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Review

Adaptive plasticity of skeletal muscle energetics in hibernating frogs: mitochondrial proton leak during metabolic depression

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Summary

The common frog (*Rana temporaria*) spends the coldest months of each year overwintering in ice-covered ponds where temperatures can vary from 0.5 to 4.0 °C. Over the course of a winter season, the animals enter progressively into a state of metabolic depression that relies almost exclusively on aerobic production of ATP. However, if aerobic metabolism is threatened, for example by increasingly hypoxic conditions, decreases in the animal's metabolic rate can reach upwards of 75 % compared with the 50 % decrease seen during normoxia. Under these conditions, the major proportion of the overall reduction in whole-animal metabolic rate can be accounted for by metabolic suppression of the skeletal muscle (which makes up approximately 40 % of body mass). Little is known about the properties of mitochondria during prolonged periods of metabolic depression, so we have examined several aspects of mitochondrial metabolism in the skeletal muscle of frogs over periods of hibernation of up to 4 months. Mitochondria isolated from the skeletal muscle of frogs hibernating in hypoxic water show a considerable reorganisation of function compared with those isolated from normoxic submerged animals at the same temperature (3 °C). Both the active (state 3) and resting (state 4) respiration rates of mitochondria decrease during hypoxic, but not normoxic, hibernation. In addition, the affinity of mitochondria for oxygen increases during periods of acute hypoxic stress during normoxic hibernation as well as during long-term hibernation in hypoxic water. The decrease in mitochondrial state 4 respiration rates during hypoxic hibernation evidently occurs through a reduction in electron-transport chain activity, not through a lowered proton conductance of the mitochondrial inner membrane. The reduced aerobic capacity of frog skeletal muscle during hypoxic hibernation is accompanied by lowered activities of key enzymes of mitochondrial metabolism caused by changes in the intrinsic properties of the mitochondria. In the absence of oxygen, the mitochondrial F_1F_o-ATPase (the ATP synthase) begins to run backwards as it actively pumps protons from the matrix in an attempt to maintain the mitochondrial membrane potential. At this time, the ATP synthase functions as an ATPase to preserve a certain proton-motive force. Frogs limit ATP wastage during anoxia by a profound inhibition of the ATP synthase. Taken together, our studies show that proton-motive force is lowered aerobically by restricting electron supply and during anoxia by restricting mitochondrial ATPase activity.

Key words: frog, skeletal muscle, mitochondria, proton leak, hibernation, hypoxia, oxyconformation, metabolic depression, *Rana temporaria*.

Introduction

Metabolic depression is a common organismal response to environmental pressures such as cold, desiccation, hypoxia and food shortage (Hochachka and Guppy, 1987; Storey and Storey, 1990; Boutilier et al., 1997; Guppy and Withers, 1999; Brand et al., 2000). The cold-submerged frog (*Rana temporaria*) serves as a useful model for many hibernating ectotherms that take refuge in ice-covered ponds and lakes until more favourable conditions of climate and food availability return. Because the ice and snow cover inhibits both the equilibration of surface waters with the atmosphere and light penetration for photosynthesis, oxygen consumption by the resident organisms often exceeds replenishment. Under these effectively 'closed' conditions for gas exchange, freshwater systems can become increasingly hypoxic throughout the winter. Our experimental approach has been to submerge frogs at a constant temperature of 3 °C so as to mimic the overwintering conditions under ice cover. Over the course of 2–4 weeks, the animals enter gradually into a state of

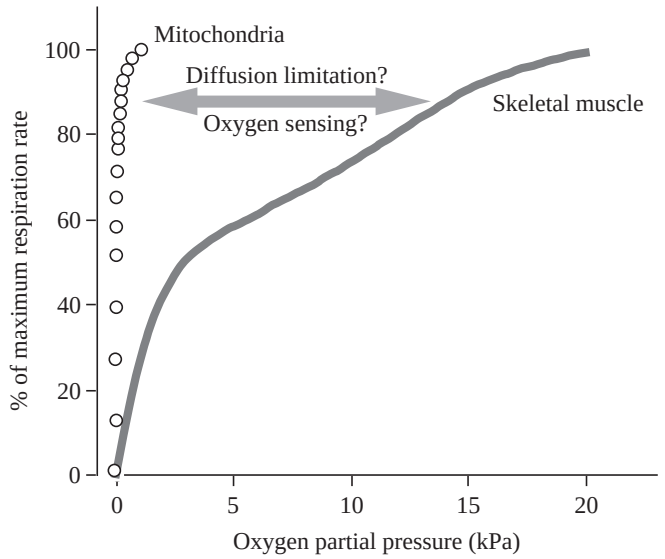

Fig. 1. Oxygen-dependence of respiration at 20 °C for superfused sartorius muscle and for skeletal muscle mitochondria isolated from the frog *Rana temporaria*. Measurements were made using high-resolution respirometry (Oroboros Oxygraph, Paar, Garz, Austria) that enables sensitive measurements of oxygen kinetics at low oxygen partial pressures (see St-Pierre et al., 2000c). Mitochondria show strict oxyregulation over a broad range of O_2 tensions, while skeletal muscle begins to oxyconform at P_{O_2} levels that are far in excess of the K_m of isolated mitochondria. Although oxyconformation is seldom seen in isolated cell preparations (see, however, Brand et al., 2000; Bishop and Brand, 2000; Guppy et al., 2000; Bishop et al., 2002), it does operate at the level of intact skeletal muscle (Hochachka and Guppy, 1987; West and Boutilier, 1998). One possibility is that the P_{O_2} of localised (hypoperfused) regions of tissue might fall below the critical P_{O_2} (P_{crit}) at which diffusion of oxygen to the mitochondria begins to limit oxidative phosphorylation. The metabolic rate of such localised regions could therefore become suppressed even though the mixed venous blood continues to exit the tissue at P_{O_2} levels higher than the P_{crit}. This so-called 'diffusion limitation' could be one explanation for the well-known oxyconformation response seen in the intact skeletal muscle of cat (Whalen et al., 1973) and frog (Boutilier et al., 1997; West and Boutilier, 1998). Alternatively, oxyconformation could occur through some oxygen-sensing elements that trigger a reduction in the rate of mitochondrial respiration. From Boutilier (2001) with permission.

metabolic depression, the extent of which is much more pronounced in animals exposed to hypoxic water (up to 75 % suppression; Donohoe and Boutilier, 1998) than during normoxia (approximately 50 %; Donohoe et al., 1998).

The frog's skeletal muscle is thought to be the most important tissue contributing to the overall metabolic depression during hibernation because (i) it makes up the largest proportion of the animal's body mass and (ii) its metabolic rate conforms to O_2 availability such that reduced blood supply leads to a marked suppression of the rate of oxygen consumption (Fig. 1). During hibernation, cold-submerged frogs drastically reduce the blood supply to their skeletal muscle in order to shunt more blood to the skin for the extraction of oxygen (Boutilier et al., 1986, 1997; Pinder et al., 1992). Given that the metabolic rate of isolated frog skeletal muscle decreases with perfusate oxygen concentration (i.e. the oxyconformation response as shown in Fig. 1) and that 35–40 % of the frog's body mass is skeletal muscle, the reduction in blood supply (and therefore O_2 supply) to the skeletal muscle mass leads to a considerable decrease in whole-animal metabolic rate (Donohoe and Boutilier, 1998). The advantage of a metabolic depression during hibernation is that it slows the rate of utilisation of 'on-board' fuels until the environmental conditions are more favourable and activity can be resumed. Since the mitochondrion is the major contributor to total energy production during aerobic metabolism and frog survival over winter depends critically on entry into a hypometabolic state, the question we asked is whether overwintering frogs will produce changes in the properties of their mitochondria to preserve or increase their efficiency of energy production. As very few studies have looked at the intrinsic properties of mitochondria during metabolic depression, our main objective was to examine the role that mitochondria play in the development of hypometabolic states.

Recent estimates indicate that 20 % of the standard metabolic rate (SMR) in rats can be accounted for by an intracellular futile cycle of proton pump and leak across the mitochondrial inner membrane (Brand et al., 1994, 2000; Rolfe and Brand, 1996; Rolfe et al., 1999). This 'proton cycling' or 'proton leak' partially uncouples oxygen consumption from ATP synthesis, thereby leading to less effective energy conservation. When isolated mitochondria are operating at maximal rates of oxygen consumption in the presence of saturating amounts of substrate and ADP (i.e. so-called 'state 3' respiration), the proportion of respiration used to drive proton cycling is of the order of 10 % (Brand et al., 2000). However, in the absence of any ATP production (i.e. when all the ADP has been used or when the F_1F_o-ATPase has been blocked with the highly specific inhibitor oligomycin), all of the low residual respiration (state 4) drives the proton leak. Intact cells and tissues are almost certainly operating closer to 'state 4' conditions at SMR, and this is presumably also the case at minimal metabolic rates (MMRs). Mitochondrial proton cycling makes up approximately 20 % of the cellular respiration rates of the hepatocytes of the frog, the bearded dragon (reptile) and the rat and of the hepatopancreas cells of the snail (Brand et al., 1991, 2000; Bishop and Brand, 2000; Bishop et al., 2002). Thus, the amount of energy dissipated by proton cycling appears to be very similar across phylogenetic lines. If proton cycling were to make up 20 % of the standard metabolic rate of an animal such as the frog, which can depress its metabolic rate by 75 %, one could argue that proton cycling would have to be decreased during metabolic depression. Otherwise, proton cycling would dominate the residual oxygen consumption, and the efficiency of mitochondrial energy conservation would fall towards zero. This would effectively negate most, if not all, of the energy-sparing advantages associated with entry into a hypometabolic state.

Adenosine triphosphate (ATP) is often referred to as the energy currency of the cell because it is the price to pay to

carry out the vast majority of cellular transactions. Most organisms rely on mitochondrial metabolism (aerobic metabolism) to produce energy in the form of ATP. The capacity for producing ATP aerobically can vary throughout an animal's life history according to changes in body mass and/or age as well as in response to environmental conditions such as temperature, oxygen levels and food intake. Modifications in the aerobic capacity of an organism can occur at different levels of the O_2 cascade, representing different levels of biological organisation. In the face of acute environmental stress, first lines of physiological defence take place within seconds in order to provide immediate compensation for any adverse effects on cellular functions. If the environmental insult persists, however, more profound changes at the molecular level may be needed to provide for a reorganisation of cellular metabolism. Prolonged physiological stresses, such as those seen during overwintering, can lead to marked changes in the aerobic capacity at the cellular level either (i) by altering the number of mitochondria or (ii) by changing the intrinsic properties of the mitochondria.

The contribution of mitochondrial proton cycling to standard metabolic rate

Mitochondrial proton leak is correlated with a whole host of factors that determine SMR. For example, proton leak and SMR change in parallel with respect to body size, phylogeny and metabolic status (Brand et al., 1991; Brand and Murphy, 1987; Hafner et al., 1988; Porter and Brand, 1993; Porter et al., 1996; Ramsey et al., 1996). There are four ways to alter mitochondrial proton leak inside cells: (i) by changing the kinetics of the proton leak, (ii) by modifying the mitochondrial membrane potential through changes in electron-transport chain activity and/or in ATP turnover, (iii) by changing the volume density of mitochondria and (iv) by altering the cristae surface density and/or surface area within the mitochondrion. Two principal factors can explain the differences in the proton-leakiness of isolated mitochondria; namely, an increase in the surface area of the mitochondrial inner membrane and a modification in the fatty acid composition of the mitochondrial phospholipid membrane (Brand et al., 1994). Porter et al. (1996) suggested that changes in the surface area of the inner membrane might dominate in allometric differences in proton leak and that modifications in membrane composition might dominate in phylogenetic differences, whereas shifts in metabolic status might display a mixed pattern.

The extent to which proton leak uncouples cellular ATP synthesis determines quantitatively the number of moles of ATP produced per mole of oxygen consumed (i.e. the so-called P/O ratio). To obtain a realistic P/O value, it is necessary to compute an 'effective' P/O ratio by multiplying the 'mechanistic' P/O ratio of the mitochondrial ATP-synthesizing reactions (estimated to be 2.5 for NADH substrate; Rolfe and Brown, 1997) by the fraction of the total tissue O_2 consumption rate that is used to drive mitochondrial ATP synthesis. Given that non-mitochondrial respiration accounts for 10 % of mammalian SMR and proton leak for 20 % of SMR, Rolfe and Brown (1997) arrive at an effective whole-body P/O value of 1.8 (i.e. 0.7×2.5). Even lower values of effective P/O are found in individual tissues. For example, recent estimates of the contribution of oxidative phosphorylation to resting oxygen consumption in isolated rat hepatocytes (69 %) and perfused hindlimb (57 %), predict effective P/O ratios of 1.7 (i.e. 0.69×2.5) and 1.4 (0.57×2.5) for liver and skeletal muscle, respectively (Rolfe et al., 1999).

On the face of it, proton leak would therefore seem to make cellular metabolism at SMR rather inefficient, and intense efforts are under way to discover the functional significance of this so-called 'futile cycle'. A number of physiological functions for proton leak have been proposed, namely (i) heat generation, (ii) increasing the sensitivity of the oxidative pathway to effectors, (iii) reducing the rate of production of reactive oxygen species and (iv) regulation of carbon flow (Rolfe and Brand, 1997). It is unlikely that the main role of proton leak is heat generation since ectotherms ranging from snails to lizards seem to devote the same proportion of their cellular respiration to drive proton leak as do mammals (Brand et al., 1991, 2000; Bishop and Brand, 2000).

It is well known that mitochondrial proton leak in brown adipose tissue is catalysed by uncoupling protein 1 (UCP1), which diverts energy from ATP synthesis to heat generation (see Brand et al., 1999). The more recent discovery of homologues of UCP1, namely UCP2 and UCP3, has engendered debate as to their probable role in the regulation of energy balance (Ricquier and Bouillaud, 2000). UCP2 is widely expressed in a number of mammalian organs, whereas UCP3 is mainly expressed in skeletal muscle. Despite their widespread expression in mammalian tissues, there is little evidence that their function is tied primarily to thermogenesis. Indeed, the occurrence of UCP2 and UCP3 in ectotherms (Stuart et al., 2001) suggests that functions other than heat generation may have provided the initial selection advantages for the early evolution of this class of proteins (Hochachka and Somero, 2002). Recent evidence indicates that UCPs may play an important role in decreasing the mitochondrial production of reactive oxygen species (ROS) such as superoxide (Ricquier and Bouillaud, 2000). Greater uncoupling would facilitate increased rates of electron transfer to molecular oxygen, thereby preventing any back-up of electrons in the electron-transport chain (Hochachka and Somero, 2002). This would effectively reduce the lifetimes of ROS-generating centres in the electron-transport chain and ameliorate oxidative damage. Indeed, Echtay et al. (2002) have recently shown that superoxide-induced increases in mitochondrial proton conductance are effected through UCPs. Speculation continues to be rife as regards the evolution and adaptive significance of the mitochondrial UCPs.

Mitochondrial proton leak in metabolic depression

Proton leak is estimated to account for 34–52 % of the resting SMR of rat skeletal muscle, depending on the tonic

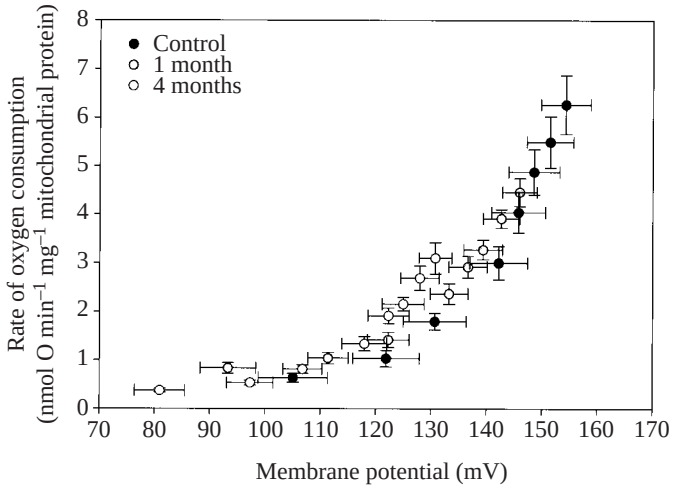

Fig. 2. Kinetics of the mitochondrial proton leak at 25 °C for hypoxic submerged frogs (*Rana temporaria*). The proton conductance of the mitochondrial inner membrane is assayed by measuring the rate of proton cycling (measured as rate of oxygen consumption) through non-coupled pathways at defined values of the proton-motive force (measured as membrane potential). Stepwise changes in respiration rate and membrane potential were achieved by adding increasing amounts of malonate. The control, 1-month-submerged and 4-month-submerged groups of frogs are represented by black, grey and white circles, respectively. Values are means ± S.E.M.; *N*=5 for control and 1-month-submerged frogs, *N*=4 for 4-month-submerged frogs. From St-Pierre et al. (2000a).

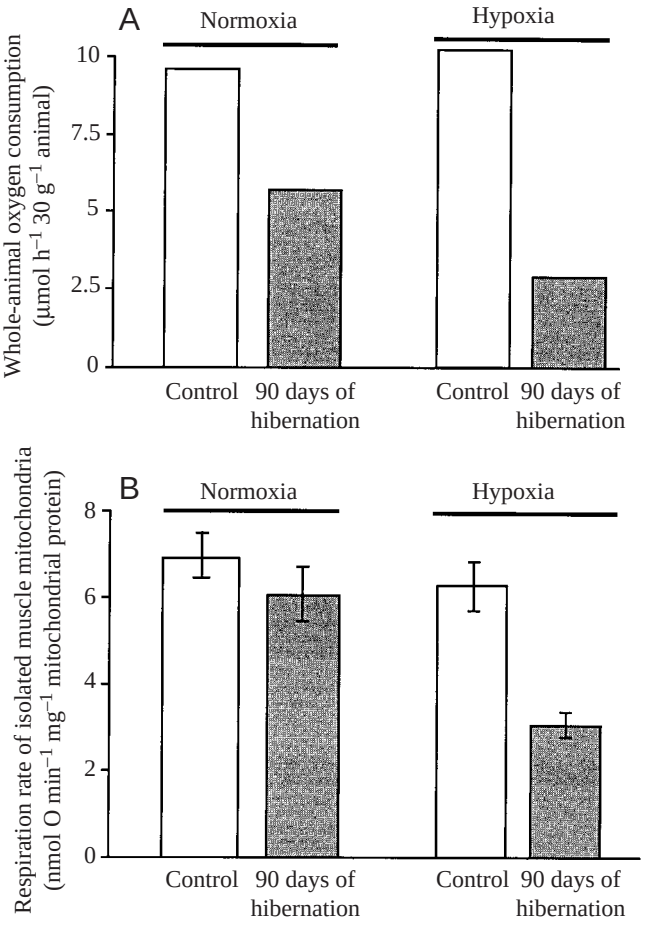

Fig. 3. (A) Measurements of whole-animal oxygen consumption of *Rana temporaria* under control conditions (in water but with access to the surface for air-breathing) and after 90 days of cold-submergence in normoxic (P_{O_2}=21 kPa) or hypoxic (P_{O_2}=8 kPa) water at 3 °C. (B) Respiration rates of skeletal muscle mitochondria isolated from frogs during normoxic and hypoxic hibernation, in state 4 and at 125 mV. Frog mitochondrial proton leak rate was measured at 25 °C because the respiration rate of frog mitochondria was so low at 3 °C that it was impossible to carry out accurate measurements with the apparatus used (St-Pierre et al., 2000a). Since that time, Q_{10} values between 3 and 20 °C have been determined for the state 4 respiration rate of mitochondria (St-Pierre et al., 2000c). As the Q_{10} values do not differ between control and 4-month-hypoxic-submerged frogs (St-Pierre et al., 2000c), the proton leak rate comparisons at 25 °C between the two groups of frogs should be adequate. Values are means ± S.E.M.; *N*=5 for control frogs, *N*=4 for 4-month-submerged frogs. From Boutilier (2001) with permission.

state of the muscle (Rolfe and Brand, 1996; Rolfe et al., 1999). Given that skeletal muscle accounts for approximately 40 % of the total body mass, the contribution of proton leak within the skeletal muscle itself can account for a substantial proportion of the whole-animal SMR (e.g. as much as 15 % of SMR; Rolfe and Brand, 1996). Since the ratio of skeletal muscle mass to total body mass in frogs is similar to that in mammals (Boutilier et al., 1997; Rolfe and Brown, 1997), modulation of muscle mitochondrial proton leak could therefore be an important phenomenon regulating metabolic depression in overwintering frogs. Only recently have studies begun to examine whether hypometabolic states can be effected, at least in part, through reductions in proton leak rates (St-Pierre et al., 2000a,c; Bishop and Brand, 2000; Bishop et al., 2002; Brand et al., 2000, 2001).

If the proton leak rates we have measured in frog skeletal muscle mitochondria (Fig. 2) were to produce in intact muscle as large an uncoupling reaction as in mammals (Rolfe and Brand, 1996; Rolfe et al., 1999), the sheer size of the skeletal muscle mass would make a reduction in proton leak during deep hypoxic hibernation almost essential. The reason being that, with a 75 % reduction in whole-animal oxygen consumption during deep hibernation (Fig. 3A), almost all the remaining energy budget would have to serve the proton leak pathway at a time when one might predict that metabolic efficiency needed to be maintained or even enhanced. For example, whereas the skeletal muscle mass of rat (42 % of total body mass) makes up 30–40 % of its SMR (Rolfe and Brand, 1996), the 35 % fractional muscle mass of the aestivating frog *Neobatrachus kunapalari* accounts for 50–65 % of its SMR and up to 85 % of its MMR during deep metabolic depression (Flanigan and Guppy, 1997). With the liver making up an additional 5 % of the SMR in this frog (Flanigan et al., 1991), fully 70 % of the SMR could be accounted for by these two tissue types and perhaps up to 90 % of the MMR in hypometabolic states. Any reduction in mitochondrial proton

cycling during hibernation would have the effect of conserving substrate and extending survival time. Thus, to preserve the efficiency of their aerobic energy production during metabolic depression, overwintering frogs would have to decrease their proton cycling by the same proportion as cellular respiration. In the absence of a downregulation of proton leak rate, hibernating frogs at MMR would be in the undesirable position of burning more substrates than control animals to produce equivalent amounts of ATP.

To test whether proton cycling might be downregulated as a part of a coordinated response to energy conservation during metabolic depression, we measured the proton leak rate of mitochondria isolated from the thigh musculature of frogs at different stages of hibernation (St-Pierre et al., 2000a), when whole-animal MMRs are 50–75 % lower than SMR (Fig. 3A; Donohoe and Boutilier, 1998). Although the proton leak rates of isolated skeletal muscle mitochondria were unaltered in fully aerobic frogs (when MMR is 50 % of SMR), those isolated from animals in chronic hypoxia (when MMR is only 25 % of SMR) exhibited a 50 % reduction in their state 4 respiration rate (Fig. 2).

If the state 4 respiration rates of isolated mitochondria are similar to the respiration rates of mitochondria in intact resting muscle, these results suggest that the proton leak rates of mitochondria *in vivo* may be reduced during hypoxic submergence. The reduction in proton leak rate during hibernation (Fig. 2) was caused by a decrease in the activity of the electron-transport chain and not by a reduction in the proton conductance of the mitochondrial inner membrane. Indeed, the proton conductance of frog mitochondria was unaltered throughout normoxic and hypoxic submergence. This is illustrated by the fact that there were no significant differences between the respiration rates at any given membrane potential (Fig. 2). However, the state 4 respiration rate achieved by mitochondria from metabolically depressed animals was only 50 % of control, and the state 4 membrane potential was also lower, showing that the rates of substrate oxidation were significantly downregulated. Overall, these results suggest that overwintering frogs effectively preserve their metabolic efficiency by reducing mitochondrial proton cycling in parallel with metabolic rate (Fig. 3) and that they do so by decreasing the rate of substrate oxidation and the size of the proton-motive force rather than by decreasing the proton conductance of the mitochondrial membrane.

One might ask how realistic it is for us to expect that mitochondria isolated *in vitro* should function the same way as *in vivo*? This same question was posed by Bishop and Brand (2000), who developed an isolated hepatopancreas cell preparation from the snail *Helix aspersa* after earlier studies revealed chronically lower respiration rates of mitochondria isolated from the kidney of aestivating animals (Brand et al., 2000). As was the case for hibernating frogs, the lower rates of oxygen consumption in mitochondria isolated from aestivating compared with awake animals were caused by a decrease in substrate oxidation, not by a decrease in proton conductance (Brand et al., 2000; St-Pierre et al., 2000a). Bishop and Brand (2000) found that the respiration rate of hepatopancreas cells isolated from aestivating snails was approximately 30 % of that of awake snails. This cellular metabolic depression was brought about by proportional decreases in mitochondrial and non-mitochondiral respiration rates. Further investigation of the primary and secondary causes of the decreased mitochondrial respiration led to studies in which cellular respiration could be experimentally divided between the producers of the proton-motive force (substrate oxidation) and the processes that consume the proton-motive force (i.e. proton leak and ATP turnover). Bishop et al. (2002) estimate that 75 % of the total response of mitochondrial respiration to aestivation occurs through primary changes in the kinetics of substrate oxidation, the remaining 25 % or less being attributed to ATP turnover (proton leak remains constant). These primary changes resulted in a lower mitochondrial membrane potential, which led to secondary decreases in the cellular respiration rates driving ATP turnover and proton leak (Bishop et al., 2002). Thus, both in the isolated mitochondria from hibernating frogs (Fig. 2) and in the hepatopanceas cells of aestivating snails, decreases in the rate of substrate oxidation result in a fall in membrane potential (Fig. 2; Bishop et al., 2002), which decreases proton leak rates during metabolic depression. As the kinetics of proton leak did not change in either case of metabolic depression, changes in proton conductance of the inner mitochondrial membrane do not play a primary role in the response of mitochondrial respiration to metabolic depression. Similarities in the metabolic responses of isolated cells and isolated mitochondria give some credence to our earlier suggestion that the results obtained using mitochondria isolated from the skeletal muscle of hibernating frogs may reflect the metabolic responses of intact tissue.

Mitochondrial proton leak during anoxia

The reduction in blood flow to the frog skeletal muscle during overwintering submergence may also lead to transient periods of ischaemia. In the absence of oxygen, the mitochondrial F_1F_o-ATPase (the ATP synthase) begins to run backwards as it actively pumps protons from the matrix in an attempt to maintain the mitochondrial membrane potential. In this state, mitochondria change from being ATP producers to potentially powerful ATP consumers (Lisa et al., 1998). Animals that can withstand anoxia for considerable periods must have very efficient ways to reduce ATP utilisation by this pathway. If not, most of the ATP generated by glycolysis could be needed to fuel that one process alone. Thus, when O_2 supplies are exhausted, the proton-motive force is generated by the F_1F_o-ATPase rather than by the electron-transport chain. Our studies on mitochondria isolated from frog skeletal muscle have shown that the proton leak is reduced by a slowing-down of the F_1F_o-ATPase and not by a decrease in the proton conductance of the inner membrane (Fig. 4; St-Pierre et al., 2000b; Boutilier and St-Pierre, 2000). This mechanism is analogous to that observed during hibernation in hypoxia (St-Pierre et al., 2000a,c). Thus, whether in the presence or absence

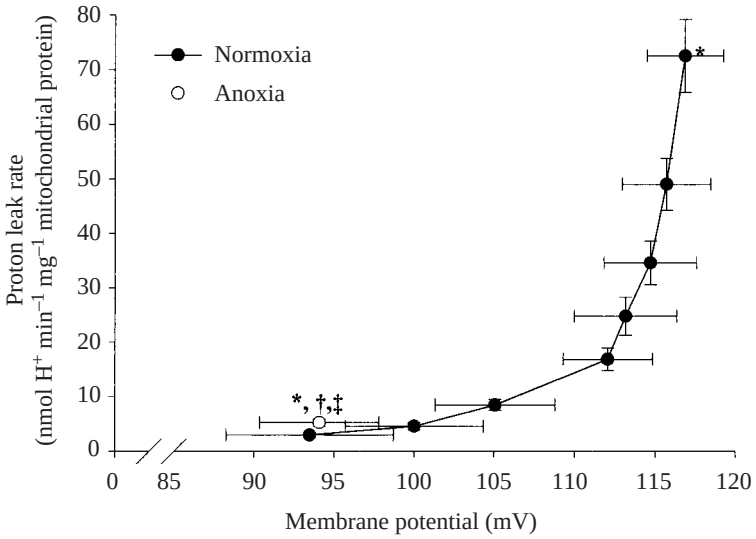

Fig. 4. Kinetics of frog skeletal muscle mitochondrial proton leak during normoxia and in anoxia. During normoxia, the membrane potential is generated by the electron-transport chain; however, during anoxia, the electron-transport chain is inhibited, and the ATP synthase functions as an ATPase, pumping protons from the matrix to the cytosol to preserve a certain membrane potential. The normoxic and anoxic conditions are represented by filled and open circles, respectively. Values are means ± S.E.M.; N=6 for both normoxia and anoxia. *Resting proton leak rate and membrane potential. Significant difference from the resting proton leak rate during normoxia (P<0.05; Student's t-test). ‡Significant difference from the resting membrane potential during normoxia (P<0.05; Student's t-test). From St-Pierre et al. (2000b) with permission.

of oxygen, the proton leak of mitochondria isolated from the skeletal muscle of frog is decreased by a reduction in the rate of the appropriate producer of the proton-motive force.

Adaptive increases in the O_2-affinity of cells and mitochondria during metabolic depression

There is no general agreement as to whether mitochondria ever become O_2-limited in steady states of normal function (for a review, see Gnaiger et al., 1995). Several studies have been carried out on mammalian heart, brain and skeletal muscle, in which the volume-average intracellular O_2 partial pressure corresponds to approximately 0.3 kPa at 37 °C (Wittenberg and Wittenberg, 1989; Erecinska and Silver, 2001). To appreciate the normal functioning of mitochondrial energetics at these low P_{O_2} levels, one needs to determine the range over which mitochondrial respiration is independent of intracellular P_{O_2}. Respiratory control by oxygen has been described for many cell types by a hyperbolic relationship in the low oxygen range (Gnaiger et al., 1995, 1998). Using such relationships, the cellular or mitochondrial oxygen affinity can be expressed as a P_{50} value (i.e. the P_{O_2} at half-maximal respiration, sometimes called K_m). Thus, the rate of mitochondrial oxygen consumption is independent of oxygen availability over a broad range of P_{O_2} levels. It is not until P_{O_2} levels begin to fall below the normal intracellular P_{O_2} values of approximately 0.3 kPa that mitochondrial respiration starts to conform to O_2 availability (i.e. when the intracellular P_{O_2} falls in the oxyconforming part of the hyperbolic relationship between P_{O_2} and respiration rate). To date, it has been impossible to know whether oxygen exerts an important control on resting respiration rates in cells from ectotherms because of a lack of data on mitochondrial O_2-affinity and intracellular O_2 partial pressures.

A number of studies on mammalian tissues have questioned whether the P_{50} of mitochondria can be reduced as an adaptation to hypoxia (i.e. effectively expanding the range over which the rate of mitochondrial respiration would be independent of P_{O_2}). The results of such studies show that, while mitochondrial O_2-affinity is unchanged during hypoxia, cellular O_2-affinity actually increases. The increased O_2-affinity at the cellular level has been ascribed to a redistribution of mitochondria within the cells (for a review, see Jones et al., 1991). We were interested to determine whether the skeletal muscle mitochondria from metabolically depressed frogs would modulate their O_2-affinity at the low P_{O_2} levels that characterise the hypoperfused muscle of hibernating frogs. To this end, we measured the active (state 3) and resting (state 4) respiration rates of mitochondria isolated from the skeletal muscle of frogs hibernating for up to 4 months.

When frogs were hibernating in normoxic water, no significant differences occurred in either the resting or active respiration rates of their skeletal muscle mitochondria. Although we have no direct estimates of skeletal muscle P_{O_2} in hibernating frogs, there is no indication that tissue P_{O_2} levels ever reach mitochondrial state 3 or state 4 P_{50} values during normoxic hibernation (St-Pierre et al., 2000c). Cutaneous gas exchange during normoxia is evidently sufficient to maintain all of the animals' aerobic metabolic requirements for periods of up to 4 months; i.e. haemoglobin O_2-saturation remains high, there is no build-up of lactate, high-energy phosphate levels remain stable and there is only modest use of glycogen reserves in liver and muscle (Donohoe et al., 1998; Donohoe and Boutilier, 1998). In contrast, after 1 month and for up to 4 months of hibernation in hypoxic water, the isolated muscle mitochondria display reduced resting and active respiration rates at any given intracellular oxygen partial pressure (Fig. 5). It seems likely that the intracellular P_{O_2} values inside frog skeletal muscle during hypoxic submergence are near state 3 and state 4 mitochondrial P_{50} values or even lower. Indeed, frogs recruit anaerobic metabolism during the first 2 months of submergence in hypoxic water, as indicated by the marked increase in plasma lactate concentration over this period (Donohoe and Boutilier, 1998). Thus, while the skeletal muscle of overwintering frogs is largely aerobic, it must rely at times on anaerobic glycolysis for its energy requirements. In fact,

frog skeletal muscle might become transiently ischaemic at various stages during hibernation, especially during periods of hypoxic stress or heightened activity.

The mean state 3 and state 4 P_{50} values for mitochondria from non-hibernating control groups of frogs (0.077 and 0.017 kPa, respectively, at 20 °C) are similar to those of rat liver (0.057 kPa and 0.020 kPa, respectively) and rat heart (0.035 kPa and 0.016 kPa, respectively) mitochondria at 30 °C (Gnaiger et al., 1998). The increased mitochondrial P_{50} values of frog skeletal muscle, seen during the transition from a resting state (state 4) to an active one (state 3), mirror those seen in other studies (Sugano et al., 1974; Costa et al., 1997; Gnaiger et al., 1998). However, our results on hibernating frogs reveal that state 3 and state 4 P_{50} values of isolated mitochondria can also change with metabolic rate (i.e. during the transition to hypometabolic states; Figs 3, 5). Unlike earlier studies on mammals, our data on frogs show that an increase in the *in vitro* O_2-affinity of mitochondria can occur following chronic *in vivo* exposure to cellular hypoxia.

Few studies have focused on the intrinsic properties of mitochondria during aerobic metabolic depression or on the possible strategies that may have evolved to increase their efficiency of energy production. From the information available, we know that mitochondrial state 3, but not state 4, respiration rates are reduced in hibernating mammals (Liu et al., 1969; Pehowich and Wang, 1984; Gehnrich and Aprille, 1988; Brustovetsky et al., 1989, 1990, 1993). We also know that the respiration rates of hepatocytes isolated from hibernating ground squirrels are the same as those obtained from hepatocytes isolated from summer 'cold-acclimated' animals (Staples and Hochachka, 1997). Taken together, these results suggest that proton leak rate is not reduced in mammals during hibernation. Even so, no studies have reported detailed proton leak titration curves in hibernating mammals to rule out this possibility. Other studies on ectotherms have shown intrinsic reductions in metabolic rate at the tissue level (aestivating frogs; Flanigan et al., 1991) and at the cellular level (hepatopancreas cells of aestivating snails; Bishop and Brand, 2000; Guppy et al., 2000). Our demonstration that metabolic depression also occurs at the mitochondrial level (St-Pierre et al., 2000a,b; Fig. 5) supports the view that hypometabolism can be reflected at all levels of biological organisation in hypoxia-tolerant animals.

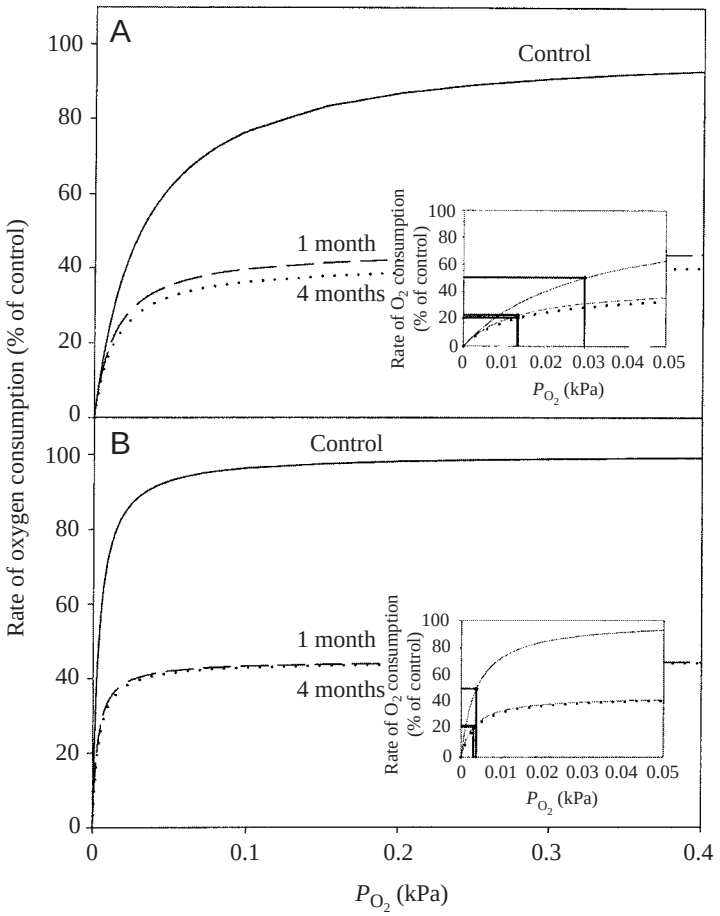

Fig. 5. Kinetics of frog mitochondrial oxygen consumption rates in the low oxygen range at 3 °C for hypoxic submerged frogs. The kinetics for state 3 (A) and state 4 (B) respiration are shown. Solid line, control; dashed line, 1-month-submerged; dotted line, 4-month-submerged groups of frogs. The insets show how P_{50} was calculated for each group of frogs. These curves were generated using the equation $\dot{V}_{O_2}=100[P_{O_2}/(P_{50}+P_{O_2})]$, where $\dot{V}_{O_2}$ (as a percentage of maximum) is the rate of oxygen consumption for state 3 or state 4. The state 3 and state 4 values from the control group of frogs were set to 100 %, and those for the 1- and 4-month-submerged groups of frogs were expressed relative to the controls. To convert P_{O_2} to μmol, kPa must be multiplied by a factor of 11.3 and mmHg by a factor of 1.5. To convert kPa to mmHg, kPa must be multiplied by a factor of 7.51. From St-Pierre et al. (2000c) with permission.

Adaptive changes in cellular and mitochondrial enzymes during metabolic depression

Many studies have focused on adaptations of glycolytic pathways during various states of metabolic depression such as aestivation, hibernation and anoxia-induced hypometabolism. The flux through the glycolytic pathways is normally reduced in these different conditions by (i) reversible phosphorylation of key enzymes of the glycolytic pathways, (ii) changes in the way enzymes bind with cellular structural components, (iii) alteration in the level of fructose 2,6-biphosphate, (iv) modifications in the allosteric control of enzymes and (v) changes in enzymes level (Storey, 1997; Brooks and Storey, 1997). Other studies have examined the properties of key components of mitochondrial metabolism during long-term aerobic metabolic depression in ectotherms.

For example, levels of citrate synthase (CS) and cytochrome *c* oxidase (CCO) decrease in the hepatopancreas of aestivating snails compared with awake controls (Stuart et al., 1998a,b). Even so, there is no reduction in CS activity in either the heart or kidney of aestivating snails (Stuart et al., 1998b). This suggests that metabolic depression at the whole-animal level

might not be reflected in all tissues by modifications in the intrinsic properties of their cell metabolism. Similarly, while the *in vitro* respiration rate of intact skeletal muscle from aestivating frogs is reduced compared with controls, no such decreases occur in the intestine, liver, skin or fat (Flanigan et al., 1991). The activity of pyruvate dehydrogenase (PDH), which regulates the entry of acetyl CoA units in the tricarboxylic acid (TCA) cycle, is also reduced during aestivation in snails by phosphorylation of the enzyme (Brooks and Storey, 1992a). Phosphorylation of PDH to produce a less active form of the enzyme also occurs during hibernation (Brooks and Storey, 1992b) and daily torpor (Heldmaier et al., 1999) in mammals.

Most studies of adaptive plasticity of glycolytic enzymes have focused on post-translational modifications and the levels of their substrates and modulators during short-term, anoxia-induced hypometabolic states (Brooks and Storey, 1997). Reductions in glycolytic enzyme levels have also been observed during long-term aestivation in the terrestrial snail and the frog *Neobatrachus pelobatoides*. The levels of hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and lactate dehydrogenase (LDH) were reduced in the foot muscle of aestivating snails compared with controls (Brooks and Storey, 1990). However, aestivating snails did not show a reduction in the activity of LDH in the kidney, heart and hepatopancreas (Stuart et al., 1998b). The liver of aestivating frogs had lower activities of aldolase and glyceraldehyde-3-phosphate dehydrogenase compared with controls, but the activities of both these enzymes remained unchanged during aestivation in the ventricle, gastrocnemius and brain (Flanigan et al., 1990). Again, these studies emphasise that metabolic depression can have quite separate and distinctly different effects on different tissues. Taken together, the results of numerous studies support the idea that changes in enzyme levels are important during long-term metabolic depression, whereas alterations in the kinetic properties of enzymes and post-translational modifications are more consequential during medium- and short-term metabolic depression (Brooks and Storey, 1990; Greenway and Storey, 1999).

We have recently shown that the profound metabolic depression of frogs hibernating in hypoxia (Donohoe and Boutilier, 1998) is accompanied by a significant decrease in the aerobic capacity of their skeletal muscle (St-Pierre and Boutilier, 2001), as indicated by the reduction in the activity of key enzymes of the TCA cycle and of the electron-transport chain (Fig. 6). Because the decreases in CS activity at the tissue level were smaller than those observed in isolated mitochondria, at least some of the decrease in aerobic capacity during hibernation can be explained by intrinsic changes in aerobic capacity at the level of the mitochondrion (St-Pierre and Boutilier, 2001). The LDH activity of the skeletal muscle of overwintering frogs was also much lower than in pre-hibernation controls, supporting the idea of a decreased flux through the glycolytic pathway during hypoxic hibernation (i.e. the so-called ‘reversed’ Pasteur effect; Hochachka and Somero, 2002; Donohoe and Boutilier, 1998). Frogs rely increasingly on a carbohydrate-based metabolism during hypoxic hibernation (Donohoe and Boutilier, 1998) and metabolic depression is associated with homeostatic concentrations of ATP, phosphocreatine and creatine inside the skeletal muscle, indicating that the decreased ATP demand is matched by a reduced ATP supply from the glycolytic pathway (glycolysis and mitochondrial pathways).

Overall, these results indicate that an important reorganisation of ATP-producing pathways occurs during

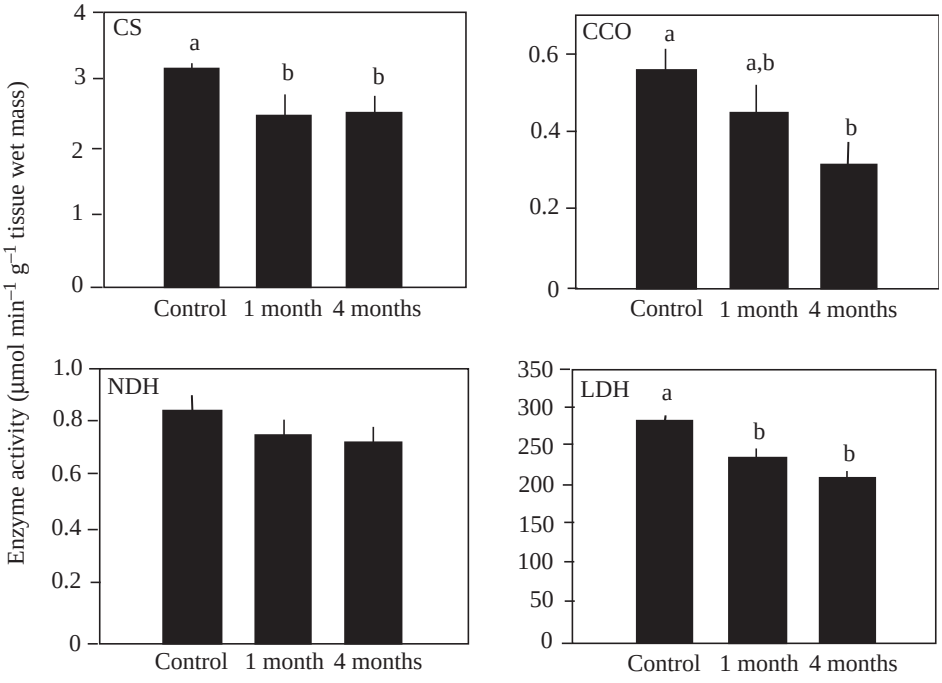

Fig. 6. Mitochondrial and glycolytic enzyme activities at 3 °C for frog skeletal muscle at different stages during hypoxic hibernation. Mitochondrial enzymes: citrate synthase (CS), cytochrome *c* oxidase (CCO) and NADH dehydrogenase (NDH). Glycolytic enzyme: lactate dehydrogenase (LDH). Values are means + S.E.M.; *N*=6 for control frogs, *N*=4 for 1-month-submerged frogs and *N*=5 for 4-month-submerged frogs. Statistically significant differences ($P<0.05$) between groups of frogs are represented by different letters (one-way ANOVA and *a posteriori* Tukey test). From St-Pierre and Boutilier (2001) with permission.

long-term metabolic depression to match the lowered ATP demand. However, recent studies on the aestivation-induced responses of mitochondrial respiration in isolated hepatopancreas cells of snail suggest that the initiation of metabolic depression is through ATP production pathways and not through the pathways of ATP utilisation (Bishop et al., 2002). These authors estimate that changes in the kinetics of substrate oxidation are three times more important than changes in the kinetics of ATP turnover for the response of mitochondrial respiration to aestivation. The implication here is that the processes we normally consider to dominate ATP demand (e.g. ion-motive ATPases and protein synthesis) may in fact not be very important in causing metabolic depression.

Concluding remarks

The overall metabolic depression of frogs during hypoxic hibernation is reflected at the mitochondrial level by an approximately 50 % decrease in their resting and active respiration rates (St-Pierre et al., 2000a,c). The lowered mitochondrial aerobic capacity is due to a decrease in the activity of the electron-transport chain (St-Pierre et al., 2000a). Recent results suggest that complex IV (CCO) and probably complex II (succinate dehydrogenase) are involved in the decrease in electron-transport chain activity during hypoxic hibernation (St-Pierre and Boutilier, 2001). Also, the flux through the TCA cycle, which feeds intermediates in the electron-transport chain, seems to be reduced during hibernation according to the lowered activity of CS in hypoxic submerged frogs compared with controls. The decrease in CS activity is less than that of the electron-transport chain enzymes. Therefore, the flux through the electron-transport chain might exert greater control over the flux through the final pathways of aerobic metabolism during metabolic depression compared with control states (St-Pierre and Boutilier, 2001). Only recently have studies begun to carry out metabolic control analyses of mitochondrial respiration in states of aerobic metabolic depression (Bishop et al., 2002). Interestingly, the decrease in mitochondrial respiration rate during metabolic depression is thought to occur mostly through changes in the kinetics of substrate oxidation, with only a small contribution for ATP turnover (Bishop et al., 2002).

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