Metabolic depression and enhanced $O_2$ affinity of mitochondria in hypoxic hypometabolism

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St-Pierre, Julie, Glenn J. Tattersall, and Robert G. Boutilier. Metabolic depression and enhanced $O_2$ affinity of mitochondria in hypoxic hypometabolism. Am J Physiol Regulatory Integrative Comp Physiol 279: R1205–R1214, 2000.—This study examined whether the steady-state hypometabolism seen in overwintering frogs (Rana temporaria) is reflected at the mitochondrial level either by a reduction in their resting (state 4) and active (state 3) respiration rates and/or by increases in $O_2$ affinity. We isolated mitochondria from the skeletal muscle of cold-submerged frogs at different times of the winter, and/or by increases in $O_2$ affinity. We isolated mitochondria from the skeletal muscle of cold-submerged frogs at different stages during their hibernation in normoxic and hypoxic water. A modest metabolic depression at the whole animal level (normoxic submergence) was not associated with a reduction in mitochondrial state 4 and state 3 respiration rates. However, mitochondria isolated from frogs that were submerged for 1 mo manifested an increase in their $O_2$ affinity compared with controls and with animals submerged for 4 mo. Hypometabolism was more pronounced at the whole animal level during hypoxic submergence and was accompanied by 1) a reduction in mitochondrial state 4 and state 3 rates and 2) an increase in the $O_2$ affinity of mitochondria. These findings demonstrate that metabolic depression can be reflected at all levels of biological organization in hypoxia-tolerant animals.

frog; hypoxia; state 3; state 4; $P_{50}$

Many species of frogs cope with harsh overwintering conditions each year. The common frog, Rana temporaria, hibernates under water, often in ice-covered ponds (40). Although the selection of an aquatic environment protects against the stresses of freezing and desiccation, overwintering submergence can last for several months and is often associated with severe hypoxia as well as inhibition of normal feeding behavior (2, 5). When R. temporaria are submerged without air access for 3 to 4 mo at 3°C, so as to mimic the overwintering conditions under ice cover, they enter gradually into a state of metabolic depression. The extent of metabolic depression is greater when frogs are exposed to hypoxic environments (75% depression at $P_{O_2} = 60 \text{ mmHg}$; Ref. 14) than in normoxia (39% depression at $P_{O_2} = 155 \text{ mmHg}$; Ref. 15). The key to their survival during long periods of cold submergence in hypoxic environments is their ability to enter slowly into a hypometabolic state so that their energetic needs can be met aerobically. Indeed, R. temporaria deplete their substrate reserves and perish when they are presented with rapid or prolonged exposure to severe hypoxia ($P_{O_2} < 40 \text{ mmHg}$) because they lack the capacity to acutely suppress their metabolic rate to match such drastic reductions in ambient oxygen supply (13).

Metabolic depression is the strategy of choice for many overwintering ectotherms facing energy limitations because it spares substrate reserves and avoids the accumulation of toxic end-products from anaerobic metabolism (31). At the cellular level, metabolic depression may be brought about by decreasing ATP consuming processes and/or by increasing the efficiency of ATP producing pathways (reviewed in Refs. 24, 26, 28, 30, 44). Among the ATP consuming processes, the rates of protein synthesis and of Na$^+-K^+$-ATPase are often reduced in metabolically depressed ectotherms (24, 26, 28, 30). For example, when R. temporaria were submerged in normoxic or hypoxic water, the activity of their skeletal muscle Na$^+-K^+$-ATPase was reduced by as much as 50% (16). In addition to a reduced ATP demand for protein synthesis and for Na$^+-K^+$-ATPase activity, the rates of protein breakdown, ureagenesis, and gluconeogenesis were found to be reduced when normoxic turtle hepatocytes were exposed to anoxia (reviewed in Ref. 30). Most studies concerning the increased efficiency of energy production during metabolic depression have focused on anaerobic metabolism. For example, some invertebrate species display energetically improved fermentative pathways during anoxia (31). In addition, enzyme phosphorylation events can play important roles in regulating glycolytic ATP production (reviewed in Refs. 6, 43, 44). However, despite the fact that many organisms entering into metabolic depression maintain an overall aerobic metabolism, little information exists concerning the intrinsic properties of mitochondria in such hypometabolic states.

During prolonged submergence, frogs hyperperfuse their cutaneous vasculature to facilitate transcutaneous $O_2$ uptake (4). As a consequence of preferentially redistributing the $O_2$-enriched blood at the skin to the hypoxia-sensitive central organs, the largely inactive...
and hypoxia-tolerant skeletal muscle mass becomes hypoperfused (4, 40). It appears, therefore, that the skeletal muscle of overwintering frogs may become severely hypoxic for prolonged periods during hibernation. Given that experiments on isolated frog muscle show that step decreases in perfuse oxygen lead to step decreases in muscle metabolic rate (3, 49) and given that 35–40% of the frog’s body mass is skeletal muscle, we can conclude that blood flow and therefore O₂ flow limitations to the oxyconforming muscle mass probably serve to bring about a major proportion of the overall reduction in whole animal metabolic rate (14).

The aim of this study was to test whether the metabolic depression observed in overwintering frogs is reflected at the mitochondrial level either through changes in respiration and/or mitochondrial O₂ affinity. To do so, we examined the intrinsic properties of respiration in mitochondria isolated from the skeletal muscle of frogs at various stages of their hibernation.

**MATERIALS AND METHODS**

**Animals.** All animals used in these experiments were adult male *R. temporaria* (~25–30 g) collected by a local supplier (Blades Biological) during the winters of 1998 and 1999. At the start of each winter, frogs were acclimated to 3°C water for 4 wk, during which time they had direct access to air. After this acclimation period, 15 frogs were then taken for experiments (control groups) while another 30 were submerged in either normoxic water (P O₂ = 155 mmHg; winter 1998) or hypoxic water (P O₂ = 60 mmHg; winter 1999) in a temperature-controlled recirculated water system (Living Stream, Frigid Units, Cleveland, OH) maintained at 3°C, as described previously (14). The normoxic and hypoxic submerged frogs were sampled after 1 and 4 mo.

**Isolation of mitochondria.** Frogs were killed by concussion and heparin at a concentration of 25–30 mg of mitochondrial protein/ml heparin and 0.5% BSA adjusted to pH 7.3 at room temperature. The experiments were carried out at 3 and 20°C. The main purpose of the experiments carried out at 20°C was to facilitate comparisons with previous data. The temperature of the Oxygraph was regulated to ±0.05°C by a Peltier heat pump. The oxygen solubility of the assay medium was considered to be 18.194 and 12.135 μM/kPa at 3 and 20°C respectively. Calibration of the system, including signal correction for electrode response time, blank controls, internal zero calibration as well as data acquisition and analyses, was carried out as described elsewhere (22, 27, 37). The signals from the oxygen electrode were recorded at 1-s intervals on a computer-driven data acquisition system (DatLab software; Oroboros, Innsbruck, Austria).

The oxygen concentration in the Oxygraph chamber was reduced to ~20% of air saturation at each experimental temperature by blowing nitrogen on the surface of the assay medium before the addition of mitochondria at a final concentration of 1 mg mitochondrial protein/ml. First, malate (1.2 mM) was added to spark the Krebs cycle followed by the addition of pyruvate (5 mM). The respiration rate of mitochondria under these conditions is called state 2. The state 3 respiration rate was then obtained by the addition of 0.428 mM ADP. The mitochondria subsequently consumed all of the oxygen and entered anoxia while still in state 3. After 10 min of anoxia, the oxygen concentration in the chamber was raised again to roughly 20% of air saturation by lifting the lid for a few seconds. The state 4 respiration rate was then reached (state 4 is reached when all the ADP is phosphorylated into ATP) and was recorded until the mitochondria entered a second anoxic period. The experimental protocol is represented graphically in Fig. 1. The respiratory control ratio (RCR) was calculated by dividing the state 3 respiration rate by the state 4 respiration rate.

**Calculations and statistical analyses.** All data are presented as means ± SE. Statistical analyses were performed with SigmaStat (version 2.0). Comparisons of state 3 rates, state 4 rates, state 3 O₂ affinity (1/P₅₀) values, state 4 P₅₀ values, RCR values, and Q₁₀ values between the three groups of frogs for each year were performed with one-way ANOVA and the a posteriori test of Tukey. Comparisons of state 3 rates, state 4 rates, state 3 P₅₀ values, state 4 P₅₀ values, and RCR values between the two groups of control frogs used the Student’s t-test. Comparisons of RCR values between experimental temperatures and of P₅₀ values between state 4 and state 3 rates were carried out with Student’s paired t-test. The level of significance was P = 0.05.

**RESULTS**

**General characteristics of mitochondria.** For the experiments carried out in 1998 (normoxic hibernation), there were no significant differences in the RCR values of control, 1-mo-submerged, and 4-mo-submerged groups of frogs at either of the experimental temperatures (Table 1). However, there was a general tendency toward higher RCR values at 20°C compared with 3°C, but this only reached statistical significance for the 1-mo-submerged group of frogs (P = 0.046; Table 1). There was no difference in the Q₁₀ values for state 3 and state 4 respiration rates between the three groups of animals (Table 1).

In the experiments carried out during winter 1999 (hypoxic hibernation), there was no difference in the
RCR values at 3°C between the three groups of animals (Table 2). However, the mitochondria isolated from frogs submerged in hypoxic water for both 1 mo and 4 mo displayed lower RCR values at 20°C compared with those obtained with the mitochondria isolated from the control group (Table 2). There was no significant difference in the Q10 values for state 3 and state 4 respiration rates between the three groups of frogs (Table 2).

There were some differences between the two groups of control frogs. The state 4 respiration rates and state 4 P50 values at 20°C were lower in winter 1999 compared with 1998 (Tables 3 and 4). The state 3 P50 values at 3°C were lower in winter 1998 than in 1999, although it did not reach statistical significance ($P = 0.053$; Figs. 2 and 4). Moreover, the RCR values at 20°C were higher in winter 1999 compared with 1998 (Tables 1 and 2). These differences between the two groups of control animals might be explained by the fact that physiological parameters can vary considerably between different groups of frogs brought into the laboratory (14, 15). For that reason, we decided to study a given cohort of frogs for each year. However, we cannot exclude the possibility that there were differences in the quality of the mitochondrial preparations for the two groups of control frogs that would contribute to, or maybe even explain, the difference in RCR values obtained at 20°C.

Overall, the general characteristics of the mitochondria were mostly unaltered between the three experimental groups of frogs for both winter 1998 and winter 1999. In addition, the mitochondrial preparations were of good quality, displaying RCR values above 4 at almost every sampling period.

**Respiration rates and O2 affinity of mitochondria from normoxic submerged frogs.** At 3°C, the active (state 3) and resting (state 4) oxygen consumption rates were unchanged between the control, 1-mo-submerged and 4-mo-submerged groups of frogs (Fig. 2). The P50 values for state 3 and state 4 tended to decrease in the 1-mo-submerged frogs compared with controls, but the tendency was significant only for the state 3 P50 values (Fig. 2; $P = 0.077$ for P50 values in state 4). The P50 values for state 3 and state 4 respiration rates from the 4-mo-submerged group were similar to those of the control group (Fig. 2). At 20°C, there was no difference in the state 3 and state 4 rates or their corresponding P50 values between the three groups of animals (Table 3). At both experimental temperatures and for each group of frogs, the P50 values were always above 10.

**Table 1.** RCR and Q10 values of frog skeletal muscle mitochondria isolated from animals before and at stages during hibernation in normoxic water at 3°C

<table>
<thead>
<tr>
<th>Group</th>
<th>RCR</th>
<th>Q10</th>
<th>Q10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3°C</td>
<td>20°C</td>
<td>State 3</td>
</tr>
<tr>
<td>Control</td>
<td>5.0</td>
<td>5.6 ± 1.4(3)</td>
<td>2.5 ± 0.2(4)</td>
</tr>
<tr>
<td>1 mo Submerged</td>
<td>4.6 ± 0.6(4)</td>
<td>6.7 ± 0.4* (5)</td>
<td>2.5 ± 0.1(5)</td>
</tr>
<tr>
<td>4 mo Submerged</td>
<td>3.5 ± 0.5(5)</td>
<td>4.8 ± 0.5(5)</td>
<td>2.3 ± 0.1(5)</td>
</tr>
</tbody>
</table>

Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses. *Significant difference from the corresponding 3°C respiratory control ratio (RCR) value: $P < 0.05$ (Student's paired t-test).

**Table 2.** RCR and Q10 values of frog skeletal muscle mitochondria isolated from animals before and at stages during hibernation in hypoxic water at 3°C

<table>
<thead>
<tr>
<th>Group</th>
<th>RCR</th>
<th>Q10</th>
<th>Q10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3°C</td>
<td>20°C</td>
<td>State 3</td>
</tr>
<tr>
<td>Control</td>
<td>6.4 ± 1.2(4)</td>
<td>10.8 ± 1.3(5)</td>
<td>2.5 ± 0.3(4)</td>
</tr>
<tr>
<td>1 mo Submerged</td>
<td>5.5 ± 0.9(5)</td>
<td>5.2 ± 0.2* (4)</td>
<td>2.2 ± 0.1(4)</td>
</tr>
<tr>
<td>4 mo Submerged</td>
<td>6.1 ± 1.4(4)</td>
<td>5.1 ± 0.4* (4)</td>
<td>2.4 ± 0.1(4)</td>
</tr>
</tbody>
</table>

Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses. *Significant difference from the corresponding control group: $P < 0.05$ (1-way ANOVA and a posteriori test of Tukey).
values were lower in the resting state compared with the active state, but this difference did not reach statistical significance for the group of control frogs at 20°C (Fig. 2 and Table 3).

The kinetics of resting (state 4) and active (state 3) oxygen consumption rates at 3°C of frog mitochondria from all groups of frogs in the low oxygen range are illustrated in Fig. 3. The decrease in state 3 and state 4 P50 values observed in the 1-mo-submerged frogs is illustrated in Fig. 3 as a shift of the state 3 and state 4 respiration curves to the left compared with the control and 4-mo-submerged ones (see Fig. 3, insets).

Respiration rates and O2 affinity of mitochondria from hypoxic submerged frogs. After 1 and 4 mo of hypoxic submergence at 3°C, the active and resting oxygen consumption rates were both reduced by ~60% compared with controls (Fig. 4). This decreased active respiration rate in mitochondria from the hibernating frogs was paralleled by a ~60% diminution of the state 3 P50 value at 1 and 4 mo compared with controls (Fig. 4). On the other hand, there were no significant differences observed for the P50 values at state 4 among the three different groups of animals (Fig. 4). However, the state 4 P50 values obtained at 3°C were extremely low (~0.0034 kPa) and probably near the limit of detection of the apparatus, thus making it difficult to rule out any possible decrease in mitochondrial P50 during submergence. At 20°C, the state 3 and state 4 respiration rates were reduced by ~70 and ~30%, respectively, in both the 1- and 4-mo-submerged groups compared with controls (Table 4). Moreover, the state 3 P50 values of the two submerged groups were ~75% lower than the control values (Table 4). In contrast, the state 4 P50 value after 4 mo of hibernation was higher than those of both the control and 1-mo-submerged groups of frogs (Table 4). As was the case for normoxic hibernation, the P50 values were lower in the resting state compared with the active state at both experimental temperatures and for each group of frogs, but this difference did not reach statistical significance for the 1- and 4-mo-submerged groups of frogs at 3°C and for the 4-mo-submerged group of frogs at 20°C (Figs. 4 and Table 4).

The kinetics of mitochondrial state 4 and state 3 respiration rates in the low oxygen range during hypoxic hibernation are represented in Fig. 5. The matched decrease in state 3 P50 values and state 3 respiration rates for the two groups of hibernating animals is shown in Fig. 5A, inset, and illustrates that the shift in P50 values toward higher affinity is much greater in the animals submerged in hypoxic water (Fig. 5) than in those submerged in normoxic water (Fig. 3). Figure 5 also reveals that the mitochondrial respiration rates of mitochondria from hypoxic submerged frogs are metabolically depressed in both the resting and active states with respect to control frogs at any given intracellular PPO2.

In conclusion, the mitochondria from frogs submerged in hypoxic water display more profound intrinsic changes in P50 values and respiration rates compared with mitochondria from frogs submerged in normoxic water.

### DISCUSSION

The results presented in this paper show for the first time that an increase in the in vitro O2 affinity of mitochondria can occur after chronic in vivo exposure to cellular hypoxia. Moreover, the results demonstrate that metabolic depression at the whole animal level can be reflected at the mitochondrial level. Taken together, these findings illustrate the plasticity of mitochondria under physiological constraints.

The moderate metabolic depression at the whole animal level in frogs submerged in normoxic water (15)

<table>
<thead>
<tr>
<th>Group</th>
<th>Oxygen Consumption Rate, nmol O2·min⁻¹·mg mitochondrial protein⁻¹</th>
<th>P50, kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td>Control</td>
<td>50.38 ± 10.29(5)</td>
<td>9.91 ± 1.98(3)</td>
</tr>
<tr>
<td>1 mo Submerged</td>
<td>42.65 ± 11.74(5)</td>
<td>6.23 ± 1.65(5)</td>
</tr>
<tr>
<td>4 mo Submerged</td>
<td>35.51 ± 7.81(5)</td>
<td>7.69 ± 1.92(5)</td>
</tr>
</tbody>
</table>

Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses.

### Table 4. Oxygen consumption rate and O2 affinity of skeletal muscle mitochondria from frogs hibernating in hypoxic water under resting (state 4) and active (state 3) conditions at 20°C

<table>
<thead>
<tr>
<th>Group</th>
<th>Oxygen Consumption Rate, nmol O2·min⁻¹·mg mitochondrial protein⁻¹</th>
<th>P50, kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td>Control</td>
<td>56.40 ± 7.55(5)</td>
<td>5.20 ± 0.25(5)</td>
</tr>
<tr>
<td>1 mo Submerged</td>
<td>15.66 ± 1.16(4)</td>
<td>3.04 ± 0.32(5)</td>
</tr>
<tr>
<td>4 mo Submerged</td>
<td>20.60 ± 1.38(4)</td>
<td>4.12 ± 0.26(4)</td>
</tr>
</tbody>
</table>

Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses. *Significant difference from the respiration rate of the corresponding control group; P < 0.05 (1-way ANOVA and a posteriori test of Tukey). †Significant difference from the P50 of the corresponding control group; P < 0.05 (1-way ANOVA and a posteriori test of Tukey).
Fig. 3. Kinetics of frog mitochondrial oxygen consumption rates in the low oxygen range at 3°C for normoxic submerged animals. The kinetics for state 3 (A) and state 4 (B) are shown. Solid line, control (C); dashed line, 1-mo-submerged; dotted line, 4-mo-submerged groups of frogs. The insets show the $P_{50}$ for each group of frogs. These curves were generated using the equation: $V_{O2} (\% max) = [PO2/(P_{50} - PO2)] \times 100$ (21), where $V_{O2}$ represents the oxygen consumption rate for state 3 or state 4. The state 3 and state 4 values from the control group of frogs were assigned to 100%, and the ones obtained from the 1- and 4-mo-submerged groups of frogs were expressed relative to the controls. The variability in state 3 rates, state 4 rates, state 3 $P_{50}$ values and state 4 $P_{50}$ values is presented in Fig. 2.

Fig. 2. Oxygen consumption rate and $P_{50}$ of mitochondria under resting (state 4) and active (state 3) conditions at 3°C for normoxic submerged animals. Squares, state 4 rates, circles, state 3 rates. The control, 1-mo-submerged and 4-mo-submerged groups of frogs are represented by a solid, open, and shaded filling, respectively. Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses. *Significant difference from the $P_{50}$ of the control group; $P < 0.05$ (1-way ANOVA and a posteriori test of Tukey). **Significant difference from the $P_{50}$ of the 1-mo-submerged group; $P < 0.05$ (1-way ANOVA and a posteriori test of Tukey).
Fig. 4. Oxygen consumption rate and P_{50} of mitochondria under resting (state 4) and active (state 3) conditions at 3°C for hypoxic submerged animals. Squares, state 4 rates; circles, state 3 rates. The control, 1-mo-submerged and 4-mo-submerged groups of frogs are represented by solid, open, and shaded fillings, respectively. Data are means ± SE. The number of mitochondrial preparations is indicated in parentheses. *Significant difference from the P_{50} of the control group; P < 0.05 (1-way ANOVA and a posteriori test of Tukey). †Significant difference from the respiration rate of the control group; P < 0.05 (1-way ANOVA and a posteriori test of Tukey).

Fig. 5. Kinetics of frog mitochondrial oxygen consumption rates in the low oxygen range at 3°C for hypoxic submerged animals. The kinetics for state 3 (A) and state 4 (B) are shown. Solid line, control; dashed line, 1-mo-submerged; dotted line, 4-mo-submerged groups of frogs. The insets show the P_{50} for each group of frogs. These curves were generated using the equation 
\[ \text{V}_\text{O}_2 \text{%max} = \frac{\text{PO}_2}{(\text{P}_{50} + \text{PO}_2)} \times 100 \] (21), where \( \text{V}_\text{O}_2 \) is the oxygen consumption rate for state 3 or state 4. The state 3 and state 4 values from the control group of frogs were assigned to 100%, and the ones obtained from the 1- and 4-mo-submerged groups of frogs were expressed relative to the controls. The variability in state 3 rates, state 4 rates, state 3 P_{50} values, and state 4 P_{50} values is presented in Fig. 4.
was not reflected at the mitochondrial level (Fig. 3). In fact, the active and resting respiration rates of mitochondria stayed more or less constant throughout normoxic submergence. However, the mitochondria from the 1-mo-submerged group of frogs showed an increase in their state 3 P50 value reverts to the levels observed in control animals (Fig. 3). The corresponding state 4 results (respiration rate and P50) reveal similar trends without reaching statistical significance (Fig. 3B, inset).

The increase in mitochondrial O2 affinity after 1 mo of submergence in normoxic water might not be physiologically relevant. In fact, we have no indication to believe that the intracellular PO2 inside frog skeletal muscle during normoxic submergence are around mitochondrial state 3 or state 4 P50 values. Frogs submerged in normoxic water do not display an increase in plasma or skeletal muscle lactate levels, thereby indicating that blood supply (i.e., oxygen supply) to the skeletal muscle, despite being drastically reduced, is sufficient to maintain aerobic metabolism (15). Moreover, the ATP, ADP, AMP, phosphocreatine, and creatine levels inside the skeletal muscle stay constant throughout normoxic submergence, indicative of a balance between ATP supply and demand (15).

The O2-dependent properties of mitochondria isolated from hypoxic animals show a more profound reorganization of function. For example, mitochondria isolated from frogs submerged in hypoxic water for 1 and 4 mo display reduced resting and active respiration rates and thus metabolic rate at any given intracellular PO2 (Fig. 5). The mechanism responsible for the decrease in mitochondrial state 4 respiration rates during submergence in hypoxic water is a reduction in the activity of the electron transport chain (45). This reduction in electron transport chain activity is at least partly, and might be even entirely, responsible for the decrease in state 3 respiration rates. However, it is also possible that a reduction in the activity of the phosphorylation system plays a role in the decrease of mitochondrial state 3 respiration rates. The intracellular PO2 inside frog skeletal muscle during hypoxic submergence are probably around mitochondrial state 3 and state 4 P50 values and even lower. In fact, frogs recruit anaerobic metabolism during the first 2 mo of submergence in hypoxic water as indicated by the marked increase in plasma lactate concentration (14). A large proportion of the increase in plasma lactate concentration is thought to come from the hypoperfused muscle mass (13). The plasma lactate concentration decreases steeply after 1 mo of submergence in hypoxic water and returns to presubmergence values after 2 mo when the metabolic rate of the frog is dramatically reduced compared with controls (14). In fact, the metabolic depression after 2 mo of submergence is so profound that the energetic needs can now be met through an entirely aerobic metabolism (14).

The reduction in the resting and active respiration rates of frog skeletal muscle mitochondria during hypoxic submergence will lead to a decrease in the rate of ATP production. Even so, the rate of ATP consuming processes seems to be reduced accordingly, as indicated by the maintenance of ATP, phosphocreatine, and creatine levels similar to controls throughout hypoxic submergence (14). However, there is an increase in the adenylate energy charge inside the skeletal muscle during hypoxic submergence owing to significant decreases in ADP and AMP levels (14). This might indicate lowered ATP demand by the skeletal muscle. In fact, the activity of the skeletal muscle Na+–K+-ATPase is reduced by 50% during hibernation in hypoxic water (16). The profound metabolic depression observed in frogs after 2 mo of submergence also has the advantage of reducing the rate of depletion of glycogen inside the skeletal muscle (14). Overall, the steady-state metabolic depression at the whole animal level during hypoxic submergence (14) is reflected at the cellular level by a maintenance of energy balance; a situation similar to that during normoxic hibernation.

Modifications in mitochondrial properties also occur in mammals during chronic exposure to hypoxia. Hypoxia in mammalian cells is often correlated with a reduction in cytochrome levels and mitochondrial enzyme activities. In addition, respiration rates decrease in brain mitochondria of rats and mice exposed to intermittent hypobaric hypoxia (10, 17, 35, 38). However, the resting and active respiration rates of liver and heart mitochondria isolated from rats acclimatized to hypobaric hypoxia did not differ from those of control rats (11), not even when measured at more physiological low oxygen concentrations (12). Previous studies concerning the affinity of cells and of mitochondria for oxygen have produced contrasting results. On the one hand, studies carried out on liver mitochondria isolated from hypoxic rats revealed no change in P50 values (12, 32). On the other hand, the cellular P50 values for hepatocytes isolated from hypoxic rats were lower than those from control rats (32). Such changes in cellular P50 values have been ascribed to a redistribution of mitochondria within the cell (11; reviewed in Ref. 32). Similarly, the P50 values of rat mitochondria isolated from hypoxia-tolerant newborns are not different than those of their hypoxia-sensitive adults, whereas hepatocytes isolated from newborn rats manifest lower P50 values than those of their adult counterparts (1). Again the differences in cellular P50 values were ascribed to differences in the density and distribution of mitochondria within the cellular network (32). The differences in mitochondrial and cellular responses to hypoxia in the ectotherm and mammal may reflect fundamental differences in their tolerance to hypoxia and/or their relative capacity to exploit metabolic suppression at the whole animal level.

Entering into a new viable hypometabolic state implies that energy supply remains balanced with energy demand. Many studies have looked at cellular adaptations, focusing mainly on the energy demand processes.
that can be turned down during metabolic depression (reviewed in Refs. 24, 26, 28, 30). However, few studies have looked at the intrinsic properties of mitochondria during hypometabolic states. From the information available, we know that mitochondrial state 3, but not state 4, respiration rates are reduced in hibernating mammals (7–9, 19, 34, 36, 39). We also know that respiration rates of hepatocytes isolated from hibernating ground squirrels are the same as the rates obtained from hepatocytes isolated from summer “cold-acclimated” animals (41). Indeed, there is no evidence that state 4 rates become altered during hibernation in mammals. With regard to ectotherms, citrate synthase (CS) activities are unchanged during metabolic depression in most tissues of the terrestrial snail, with the exception of the hepatopancreas (47). Similarly, cytochrome c oxidase activity is considerably reduced in the hepatopancreas of estivating snails compared with control animals (46). Also, mitochondrial protein synthesis is markedly decreased during anoxia-induced quiescence in brine shrimp embryos (33). Other studies have shown intrinsic reductions in metabolic rate at the tissue level (18) and at the cellular level (25). The present study demonstrates metabolic depression at the mitochondrial level (Fig. 5), supporting the view that hypometabolism can be reflected at all levels of biological organization in hypoxia-tolerant animals.

This study reports state 3 and state 4 $P_{50}$ values from an ectotherm using high-resolution respirometry. The average state 3 and state 4 $P_{50}$ values for the two control groups of frogs are approximately 0.077 and 0.017 kPa, respectively, at 20°C. These values are similar to the control groups of frogs (0.057 and 0.020 kPa) and rat heart mitochondria (0.077 and 0.017 kPa) and rat liver mitochondria (0.035 and 0.016 kPa) at 30°C (20). The mitochondrial $P_{50}$ values for all groups of frogs increase during transition from a resting state (state 4) to an active one (state 3). Other studies have shown similar increases in $P_{50}$ values in active states in isolated mitochondria (12, 20, 48). The present study shows that state 3 and state 4 $P_{50}$ values can change with metabolic rate. By varying the mitochondrial concentration in their experimental chamber, Steinlechner-Maran et al. (42) noted an incidental positive correlation between respiration rate and $P_{50}$ in endothelial cells. However, the $P_{50}$ values of isolated mitochondria were shown to be constant at various mitochondrial concentrations in the same experimental chamber as we used (20). This, in conjunction with the fact that we carried out all our experiments at the same concentration of mitochondria (see MATERIALS AND METHODS), supports the conclusion that the parallel decrease in state 3 respiration rates and state 3 $P_{50}$ values during hypoxic submergence (Fig. 5) was due to different active metabolic states of the mitochondria.

There is an inverse relationship between the turnover rate of cytochrome c oxidase and the $O_2$ affinity of mitochondria that can explain the differences in $P_{50}$ values between state 3 and state 4 respiration rates and between different types of mitochondria (21). The turnover rate of cytochrome c oxidase is reduced under state 4 conditions, which leads to a decrease in $P_{50}$ value compared with state 3 conditions. Rat heart mitochondria have a higher excess capacity of cytochrome c oxidase compared with rat liver mitochondria, which leads to a lower turnover rate of cytochrome c oxidase at high flux through the electron transport chain (state 3) by distributing electron input flux through a higher number of enzymes. This result correlates with the higher $O_2$ affinity of heart mitochondria compared with liver mitochondria under state 3 conditions (21).
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