The Journal of Experimental Biology 203, 1469–1476 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2654

THE EFFECT OF METABOLIC DEPRESSION ON PROTON LEAK RATE IN MITOCHONDRIA FROM HIBERNATING FROGS

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Accepted 17 February; published on WWW 6 April 2000

Summary

Futile cycling of protons across the mitochondrial inner membrane accounts for 20 % or more of the total standard metabolic rate of a rat. Approximately 15 % of this total is due to proton leakage inside the skeletal muscle alone. This study examined whether the rate of proton leak is down-regulated as a part of a coordinated response to energy conservation during metabolic depression in cold-submerged frogs. We compared the proton leak rate of skeletal muscle mitochondria isolated from frogs at different stages of hibernation (control, 1 month and 4 months of submergence in normoxia and hypoxia). The kinetics of mitochondrial proton leak rate was unaltered throughout normoxic and hypoxic submergence. The state 4 respiration rates did not differ between control animals and frogs hibernating in normoxia. In contrast, the state 4 respiration rates obtained from frogs submerged in hypoxic water for 4 months were half those of control animals. This 50 % reduction in respiration rate in hypoxic hibernation was due to a reduction in electron transport chain activity and consequent decrease in mitochondrial membrane potential. We conclude that proton leak rate is reduced during metabolic depression as a secondary result of a decrease in electron transport chain activity, but that the proton conductance is unchanged. In addition, we show that the rate of proton leakage and the activity of the electron transport chain are lower in frogs than in rats, strengthening the observation that mitochondria from ectotherms have a lower proton conductance than mitochondria from endotherms.

Key words: proton leak, mitochondria, metabolic depression, skeletal muscle, hypoxia, normoxia, hibernation, electron transport chain, frog, *Rana temporaria*, state 4, proton conductance.

Introduction

Faced by harsh overwintering conditions, many species of frog, including *Rana temporaria*, hibernate under water, often in ice-covered ponds (Pinder et al., 1992). This overwintering submergence is associated with three physiological stresses: hypoxia, low temperature and inhibition of normal feeding behaviour (Barica and Mathias, 1979; Bradford, 1983). When *R. temporaria* are cold-submerged in the laboratory at 3 °C, to mimic the overwintering conditions under ice cover, they gradually enter a state of metabolic depression that spares substrate reserves and avoids the accumulation of toxic end-products from anaerobic metabolism (Donohoe et al., 1998). The extent of metabolic depression in overwintering frogs is dependent on the concentration of oxygen in the water. For example, frogs submerged for 90 days in normoxia (P_{O_2}=155 mmHg; 20.7 kPa) exhibit a metabolic rate that is 61 % of that of control frogs that have direct access to air (Donohoe et al., 1998). In contrast, frogs submerged in hypoxia (P_{O_2}=60 mmHg; 8.0 kPa) for up to 4 months manifest an aerobic metabolic rate that is only 25 % of that of control animals (Donohoe and Boutilier, 1998). The key for frogs such as *R. temporaria* to survive long winters is to enter into a hypometabolic state that relies on aerobic metabolism for the production of energy. Indeed, frogs die when facing either a rapid transition to severe hypoxic conditions or prolonged periods at low oxygen levels because they do not have enough time or sufficient capacity to decrease their rates of energy-consuming processes so that they match the rates of aerobic energy supply.

The relative contributions of different biochemical processes to standard metabolic rate have been extensively studied in mammals (primarily in rats). Recently, it has been shown that 90 % of mammalian respiration is mitochondrial, 20 % of which is due to proton leak (futile cycling of protons across the inner membrane of the mitochondria) and 80 % of which is coupled to ATP synthesis. Of the 80 % coupled to ATP synthesis, 25–30 % is used by protein synthesis, 19–28 % by the Na^+/K^+-ATPase, 4–8 % by the Ca^{2+}-ATPase, 2–8 % by the actinomyosin ATPase, 7–10 % by gluconeogenesis and 3 % by ureagenesis,

with substrate cycling and mRNA synthesis making significant contributions (for a review, see Rolfe and Brown, 1997). Among these ATP-consuming processes, the rate of protein synthesis and the activity of the Na^+/K^+-ATPase have frequently been shown to be reduced in diverse ectotherms during metabolic depression (Guppy et al., 1994; Hand and Hardewig, 1996; Hochachka et al., 1997; Guppy and Withers, 1999). Similarly, the ATP demand for protein synthesis, protein breakdown, ureagenesis, gluconeogenesis and the Na^+/K^+-ATPase was shown to be reduced when normoxic turtle hepatocytes were exposed to anoxia (for a review, see Hochachka et al., 1997). In addition, mitochondrial protein synthesis is reduced during anoxia-induced quiescence in brine shrimp embryos (Kwast and Hand, 1996). However, no studies have examined whether hypometabolic states in ectotherms can be effected, at least in part, through reductions in proton leak rates. Although proton leak can account for up to 15 % of active metabolic rate and 20 % of resting metabolic rate in mammals (Rolfe et al., 1999), we have no information about the contribution of the mitochondrial proton leak to standard metabolic rate in ectotherms. The only data available show that the rat and the bearded dragon (reptile) devote a similar proportion of their hepatocyte respiration to drive proton leak (Brand et al., 1991).

Mitochondrial proton leak is correlated with a whole host of factors that determine basal metabolic rate. For example, proton leakiness changes with body size, phylogeny and metabolic status, such that it increases with metabolic rate (Brand and Murphy, 1987; Hafner et al., 1988; Brand et al., 1991, 1994b; Porter and Brand, 1993; Porter et al., 1996; Ramsey et al., 1996). Changes in apparent proton leakiness can be brought about by changing the intrinsic properties of the mitochondria or by altering the number of mitochondria within a tissue. Two principal factors have been put forward to explain the differences in the proton leakiness of isolated mitochondria: an increase in the surface area of the mitochondrial inner membrane and a modification in the fatty acid composition of the mitochondrial phospholipid membrane (i.e. modification of the mitochondrial membrane properties *per se*; Brand, 1990). Since skeletal muscle represents a substantial proportion of an animal's body mass (approximately 30 % in rat, Rolfe and Brown, 1997; approximately 40 % in frogs, Boutilier et al., 1997), it is an important contributor to standard metabolic rate. It has also been shown that as much as 50 % of the oxygen consumption of resting muscle in rat is due to proton leak (the contribution of the proton leak in muscle to standard metabolic rate is approximately 15 %; Rolfe and Brand, 1996). Since the proportion of skeletal muscle to total body mass in frogs is roughly the same as in mammals, modulation of muscle mitochondrial proton leak could therefore be an important phenomenon regulating metabolic depression in overwintering frogs. Moreover, the extent of metabolic depression in the muscle of overwintering frogs may be higher than in other tissues since the blood flow to the muscle in the cold-submerged animal is greatly reduced to shunt more blood to the skin for the extraction of oxygen (Pinder et al., 1992).

To determine whether proton leak rate can be down-regulated as a part of a coordinated response to energy conservation in frog skeletal muscle during overwintering, we measured the proton leakiness of mitochondria isolated from the thigh musculature of frogs at different stages during their hibernation.

Materials and methods

Animals

The frogs used in these experiments were adult male *Rana temporaria* (approximately 25–30 g) collected by a local supplier (Blades Biological Co., UK) during the winters of 1998 and 1999. At the start of each winter, frogs were acclimated to water at 3 °C for 4 weeks, during which time they had direct access to air. After the acclimation period, 15 frogs were then taken for experiments (control groups) and another 30 were submerged in either normoxic water (P_{O_2}=155 mmHg, 20.7 kPa; winter 1998) or hypoxic water (P_{O_2}=60 mmHg, 8.0 kPa; winter 1999) in a temperature-controlled recirculated water system (Living Stream, Frigid Units Inc., Cleveland, Ohio, USA) maintained at 3 °C as described in Donohoe and Boutilier (1998). The frogs submerged in normoxia and hypoxia were sampled after 1 month and 4 months.

For each set of experiments carried out on frogs, we measured the proton leak rate of rat liver mitochondria as an internal control. Although the utilisation of rat skeletal muscle mitochondria would have facilitated comparisons with the results obtained using frogs, the main purpose of this control was to verify that the experimental arrangement gave a constant response over time, thereby lessening the importance of the tissue choice. Female Wistar rats (approximately 250 g; 2–3 months old) were used to prepare liver mitochondria. The results obtained with the rat mitochondria were identical between the different sampling periods of each year, confirming that the system was giving a consistent response over time. Given that, we averaged the six proton leak curves that we obtained with the rats during the 2 year study in order to compare the kinetics of proton leak rate between rats and frogs.

Isolation of mitochondria

Frogs were killed according to the Schedule 1 Home Office protocol. Wistar rats were maintained at 25 °C and also killed according to the Schedule 1 Home Office protocol. Rat liver mitochondria were isolated as described previously (Brookes et al., 1997b). Frog mitochondria were isolated according to a modification of the method of Hillman et al. (1991). The thigh muscles of three frogs were pooled for each mitochondrial preparation. Each thigh muscle was rapidly excised and finely minced with razor blades. The tissue was then placed in a beaker containing the isolation medium and further homogenised using micro-scissors. The isolation medium contained 170 mmol l^{-1} mannitol, 55 mmol l^{-1} sucrose, 5 mmol l^{-1} EGTA, 20 mmol l^{-1} Hepes, 50 i.u. ml^{-1} heparin and 0.5 % bovine serum albumin (BSA) adjusted to pH 7.3 at room temperature (20 °C). The

homogenate was transferred to a Potter-type glass homogenizer and ground (200 revs min^{-1}) consecutively with three pestles of increasing size (two strokes with each pestle). The homogenate was then centrifuged at 755 ***g*** for 5 min, and the supernatant was subsequently filtered through medical gauze and centrifuged at 9800 ***g*** for 11 min. The resulting pellet was then washed with isolation medium lacking heparin, resuspended and centrifuged at 9800 ***g*** for 8 min. The final pellet was resuspended in isolation medium lacking heparin at a concentration of 25–30 mg mitochondrial protein ml^{-1}. Rat and frog mitochondrial protein concentrations were determined using the Biuret method (Gornall et al., 1949), and sodium deoxycholate was added to disrupt the membranes.

Proton leak rate measurements

Mitochondrial preparations from rat liver and frog skeletal muscle were of good quality, displaying at least a threefold increase in respiration rate upon addition of the uncoupler FCCP. Proton leak rate measurements were carried on the basis of the protocol of Brand (1995) and Rolfe et al. (1994). The mitochondrial oxygen consumption rate and membrane potential were measured simultaneously at 25 °C in a thermostatically controlled chamber. We measured the frog mitochondrial proton leak rate at 25 °C because the respiration rate of frog mitochondria is so low at 3 °C that it would have been impossible to carry out accurate measurements. Since the Q_{10} values between 3 and 20 °C for the state 4 respiration rate of mitochondria are not significantly different between control, 1 month and 4 month normoxic or hypoxic submerged frogs (J. St-Pierre, G. J. Tattersall and R. G. Boutilier, unpublished results), the proton leak rate comparisons at 25 °C between the three groups of frogs for each year should be adequate. It is important to mention that, although it was possible to obtain state 4 values for frog skeletal muscle mitochondria at 3 °C, it was impossible to obtain accurate titration curves of respiration rate. Indeed, the lowest respiration rates on our proton leak titration curves generated at 25 °C were approximately seven times lower than the initial state 4 respiration rates. We used a Clark-type electrode to measure oxygen consumption rate and an electrode sensitive to the lipophilic cation triphenylmethylphosphonium ($TPMP^+$) to determine membrane potential. Frog and rat mitochondria were incubated at a concentration of 1 mg ml^{-1} in a medium containing 120 mmol l^{-1} KCl, 5 mmol l^{-1} K_2HPO_4, 3 mmol l^{-1} Hepes, 1 mmol l^{-1} EGTA, 0.3 % defatted BSA, 5 μmol l^{-1} rotenone, 1 μg ml^{-1} oligomycin (to ensure that the respiration drives only proton leak and not ATP synthesis), 80 ng ml^{-1} nigericin (to clamp ΔpH to zero) and 5 mmol l^{-1} succinate, pH 7.4 (pH 7.2 for rat), at 25 °C. The TPMP electrode was calibrated with sequential additions up to 2.55 μmol l^{-1} for frog mitochondria and 5 μmol l^{-1} TPMP for rat mitochondria. Malonate was added sequentially up to 5.3 mmol l^{-1} for frog mitochondria and 6.4 mmol l^{-1} for rat mitochondria to change the mitochondrial membrane potential. After each run, FCCP was added at a final concentration of 2.9 μmol l^{-1} to release TPMP for baseline correction. Each run was carried out at least in duplicate, and most were carried out in triplicate. The TPMP binding correction was considered to be 0.4 (μl mg^{-1} protein)$^{-1}$ for both frog and rat mitochondria. The oxygen solubility of the medium was 479 nmol O ml^{-1} assay medium.

Calculations and statistical analyses

All data are presented as means ± S.E.M. The proton leak rate was obtained by multiplying the oxygen consumption rate by six (Brand, 1994). We assumed that there was no slip in the frog and rat mitochondrial proton pumps, and there is evidence of the absence of slip in the rat mitochondrial proton pumps at 37 °C (Brand et al., 1994a; Canton et al., 1995), although there is some controversy for 25 °C (Hafner and Brand, 1991; Canton et al., 1995).

Statistical analyses were performed using SigmaStat (version 2.0). Comparisons of state 4 rates and state 4 membrane potential values between the three groups of frogs during normoxic and hypoxic hibernation were performed using one-way analysis of variance (ANOVA) and the *a posteriori* Tukey test. Comparisons of state 4 rates and state 4 membrane potentials between frog skeletal muscle mitochondria and rat liver mitochondria were carried out using Student's *t*-test. The level of significance was P=0.05.

Results

The kinetics of mitochondrial proton leak did not differ between control animals and frogs hibernating in normoxia (Fig. 1). In fact, the oxygen consumption rates at any given membrane potential were almost identical for the three groups of animals. In addition, neither the state 4 values (the highest point on the proton leak curve) nor the state 4 membrane potential values (Table 1; Fig. 1) varied significantly among the three groups of frogs (Table 1; Fig. 1).

As observed in normoxic hibernation, the kinetics of mitochondrial proton leak remained unaltered throughout all stages of hypoxic submergence (Fig. 2). However, the state 4 respiration rate obtained with the 1 month submerged group of frogs (4.46±0.29 nmol O min^{-1} mg^{-1} mitochondrial protein) was significantly lower than that observed in control animals (6.27±0.60 nmol O min^{-1} mg^{-1} mitochondrial protein; Table 1; Fig. 2). In addition, the state 4 respiration rate of the 4 month submerged animals (3.10±0.32 nmol O min^{-1} mg^{-1} mitochondrial protein) was reduced compared with both the control and the 1 month submerged frogs, but the difference reached statistical significance only between control and 4 month submerged frogs (Table 1; Fig. 2). If the state 4 respiration rate of isolated mitochondria approximates the actual respiration rate of mitochondria in intact resting muscle, these results suggest that the proton leak rate of mitochondria *in vivo* would be reduced by half in 4 month hypoxic submerged animals compared with controls. This reduction in proton leak rate in hibernating frogs was not achieved by changing the kinetics of the proton leak, but rather by altering the electron transport chain activity of the mitochondria. In fact, a reduction in the mitochondrial electron

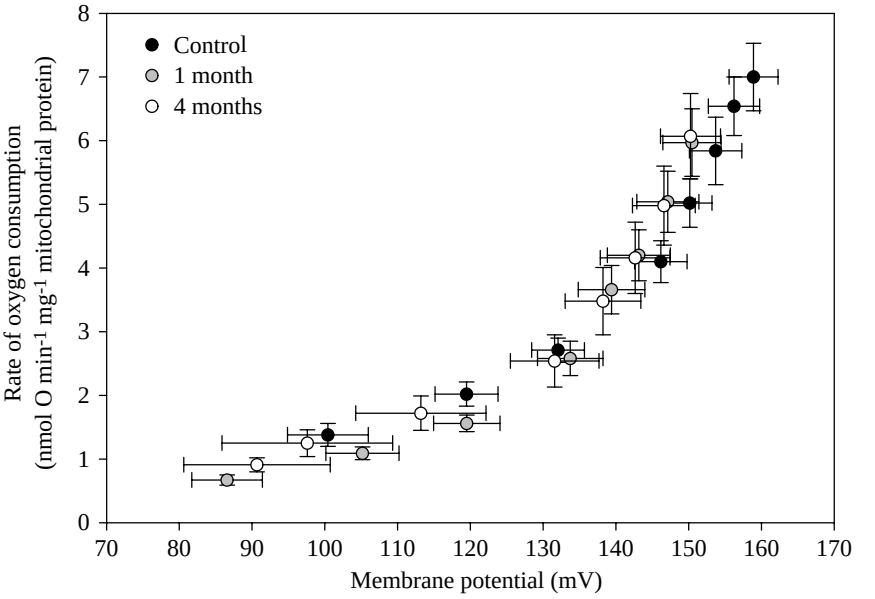

Fig. 1. Kinetics of the mitochondrial proton leak at 25 °C for normoxic submerged animals. Stepwise changes in respiration rate and membrane potential were achieved by adding increasing amounts of malonate. The control, 1 month submerged and 4 month submerged groups of frogs are represented by black, grey and white shading, respectively. Values are means ± S.E.M., *N*=5 for control and 4 month submerged frogs, *N*=6 for 1 month submerged frogs.

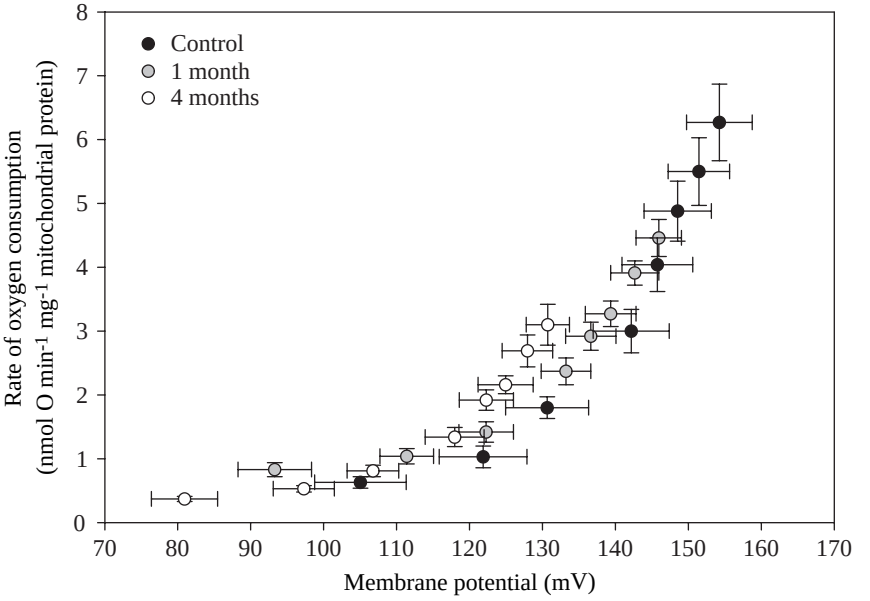

Fig. 2. Kinetics of the mitochondrial proton leak at 25 °C for hypoxic submerged animals. Stepwise changes in respiration rate and membrane potential were achieved by adding increasing amounts of malonate The control, 1 month submerged and 4 month submerged groups of frogs are represented by black, grey and white shading, respectively. Values are means ± S.E.M., *N*=5 for control and 1 month submerged frogs, *N*=4 for 4 month submerged frogs.

Table 1. *State 4 respiration rate and membrane potential at 25 °C of frog muscle mitochondria during normoxic and hypoxic hibernation*

	Normoxic hibernation		Hypoxic hibernation	
Group	State 4 respiration rate (nmol O min^{-1} mg^{-1} mitochondrial protein)	State 4 membrane potential (mV)	State 4 respiration rate (nmol O min^{-1} mg^{-1} mitochondrial protein)	State 4 membrane potential (mV)
Control	7.00±0.53 (5)	158.92±3.37 (5)	6.27±0.60 (5)	154.26±4.51 (5)
1 month submerged	5.97±0.53 (6)	150.46±4.01 (6)	4.46±0.29* (5)	145.95±3.11 (5)
4 months submerged	6.07±0.67 (5)	150.26±4.12 (5)	3.10±0.32* (4)	130.74±2.96*‡ (4)

Values are means ± S.E.M. The number of mitochondrial preparations is indicated in parentheses.
*Significant difference from the corresponding control group of frogs; $P<0.05$ (one-way ANOVA and *a posteriori* Tukey test).
‡Significant difference from the corresponding 1 month submerged group of frogs; $P<0.05$ (one-way ANOVA and *a posteriori* Tukey test).

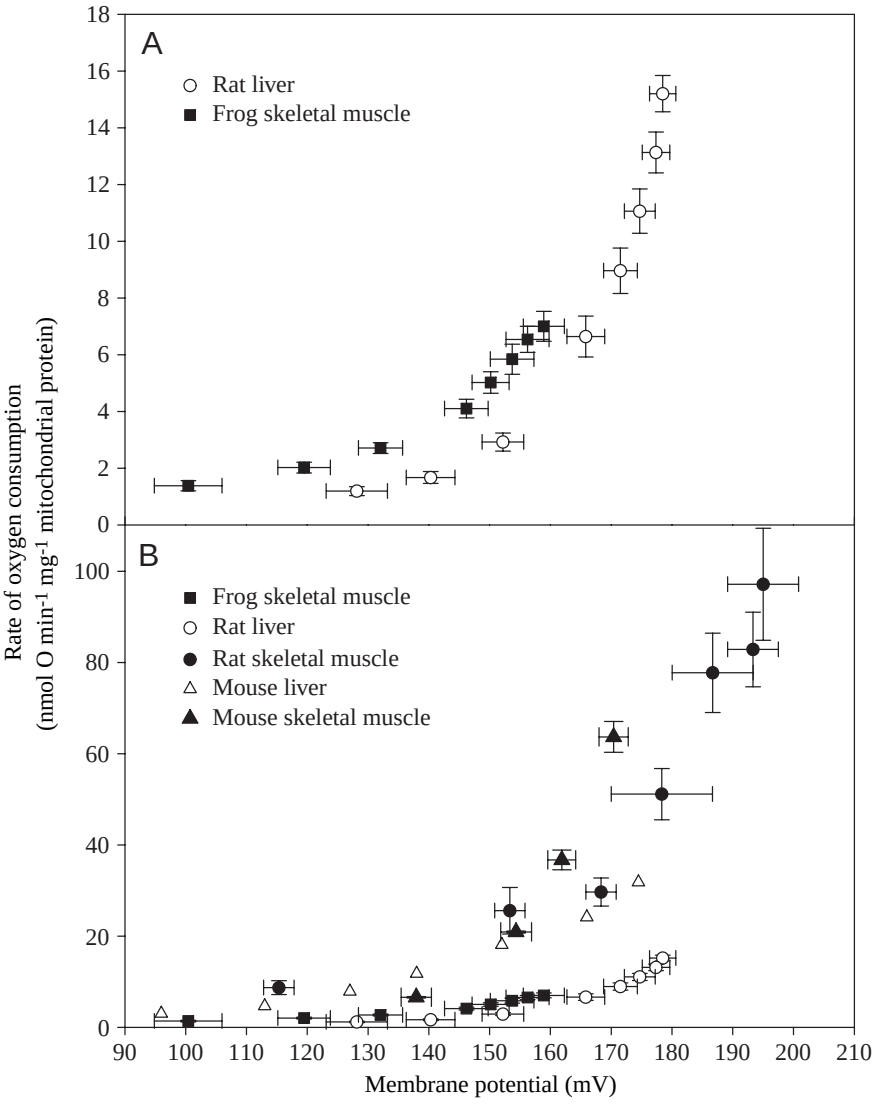

Fig. 3. (A) Kinetics of the proton leak in frog skeletal muscle mitochondria and in rat liver mitochondria at 25 °C. The frog and rat proton leak rates are represented by squares and circles, respectively. Values are means ± S.E.M., *N*=5 for frog skeletal mitochondria; *N*=6 for rat liver mitochondria. (B) Kinetics of the mitochondrial proton leak in frog skeletal muscle, in rat liver and skeletal muscle and in mouse liver and skeletal muscle at 25 °C. The mitochondrial proton leak curves for frog skeletal muscle and rat liver are those from A. The mitochondrial proton leak curves for rat skeletal muscle, mouse liver and mouse skeletal muscle are adapted from Rolfe et al. (1994), Porter and Brand (1993) and Susana Cadenas (personal communication), respectively, assuming a Q_{10} of 2 for the rate of oxygen consumption between 25 and 37 °C. We made no correction for the effect of temperature on membrane potential values. According to Dufour et al. (1996), a decrease in temperature from 37 to 25 °C will make the proton leak curve of rat liver mitochondria slightly steeper, i.e. the decrease in mitochondrial proton leakage will be more rapid at intermediate values of membrane potential. The frog, rat and mouse proton leak curves are represented by squares, circles and triangles, respectively. Liver and skeletal muscle mitochondria are represented by open and filled symbols, respectively. Values are means ± S.E.M.

transport chain activity will lower the membrane potential and thus the proton leak rate. Because of the steep dependence of proton leak rate on mitochondrial membrane potential, a small decrease in membrane potential can lead to a considerable reduction in proton leak rate. The state 4 mitochondrial membrane potential value obtained in the 4 month hypoxic submerged frogs (130.74±2.96 mV) was significantly lower than those obtained in control (154.26±4.51 mV) and 1 month hypoxic submerged (145.95±3.11 mV) frogs (Table 1; Fig. 2), but there was no significant difference between the membrane potential values obtained in control and 1 month submerged frogs (Table 1; Fig. 2).

To compare frog and rat mitochondrial proton leak rates, we chose the proton leak curve obtained from the control frogs of the normoxic hibernation experiment since there was no significant difference between the proton leak curves obtained with the control groups of frogs for the normoxic and hypoxic hibernation experiments. The proton leak rate of frog skeletal muscle mitochondria was approximately twice that of rat liver mitochondria at any given membrane potential (Fig. 3A). Since proton leak rate is higher (approximately sixfold) in rat skeletal muscle mitochondria than in rat liver mitochondria at any given membrane potential (Rolfe et al., 1994), the proton leakiness of frog muscle mitochondria was approximately three times lower than that of rat muscle mitochondria (Fig. 3B). In addition, the state 4 respiration rate and the state 4 membrane potential were significantly lower in frog mitochondria than in rat liver mitochondria (7.00±0.53 nmol O min^{-1} mg^{-1} mitochondrial protein and 158.92±3.37 mV for frogs compared with 15.20±0.64 nmol O min^{-1} mg^{-1} mitochondrial protein and 178.48±2.15 mV for rats; $P<0.001$, Student's *t*-test; Fig. 3A). The latter result indicates that the reactions of substrate oxidation are less active in frogs than in rats.

Discussion

This study examined the effect of metabolic depression in ectotherms on their mitochondrial proton leak rate. The results presented show that a modest metabolic depression (39 %) in

normoxic hibernating frogs is not accompanied by a reduction in mitochondrial proton leak rate (Table 1; Fig. 1), while the larger reduction in metabolic rate (75 %) seen in hypoxic animals (Donohoe and Boutilier, 1998) leads to at least a twofold reduction in mitochondrial electron transport chain activity and thus to a reduction in proton leak rate (Table 1; Fig. 2). This reduction in proton leak rate at the mitochondrial level may preserve or increase the efficiency of aerobic energy production by frog skeletal muscle fibres during metabolic depression. In fact, if proton leak rate were to decrease by the same proportion as cellular respiration rate during metabolic depression this would preserve the same efficiency of aerobic energy production as observed in control animals. However, the absence of a down-regulation in proton leak rate during aerobic metabolic depression would lead cells to burn more substrates to produce the same amount of energy. Since metabolic depression is associated with a lack of energy intake, it is a useful strategy to reduce mitochondrial proton leak rate to spare substrate reserves. However, we have ignored probable changes in substrate supply in intact muscle during normoxic or hypoxic hibernation.

Organisms in hypometabolic states can rely on aerobic or anaerobic metabolism to produce energy. Animals relying on glycolysis to produce energy during anaerobic metabolic depression have evolved improved fermentative pathways (Hochachka and Somero, 1984) and/or possess phosphorylation processes that regulate key enzymes involved in the production of energy (for reviews, see Storey and Storey, 1990; Brooks and Storey, 1997; Storey, 1997). However, few studies have focused on the intrinsic properties of mitochondria during aerobic metabolic depression and on the possible strategies that may have evolved to increase their efficiency of energy production. Martin et al. (1999) investigated whether proton leak rate might be reduced in hibernating mammals by measuring mitochondrial state 4 respiration, which is a rough indicator of proton leak. They found no difference in the state 4 respiration rate between hibernating and control animals (Martin et al., 1999), a result that was also obtained in previous studies carried out on hibernating mammals (Liu et al., 1969; Pehowich and Wang, 1984; Gehnrich and Aprille, 1988; Brustovetsky et al., 1989, 1990, 1993). Similarly, the resting respiration rate of hepatocytes isolated from hibernating ground squirrels did not differ from that of hepatocytes 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 carried out detailed proton leak titration curves in hibernating mammals to rule out this possibility. Nor, to our knowledge, has anyone looked at a possible down-regulation of mitochondrial proton leak in ectotherms during metabolic depression. Another study carried out on frog skeletal mitochondria showed that state 4 respiration rates at 3 and 20 °C were not reduced in normoxic hibernation, whereas the opposite result was observed during hypoxic hibernation (J. St-Pierre, G. J. Tattersall and R. G. Boutilier, unpublished results). These results coincide perfectly with the absence of reduction in mitochondrial proton leak rates during normoxic hibernation (Table 1; Fig. 1) and a decrease in the proton leak rate of mitochondria during hypoxic hibernation (Table 1; Fig. 2).

Recent studies carried out on the terrestrial snail have also looked at the intrinsic properties of mitochondria during aerobic metabolic depression (Stuart et al., 1998a,b). Hepatopancreas mitochondria isolated from aestivating snails displayed a reduction in cytochrome *c* oxidase activity as well as a different membrane phospholipid composition compared with mitochondria isolated from control animals (Stuart et al., 1998a,b). In fact, the mitochondrial membrane composition of aestivating snails is similar to that in animals with a low standard metabolic rate and proton leak rate (Stuart et al., 1998a,b), suggesting a reduction in mitochondrial proton leak rate during aestivation in terrestrial snails.

Our finding that frog mitochondria are less leaky than rat mitochondria at 25 °C (Fig. 3) has to be interpreted with caution since this temperature is not physiological either for the frog or the rat. It is possible that the frog and the rat may have different Q_{10} values for respiration that could affect the relative positions of the proton leak curves. However, our comparisons of the kinetics of mitochondrial proton leak between the frog and the rat are conservative because the rats used in the present study were larger than the frogs, and it is known that there is an inverse relationship between the proton leakiness of mitochondria and body size in mammals (Porter and Brand, 1993). In fact, if we were to compare the proton conductance of our frog skeletal muscle mitochondria with that of mitochondria isolated from a similarly sized mammal (e.g. a mouse), we would find that the frog mitochondria are much less leaky (approximately sixfold). This can be explained by the fact that mouse mitochondria are more leaky to protons than rat mitochondria by a factor of approximately 2 (Porter and Brand, 1993; see Fig. 3B) and that the proton leakiness of frog mitochondria is approximately three times lower than that of rat mitochondria (see Results section and Fig. 3B).

Previous observations have also shown that mitochondria from ectotherms are generally less leaky to protons than those from endotherms (rat, Brand et al., 1991; Brookes et al., 1998). Akhmerov (1986) has also reported lower state 4 respiration rates in frog liver and heart mitochondria compared with those of rat liver and heart mitochondria. The differences in the proton leakiness of mitochondria between ectotherms and endotherms are not correlated with the surface area of the mitochondrial inner membrane, but rather with a modification of the fatty acid composition of the mitochondrial membrane phospholipids (Brand et al., 1991; Brookes et al., 1998). Although the mitochondrial membranes of ectotherms are less permeable to protons than those of endotherms, ectotherms do not seem to display a more efficient aerobic metabolism. In fact, it has been shown that hepatocytes isolated from the bearded dragon (reptile) and the rat devote a similar proportion of their resting respiration to drive mitochondrial proton leak (Brand et al., 1991).

Because the proton leak across liposomes is approximately

5 % of the leak obtained with isolated rat liver mitochondria, proton leak pathways through bulk phospholipid bilayers make up only a small proportion of the leak observed in intact mitochondria (Brookes et al., 1997b). The fact that the membrane fatty acid composition of mitochondria is correlated with the proton leakiness in isolated mitochondria, but not in pure phospholipid membranes (Brookes et al., 1997a), is of interest in understanding the mechanism responsible for proton leak. As suggested by Brookes et al. (1998), the fatty acids inside the mitochondrial membrane might affect the behaviour of putative proteins implicated in the proton leak process. An attractive possibility is that such proteins include two homologues of the brown adipose tissue uncoupling protein UCP1, UCP2 and UCP3. Intense efforts are currently under way to determine whether these homologues are capable of catalysing the mitochondrial proton leak of other tissues. However, this remains a point of some controversy in the literature, since there has yet to be any unequivocal demonstration that UCP2 and UCP3 catalyse mitochondrial proton leak under physiological conditions (Brand et al., 1999).

In conclusion, the results presented in this manuscript strengthen the previously observed correlation between standard metabolic rate and the proton leakiness of mitochondrial membranes. There are four ways in which to change the mitochondrial proton leak inside cells: (i) by altering 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 of the cell, (iii) by changing the volume density of mitochondria inside the cell, and (iv) by altering the cristae surface density within the mitochondrion. We have now shown that the proton conductance is unchanged in normoxic and hypoxic hibernation, but that the proton leak rate in frog skeletal muscle mitochondria is reduced during hypoxic hibernation as a result of a reduction in electron transport chain activity. The next step is to investigate whether changes in the volume density of mitochondria inside skeletal muscle fibres bring about a reduction in the proton leak rate of mitochondria during normoxic submergence or act in concert with our observed intrinsic changes in the mitochondria to bring about a larger reduction in proton leak rate during hypoxic hibernation.

The authors want to thank J. A. Buckingham for excellent technical assistance. This work was funded by a Natural Environment Research Council operating grant to R.G.B. J.St-P. was a recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC), Fonds pour la formation de chercheurs et l'aide à la recherche (FCAR) and Trinity College (Cambridge, UK) scholarships.

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