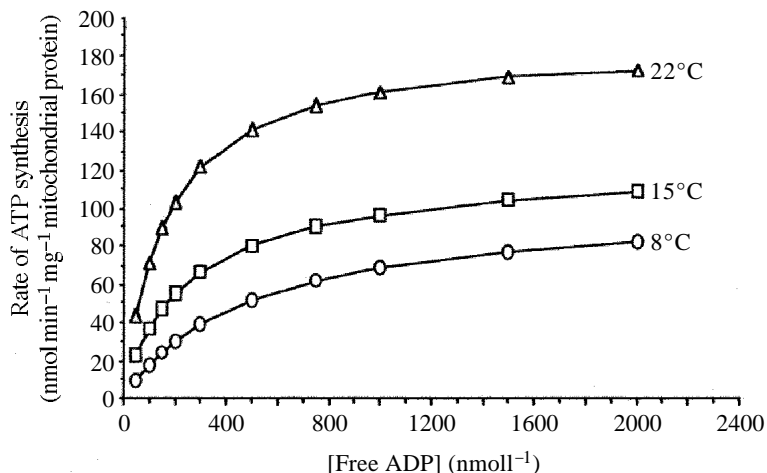


Fig. 1. The kinetics of mitochondrial ATP synthesis by rainbow trout red muscle. The rates of ATP synthesis presented in this figure were calculated using the kinetic variables given in Table 1 and equation 1.

Table 2. *Temperature and pH effects on the  $K_{m,app}$  for ADP and the  $V_{max}$  for ATP synthesis by mitochondria isolated from rainbow trout red muscle*

Temperature (°C)	pH	N	$K_m$ (total ADP)	$K_m$ (free ADP)	$V_{max}$
8	7.40	4	11.11±1.98	582±104	112±13
8	7.61	4	9.39±0.66	465±32	93±26
8	Combined	4	10.25±1.21	524±63	102±19
15	7.40	4	6.33±2.16	285±97	126±23
22	7.40	4	4.35±0.81	169±31	186±55
22	7.19	4	4.13±0.33	155±22	188±31
22	Combined	4	4.24±0.38	162±6	187±16

The apparent  $K_m$  values are expressed in  $\mu\text{mol l}^{-1}$  for total ADP and  $\text{nmol l}^{-1}$  for free ADP.

The  $V_{max}$  values are expressed as  $\text{nmol of ATP produced (NADP reduced) min}^{-1} \text{mg}^{-1}$  mitochondrial protein; values are mean  $\pm$  S.E.

The combined values represent the mean  $\pm$  S.E. of the kinetic variables determined using the data obtained at the two pH values for each preparation.

when calculated with either free ADP or total ADP (ANOVA,  $P < 0.025$ ). The  $K_{m,app}$  of mitochondria for ADP increases with decreasing temperature while the  $V_{max}$  increases with rising temperature (Table 2). The  $Q_{10}$  values obtained for the  $V_{max}$  are  $1.6 \pm 0.5$  ( $N=4$ ) between 8 and 15°C and  $2.0 \pm 0.4$  ( $N=4$ ) between 15 and 22°C.

### Discussion

The reliability of our method is demonstrated by the agreement between the ADP/O ratio estimated spectrophotometrically and that measured polarographically. The ADP/O



values are similar to those found for mitochondria from different animals for the same substrates (malate+pyruvate) and indicate an efficient coupling of oxidative phosphorylation as well as good mitochondrial quality. The ADP/O ratio for mitochondria from rainbow trout white muscle using pyruvate as a substrate is 2.6 (Moyes *et al.* 1992) and it is 2.8 for frog muscle mitochondria (Skoog *et al.* 1978) and 2.73 for rat muscle mitochondria (Davies *et al.* 1981). The other data for fish are somewhat aberrant (4.1) (Mourik, 1983).

Treatment with inhibitors shows that the reduction of NADP is tightly coupled to the oxidation of exogenous substrates. Given the facility of coupling NADP reduction to mitochondrial ADP phosphorylation in the reaction medium, we propose this method as an alternative to the polarographic method for studies of mitochondrial fuel preferences, control of mitochondrial metabolism or mitochondrial dysfunction. We believe that the spectrophotometric approach has many significant advantages. For example, we can easily follow, in a cuvette, a rate of ATP synthesis of  $10\text{nmolmin}^{-1}\text{ml}^{-1}$ . Since we can obtain about 1g of mitochondrial protein from 1kg of fish red muscle and since, at  $15^{\circ}\text{C}$ , rainbow trout red muscle mitochondria have a maximum rate of ATP synthesis of  $142\pm 19\text{nmolmin}^{-1}\text{mg}^{-1}$  of mitochondrial protein ( $N=8$ ), less than 70mg of tissue is sufficient to make a measurement. This is much less than that required for polarographic measurements (Lemaster, 1984). Furthermore, the rapidity of the spectrophotometric measurement allows one to minimize the problem of mitochondrial deterioration following isolation. Finally, it bypasses the necessity for polarographic calibration. The only restriction is that the different controls that we have used must be repeated when different tissues, organisms or substrates are used.

An acute decrease in temperature significantly reduces the sensitivity of mitochondria to control by ADP availability. Thus, the  $K_{m,\text{app}}$  for ADP increases at low temperature while the  $V_{\text{max}}$  decreases (Table 2). This decrease in sensitivity is even more marked when we express the results in relative terms. At lower temperatures, mitochondria need a bigger signal (change in ADP concentration) to reach a given relative activity (percentage of  $V_{\text{max}}$ ) than they do at higher temperature. For example, to change from 25 to 75% of  $V_{\text{max}}$ , the free ADP concentration must increase by 1.275, 0.771 and  $0.419\ \mu\text{mol l}^{-1}$  at 8, 15 and  $22^{\circ}\text{C}$ , respectively. The increase in  $K_{m,\text{app}}$  at lower temperatures could be associated with a decrease in the degree of coupling of oxidative phosphorylation. However, the RCRs at 15 and  $8^{\circ}\text{C}$  do not differ, suggesting that the permeability of the internal membrane to protons does not change significantly.

Control of mitochondrial respiration has been shown to be shared by many steps of the pathway (substrate transport, ADP/ATP translocation, cytochrome oxidase and membrane proton leak), and the distribution of the control strength coefficients is dependent upon the state of respiration (state 3 relative to state 4). As our index of mitochondrial sensitivity to control by ADP availability ( $K_{m,\text{app}}$ ) is calculated from mitochondrial activity at different ADP concentrations (which thus establish different states of respiration), the thermal sensitivity of control by ADP availability must logically reflect the cumulative impact of temperature on the steps included in the control (principally the adenine nucleotide translocator, substrate translocation and cytochrome

oxidase) and on the distribution of the control strength coefficients between state 3 and state 4.

A significant part of the control of mitochondrial respiration is ensured by the adenine nucleotide translocator (Groen *et al.* 1982; Gellerich *et al.* 1983; Westerhoff *et al.* 1987). Thus, we expect that the kinetics of ADP phosphorylation are at least partly explained by the behaviour of the adenine nucleotide translocator. The  $K_{m,app}$  for ADP that we obtained is close to that of the mammalian mitochondrial adenine nucleotide translocator. ADP/ATP transport in mammalian mitochondria shows Michaelis–Menten kinetics (Pfaff and Klingenberg, 1968; Duée and Vignais, 1969) with a  $K_{m,app}$  between 1 and  $10 \mu\text{mol l}^{-1}$  of total ADP for mitochondria and submitochondrial particles (Pfaff *et al.* 1969; Klingenberg, 1976, 1977; Brandolin *et al.* 1980). Translocation rates of 200 and  $750 \mu\text{mol min}^{-1} \text{g}^{-1}$  protein are reported for rat liver mitochondria and beef heart mitochondria at  $25^\circ\text{C}$  (Klingenberg, 1985). These rates are only slightly affected by pH and show a maximum capacity between pH 7.2 and 7.6 (Brandolin *et al.* 1980).

$\text{H}^+$ -ATPase is another potential site for the control of the rate of ADP phosphorylation, but the data of Wanders *et al.* (1984) suggest that  $\text{H}^+$ -ATPase is usually much less flux-controlling than the adenine nucleotide translocator. Decreasing temperature markedly decreased the phosphorylation of exogenous ADP by mammalian mitochondria and only slightly affected the phosphorylation of endogenous ADP (Kemp *et al.* 1969; Klingenberg, 1977; Klingenberg *et al.* 1982). Thus, nucleotide translocation imposes its strong temperature-dependence on oxidative phosphorylation by mitochondria.

A high ADP affinity of mitochondrial metabolism ensures that when  $\text{O}_2$  availability is adequate, oxidative metabolism will outcompete anaerobic pathways for limiting concentrations of ADP, thus gaining efficiency while avoiding the problems of fermentation (Hochachka and Somero, 1984). With an acute temperature decrease, the higher  $K_{m,app}$  for ADP will impair the ability of mitochondria to sustain both high catalytic rates and high ATP/ADP ratios. Given the relationship between a decrease in the energetic status of a muscle cell and the activation of the glycolytic pathway (Hochachka *et al.* 1983; Chiasotis, 1988), we suspect that, after acute decreases in temperature, the red muscle will activate glycolysis and reach fatigue without exploiting the entire catalytic potential of mitochondria.

This interpretation suggests that the ‘compression of recruitment order’ at low temperature could be dictated not only by the mechanical constraints of the contractile apparatus ( $V/V_{max}$ , Rome *et al.* 1990) but also by energetic constraints. Decreases in temperature would reduce the capacity of the oxidative pathway to compete with the anaerobic pathway for limiting concentrations of ADP. The lower speed of shortening of muscular fibres could be related not only to the direct effect of temperature on contractile proteins but also to lower rates of ATP production and to the adverse effect of a lower ATP/ADP ratio. At higher concentrations, ADP,  $\text{P}_i$  and  $\text{H}^+$  could inhibit the contractile process (Cooke and Pate, 1985; Kentish, 1986). Thus, the thermal sensitivity of red muscle power development could be higher than that predicted from the effects of temperature on contractile proteins or on the maximal catalytic capacity of mitochondria.

The general response of fish from temperate habitats to low-temperature acclimation (i.e. an increase in mitochondrial volume density) could be a response to a loss of

sensitivity to control by ADP. Dudley *et al.* (1987) demonstrated that, in rat muscle, the sensitivity of tissue O<sub>2</sub> uptake to cytosolic modulators [ADP, ATP/ADP and ATP/(ADP×P<sub>i</sub>)] went up as mitochondrial content increased. Thus, with more mitochondria, a smaller change in ADP concentration is required to bring about a given change in tissue O<sub>2</sub> uptake.

Changes in membrane phospholipids in response to low-temperature acclimation in fish mitochondria (Cossins *et al.* 1980; Van den Thillart and de Bruin 1981; Cossins and Prosser, 1982; Hazel and Zerba, 1986) could restore mitochondrial sensitivity to control by ADP. Studies on reconstituted systems demonstrate that changing the phospholipid composition of liposomes can drastically affect the activity of membrane transporters (Carruthers and Melchior, 1988). In particular, adding acid phospholipids or replacing phosphatidylcholine with phosphatidylethanolamine considerably increases the activity of the ADP/ATP translocator (Krämer and Klingenberg, 1980). Van den Thillart and de Bruin (1981) have demonstrated that, in goldfish, cold acclimation decreases the levels of phosphatidylcholine relative to those of phosphatidylethanolamine in red muscle mitochondria.

Our results are the first demonstration that temperature can affect the sensitivity of mitochondria to an important metabolite effector, i.e. ADP (Table 2). This does not invalidate the two alternative explanations of the responses of the muscle metabolic apparatus to cold acclimation. Rather, it clearly emphasizes that the coordination of catabolic processes to energy-consuming processes at the cellular level should be an important theme of studies on the thermal biology of ectotherms.

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