Mitochondria as ATP consumers: Cellular treason in anoxia

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In anoxia, mitochondria change from being ATP producers to potentially powerful ATP consumers. This change occurs, because the mitochondrial F1F0-ATPase begins to hydrolyze ATP to avoid the collapse of the proton motive force. Species that can survive prolonged periods of O2 lack must limit such ATP use; otherwise, this process would dominate glycolytic metabolism and threaten ATP delivery to essential ATP-consuming processes of the cell (e.g., ion-motive ATPases). There are two ways to limit ATP hydrolysis by the F1F0-ATPase, namely (i) reduction of the proton conductance of the mitochondrial inner membrane and (ii) inhibition of the enzyme. We assessed these two possibilities by using intact mitochondria isolated from the skeletal muscle of anoxia-tolerant frogs. Our results show that proton conductance is unaltered between normoxia and anoxia. However, ATP use by the F1F0-ATPase is limited in anoxia by a profound inhibition of the enzyme. Even so, ATP use by the F1F0-ATPase might account for ~9% of the ATP turnover in anoxic frog skeletal muscle.

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he chemiosmotic theory requires that the mitochondrial proton motive force can be generated either by the electron transport chain through respiration or by ATP hydrolysis via the F1F0-ATPase. Under normal physiological conditions, some of the energy stored as an electrochemical gradient across the mitochondrial inner membrane is recovered in the form of ATP when protons return to the matrix via the mitochondrial ATP synthase. Under these conditions, the mitochondrial ATP synthase provides the cell with energy in the form of ATP, which is then used to carry out diverse vital functions. In contrast, when cells are deprived of oxygen, the mitochondrial ATP synthase commits “cellular treason” by beginning to hydrolyze the ATP produced glycolytically in an attempt to maintain mitochondrial proton motive force (1, 2).

A category of organisms called “facultative anaerobes,” which includes frogs and turtles, can survive O2 lack for extended periods of time. The key to their survival lies with their acute ability to reduce metabolic rate drastically in the face of limited oxygen supply. This so-called metabolic depression is reflected at the cellular level by increases in efficiency of the ATP producers and by decreases in the rate of the ATP consumers (3–6). These organisms must decrease their ATP use by the F1F0-ATPase during anoxia, namely (i) reduction of the proton conductance of the mitochondrial membrane and (ii) inhibition of the enzyme. To date, most studies have focused on the inhibitor subunit IF1 of the mitochondrial ATPase as a means of reducing the rate of ATP use by mitochondria during short periods of anoxia. However, no studies have compared the proton conductance of the mitochondrial membrane between normoxia and anoxia.

Numerous studies on IF1 have compared cardiac tissue from a wide variety of animals. The species have been classified in three categories according to the activity of their IF1 and the amount of IF1 present in their heart mitochondria. Class a species, which include rabbit and pig, contain a full complement of higher affinity IF1 and display considerable inhibition of their mitochondrial ATPase during anoxia and/or ischemia. Class b species, which include rat and mouse, contain small amounts of higher affinity IF1 and show very little ATPase inhibition during ischemia. Finally, class c species, which include guinea pig, pigeon, turtle, and frog, contain a full complement of a lower affinity form of IF1 and manifest a low-to-moderate ATPase inhibition during ischemia (7, 8). However, a more recent study by Rouslin and Broge (9) has shown that class b and c species seem to resist better the ATP depletion during ischemia in the absence of uncoupler than do class a species. Therefore, it seems that species that lack functional IF1 must rely on different mechanisms to reduce the ATP use by their mitochondrial ATPase during ischemia.

The objectives of the present study were to evaluate (i) the ATP use by the F1F0-ATPase in intact mitochondria during O2-lack and (ii) the mitochondrial proton conductance in normoxia and anoxia in anoxia-tolerant species. We used intact frog skeletal muscle mitochondria as an experimental model, because frog muscle displays many of the major physiological characteristics of anoxia-tolerant systems. For example, isolated skeletal muscle of the frog is exposed to anoxia, it rapidly suppresses its heat output to 20% of the normoxic control values, while simultaneously preserving constant the cellular ATP concentration and membrane potential (10, 11).

Materials and Methods

Animals. The frogs used in these experiments were adult male Rana temporaria (~25–30 g) collected by a local supplier (Blades Biological, Kent, U.K.). Frogs were kept in normoxic water (PO2 = 155 mmHg) in a temperature-controlled recirculated water system (Living Stream, Frigid Units, Cleveland) maintained at 3°C as before (12). The frogs had direct access to air at all times.

Isolation of Mitochondria. Frogs were killed according to the Schedule 1 Home Office protocol. Frog mitochondria were isolated according to a modification of the methods from Hillman et al. (13). The thigh muscles of three frogs were pooled for each mitochondrial preparation. Each thigh muscle mass was excised rapidly and finely minced with razor blades. The tissue was then placed in a beaker containing the isolation medium and homogenized further with microscissors. The isolation medium contained 170 mM mannitol, 55 mM sucrose, 5 mM EGTA, 20 mM Hepes, 50 units/ml heparin, and 0.5% BSA adjusted to pH 7.4.
7.3 at room temperature. The homogenate was transferred to a Potter-type glass homogenizer and ground (200 rpm) 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 filtered subsequently through medical gauze and centrifuged at 9,800 × g for 11 min. The resulting pellet was then washed with isolation medium lacking heparin, resuspended, and centrifuged at 9,800 × g for 8 min. The final pellet was resuspended in isolation medium lacking heparin at a concentration of 25–30 mg of mitochondrial protein per ml. Mitochondrial protein concentrations were determined by the Biuret method (14), and sodium deoxycholate was added to disrupt the membranes.

High-Resolution Respirometry and ATP Concentration Measurements. To measure mitochondrial oxygen consumption rates in normoxia and ATP use in anoxia, we used a respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria), which enables sensitive measurements of oxygen kinetics at low oxygen partial pressure (15, 16). The air-equilibrated assay medium contained 55 mM mannitol, 24 mM sucrose, 10 mM Hepes, 10 mM K2HPO4, 90 mM KCl, and 0.5% BSA adjusted to pH 7.3 at room temperature. The experiments were carried out at 20°C. 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 12.15 μM/kPa. 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 (15–17). 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 by blowing nitrogen on the surface of the assay medium before the addition of mitochondria at a final concentration of 1 mg of mitochondrial protein per ml. First, malate (1.2 mM) was added to spark the Krebs cycle followed by the addition of pyruvate (6 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 0.404 mM ADP. The state 4 respiration rate was reached when all of the ADP was phosphorylated into ATP and consumed all of the oxygen present in the chamber. As soon as the mitochondrial suspension became anoxic, the F1F0-ATPase started hydrolyzing ATP, which led to a reduction in the pH of the assay medium. After 15 min of anoxia, we added 1.4 mM myxothiazol to verify that the suspension was truly anoxic. Because the addition of myxothiazol did not alter the activity of the ATPase, we concluded that the experimental chamber was indeed truly anoxic. We then added 1 μg/mg mitochondrial protein of oligomycin to block the F1F0-ATPase and recorded the residual rate of hydrolysis. We subtracted this residual rate from the total rate of hydrolysis to obtain the F1F0-ATPase activity. The addition of oligomycin collapsed completely the mitochondrial membrane potential, which demonstrated that the F1F0-ATPase was responsible for maintaining the membrane potential under anoxic conditions. In fact, the addition of 2.9 μM FCCP at the end of each run did not lead to any further release of TPMP+.

We also evaluated the activity of the mitochondrial ATPase under de-energized conditions in normoxia and anoxia. To do so, we recorded pH changes of mitochondria incubated in the presence of ATP (state 4) and uncoupler (2.9 μM FCCP) in the same medium described above.

Proton Leak Rate Measurements. Proton leak rate measurements were carried out based on the protocol of Brand (19). The mitochondrial oxygen consumption rates and membrane potentials were measured simultaneously at 25°C in a thermostatically controlled chamber. Again, we used a Clark-type electrode to measure oxygen consumption rates and an electrode sensitive to the lipophilic cation TPMP+ to determine membrane potentials. Mitochondria were incubated at a concentration of 1.5 mg/ml in a medium containing 90 mM KCl, 55 mM mannitol, 24 mM sucrose, 1 mM K2HPO4, 1 mM Hepes, 1 mM EGTA, 1 mM EDTA, 0.5% defatted BSA, 5 mM succinate, 50 μM diadenosine pentaphosphate (to inhibit myokinase), 5 μM rotenone, and 80 ng/ml nigericin (to clamp ΔpH to zero) at pH 7.3 at 25°C. The mitochondrial state 3 respiration rate was obtained by the addition of 0.306 mM ADP. The mitochondria entered into state 4 when all of the ADP was phosphorylated into ATP and consumed all of the oxygen present in the chamber. As soon as the mitochondrial suspension became anoxic, the F1F0-ATPase started hydrolyzing ATP, which led to a reduction in the pH of the assay medium. After 15 min of anoxia, we added 1.4 mM myxothiazol to verify that the suspension was truly anoxic. Because the addition of myxothiazol did not alter the activity of the ATPase, we concluded that the experimental chamber was indeed truly anoxic. We then added 1 μg/mg mitochondrial protein of oligomycin to block the F1F0-ATPase and recorded the residual rate of hydrolysis. We subtracted this residual rate from the total rate of hydrolysis to obtain the F1F0-ATPase activity. The addition of oligomycin collapsed completely the mitochondrial membrane potential, which demonstrated that the F1F0-ATPase was responsible for maintaining the membrane potential under anoxic conditions. In fact, the addition of 2.9 μM FCCP at the end of each run did not lead to any further release of TPMP+.

Mitochondrial ATPase Activity Measurements. Mitochondrial preparations were of good quality, displaying respiratory control ratio values of at least 3 when respiring on succinate. The lower respiratory control ratio values obtained in this series of experiments compared with those obtained in the previous one might be explained by the use of a different batch of frogs, variability in the purity of the mitochondrial suspensions, or the different assay conditions. The activity of the F1F0-ATPase was obtained by recording rates of pH change. The mitochondrial oxygen consumption rates and membrane potentials as well as the pH changes were measured simultaneously at 25°C in a thermostatically controlled chamber. We used a Clark-type electrode to measure oxygen consumption rates and an electrode sensitive to the lipophilic cation TPMP+ to determine membrane potentials. The outputs of the pH- and TPMP+-sensitive electrodes were fed to two different digital voltmeters whose reference sockets were connected together. The output of these two different voltmeters were fed to a double-channel chart recorder. The output of the oxygen electrode was fed to a separate chart recorder. The TPMP+-sensitive electrode was calibrated with sequential additions up to 1.6 μM TPMP+. The pH electrode was calibrated with 0.1 M HCl. The addition of TPMP+ did not affect the pH signal. Also, the addition of HCl did not affect the signal of the TPMP+-sensitive electrode. The TPMP+ binding correction was considered to be 0.4 (μl/mg)−1 protein.
calibrated with sequential additions up to 1.6 \mu M TPMP\(^+\). Malonate was added sequentially up to 5.3 mM to change mitochondrial membrane potential. After each run, 2.9 \mu M FCCP was added to release TPMP\(^+\) for baseline correction. The oxygen solubility of the medium was considered to be 479 nmol oxygen per ml of assay medium.

Calculations and Statistical Analyses. All data are presented as means \pm SEM. The proton leak rate in anoxia was obtained from the F\(_1\)F\(_0\)-ATPase activity measurements by multiplying the rate of pH change by the scalar H\(^+\)/ATP and by four (number of protons pumped into the intermembrane space per ATP hydrolyzed). The proton leak rate in normoxia was obtained by multiplying the oxygen consumption rate by six (20). We assumed that there was no slip in proton pumps.

Statistical analyses were performed with SIGMASTAT (version 2.0). Comparisons of ATP concentrations and ATP use/production under the different experimental conditions were performed with one way ANOVA and the \textit{a posteriori} test of Tukey. Comparisons of proton leak rate, membrane potential, and ATP use of frog mitochondria between normoxia and anoxia were carried out with Student's \(t\) test. The level of significance was \(P = 0.05\).

Results

We predicted that the F\(_1\)F\(_0\)-ATPase would decrease the ATP concentration of frog mitochondrial suspensions by around 277 \mu M over 30 min of anoxia based on estimations of proton leak rate during normoxia with the state 4 oxygen consumption rate. However, the ATP concentration present in frog mitochondrial suspensions after 30 min of anoxia was only 100 \mu M lower than that of control mitochondrial suspensions (state 4 conditions just before entry into anoxia; Fig. 1). The contribution of the F\(_1\)F\(_0\)-ATPase to the decrease in ATP concentration during anoxia was \approx 40 \mu M (compare the ATP concentration between “anoxia” and “anoxia + O + F”; Fig. 1). This decline in ATP concentration by the F\(_1\)F\(_0\)-ATPase was considerable when mitochondrial suspensions were incubated in the presence of uncoupler (de-energized condition) in anoxia (compare the ATP concentration between “anoxia + F” and “anoxia + O + F”; Fig. 1). We conclude that the mismatch between the expected decline in ATP concentration and the observed reduction in ATP concentration caused by the F\(_1\)F\(_0\)-ATPase under the energized condition (absence of uncoupler) in anoxic frog mitochondrial suspensions must be caused by (i) a reduction in the proton conductance of the inner mitochondrial membrane in anoxia, which would lead to a decline in the required activity of the F\(_1\)F\(_0\)-ATPase to maintain a given membrane potential, and/or (ii) a shutdown of the ATPase in anoxia, which would lead to a reduction in membrane potential and consequent decrease in proton leak rate.

The proton conductance of frog skeletal muscle mitochondria was not altered in anoxia, because the anoxic proton leak rate fell on the same kinetics curve as the normoxic proton leak (Fig. 2). In fact, the proton leak rate at \approx 93 mV was around 5 nmol H\(^+\) per min per mg of mitochondrial protein for both normoxic and anoxic mitochondrial suspensions (Fig. 2). This result indicates that the small ATP consumption by the frog F\(_1\)F\(_0\)-ATPase during anoxia (Fig. 1) cannot be explained by a reduction in the proton conductance of the mitochondrial membrane, thereby invalidating our first explanation.

The anoxic proton leak rate of frog skeletal muscle mitochondria was reduced by 93% compared with the normoxic state 4 proton leak rate (Fig. 2). This result can be explained by the fact that the F\(_1\)F\(_0\)-ATPase displays a low rate in anoxia, consequently generating a smaller membrane potential than does the electron transport chain under state 4 conditions (Fig. 2). The low rate of the F\(_1\)F\(_0\)-ATPase in anoxia is caused by a shutdown of the enzyme. Indeed, when we added myxothiazol (chemical anoxia) to a mitochondrial suspension that contains ATP, the rate of the F\(_1\)F\(_0\)-ATPase was fast initially and maintained a high membrane potential up to a new steady-state (data not shown). Taken together, these results show that the mismatch between the expected decline in ATP concentration and the observed reduction in ATP concentration caused by the F\(_1\)F\(_0\)-ATPase...
under energized conditions in anoxic frog mitochondrial suspensions (Fig. 1) was due to a shutdown of the enzyme in the absence of oxygen.

The results presented in Fig. 3 show that the rate of the ATPase under energized conditions in anoxia is ∼25 times lower than the rate of the mitochondrial ATPase under de-energized conditions in normoxia or anoxia as well as the rate of the ATP synthase under state 3 conditions. In anoxia, the observation that the rate of the F_{1}F_{0}-ATPase is faster under de-energized than under energized conditions agrees with the larger ATP depletion by the F_{1}F_{0}-ATPase under de-energized compared with energized conditions presented in Fig. 1. Taken together, these findings demonstrate that the depressed state of the F_{1}F_{0}-ATPase in anoxia can be reversed completely by an uncoupler, thereby implying a membrane potential-dependent mechanism for the shutdown of the enzyme. However, the F_{1}F_{0}-ATPase is not simply inhibited by membrane potential, because its rate is high at 115 mV ("anoxia"), and high at 0 mV ("anoxia + F").

The physiological importance of the reduced activity of the mitochondrial F_{1}F_{0}-ATPase in anoxia is well illustrated in Fig. 4. If the anoxic mitochondria were to maintain the same membrane potential as does the electron transport chain in normoxia, they would do so at a cost of around 18 nmol ATP per min per mg of mitochondrial protein (Fig. 4). This cost equates to 468 nmol ATP per min per g of tissue, because there is ∼26 mg of mitochondrial protein per g of tissue in frog skeletal muscle (J.S.-P., unpublished results). Knowing that the ATP turnover of anoxic frog skeletal muscle is 375 nmol ATP per min per g of tissue (calculated from direct calorimetry measurements in 10), the previous result implies that all of the energy of an anoxic frog skeletal muscle would have had to be allocated to the F_{1}F_{0}-ATPase. Instead, our results show that the rate of the ATPase is reduced dramatically in anoxia to 1.31 nmol ATP per min per mg of mitochondrial protein (Fig. 4), which equates to 34 nmol ATP per min per g of tissue. In this case, only ∼9% of the ATP turnover of an anoxic frog muscle might be used by the F_{1}F_{0}-ATPase.

**Discussion**

This study examined the contribution of the mitochondrial F_{1}F_{0}-ATPase to the ATP turnover of an anoxia-tolerant species. The results show that the F_{1}F_{0}-ATPase shuts down during O_{2} lack but that it still contributes to ∼9% of the ATP turnover in anoxic frog skeletal muscle. The shutdown of the F_{1}F_{0}-ATPase in anoxia leads to a reduction in membrane potential and a consequent decrease in proton leak rate compared with normoxic conditions. These findings are of fundamental importance to our understanding of cell energetics in anoxic hypometabolism, because anoxia-tolerant species must reduce their energy waste through the mitochondrial F_{1}F_{0}-ATPase during anoxic bouts; otherwise, this process would dominate the remaining metabolism causing the organism to exhaust rapidly fermentable substrates and eventually die. An alternative strategy that organisms might use is the possession of an F_{1}F_{0}-ATP synthase/ATPase that constantly functions at a low rate. However, because organisms must display an active F_{1}F_{0}-ATP synthase to make ATP at high rate when oxygen is present, the shutdown of the F_{1}F_{0}-ATPase during anoxia is a more sensible strategy.

Anoxia-tolerant species manifest an extraordinary capacity to decrease the rate of their ATP-consuming processes in the absence of oxygen to match the less efficient anaerobic metabolism (reviewed in refs. 3–6). Although the rate of many ATP consumers can be suppressed almost completely during anoxia, it is crucial to maintain ionic integrity to recover normal cell function on reoxygenation. The percentage of suppression for protein synthesis, protein breakdown, and gluconeogenesis in anoxic turtle hepatocytes is at least equal to the total suppression of ATP turnover (94%; ref. 5). However, turtle hepatocytes manifest a lower reduction (75%) in the rate of their Na^{+}/K^{+} ATPase during anoxic exposure compared with the total suppression of ATP turnover (94%; ref. 5). The fact that this reduction in the rate of the Na^{+}/K^{+} ATPase during anoxia in turtle hepatocytes is accompanied by the maintenance of their plasma membrane potential (21) implies a similar diminution of the influx of ions at the cell membrane, a mechanism called "channel arrest" (22).

Similarly, the skeletal muscle of the common frog manifests a reduction in the rate of the Na^{+}/K^{+} ATPase as well as channel arrest response when exposed to severe hypoxic conditions (11). An analogy of the channel arrest hypothesis at the level of the mitochondrion would be if proton conductance was reduced during anoxic exposure such that the rate required by the
F1F0-ATPase to maintain a given membrane potential would be less, some energy savings would be allowed. It is important to mention that the mechanism responsible for proton leak in mitochondria is not yet known, and we do not imply that it occurs via a channel by using the term channel arrest. Our results show that “proton conductance arrest” does not occur at the level of the mitochondrial but that the rate of the F1F0-ATPase decreases when frog skeletal muscle mitochondria are exposed to anoxia, thereby leading to the maintenance of a lower mitochondrial membrane potential (Fig. 2). The latter result implies that anoxic mitochondria would have a reduced capability to transport substrates than their normoxic counterparts. Because ATP is produced by glycolysis during anoxia, it is probably more important to preserve cell membrane potential than mitochondrial membrane potential. However, it is important to stress that mitochondrial membrane potential cannot be abolished completely during anoxia. Mitochondrial membrane potential is required during anoxia to import proteins that would then ensure mitochondrial maintenance. Moreover, the disruption of mitochondrial membrane potential in anoxia would lead to a series of events culminating eventually in cell death (23). Taken together, these results suggest that ~85% (around 75% for the Na+/K+ ATPase and 10% for the F1F0-ATPase) of the anoxic ATP turnover of anoxia-tolerant species might be used to maintain ionic integrity at the cellular and mitochondrial levels. Obviously, more studies need to be done on cell energy budgets in anoxia-tolerant species to verify the occurrence of such high ATP demands to preserve ionic balance during anoxic hypometabolism.

Mammals also reallocate energy between essential and non-essential ATP demand processes in face of energy limitations such as high ATP demands to preserve ionic balance during anoxic hypometabolism. ATPases.

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