Mitochondrial proton leak in brown adipose tissue mitochondria of Ucp1-deficient mice is GDP insensitive

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Monemdjou, Shadi, Leslie P. Kozak, and Mary-Ellen Harper. Mitochondrial proton leak in brown adipose tissue mitochondria of Ucp1-deficient mice is GDP insensitive. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E1073–E1082, 1999.—Mice deficient in mitochondrial uncoupling protein (UCP)1 are cold sensitive, despite abundant expression of the homologues, Ucp2 and Ucp3 (S. Enerback, A. Jacobsson, E. M. Simpson, C. Guerra, H. Yamashita, M.-E. Harper, and L. P. Kozak. Nature 387: 90–94, 1997). We have analyzed characteristics of mitochondrial proton leak from brown adipose tissue (BAT) of Ucp1-deficient mice and normal controls and conducted the first top-down metabolic control analysis of oxidative phosphorylation in BAT mitochondria. Because purine nucleotides inhibit UCP1 and because UCP2 and the long form of UCP3 have putative purine nucleotide-binding regions, we predicted that proton leak in BAT mitochondria from Ucp1-deficient mice would be sensitive to GDP. On the contrary, although control over mitochondrial oxygen consumption and proton leak reactions at state 4 are strongly affected by 1 mM GDP in mitochondria from normal mice, there is no effect in UCP1-deficient mitochondria. In the presence of GDP, the overall kinetics of proton leak were not significantly different between Ucp1-deficient mice and controls. In its absence, state 4 respiration in normal BAT mitochondria was double that in its presence. Leak-dependent oxygen consumption was higher over a range of membrane potentials in its absence than in its presence. Thus proton leak, potentially including that through UCP2 and UCP3, is GDP insensitive. However, our measurements were made in the presence of albumin and may not allow for the detection of any fatty acid-induced UCP-mediated leak; this possibility requires investigation.

The finding that mice having gene-targeted inactivation of uncoupling protein (UCP)1 are lean and become no more obese on a high-fat diet than do controls on the same diet was unexpected (16). It was anticipated that the Ucp1-deficient mice would be obese, based on the well-recognized importance of brown adipose tissue (BAT) toward energy balance and the development of obesity in rodents (30, 35, 38). Ucp1-deficient mice are, however, characterized by a cold-sensitive phenotype, and they have an abnormally low response in resting metabolic rate to acute treatments with the well-studied β3-adrenergic agonist CL-316,243 (16).

To assess the metabolic significance and control of proton leak in BAT mitochondria from Ucp1-deficient mice and controls, we have used top-down metabolic control analysis (11, 22) and its extension, top-down elasticity analysis (5, 8, 28). Mitochondrial proton leak in tissues other than BAT has been studied for over 10 years, and its possible role in establishing resting metabolic rate has been proposed (4). However, the mechanisms responsible were unknown, and only very recently was it realized that proteins might be involved (10). Coincident to this was the identification of two new forms of the long-known UCP1, which occurs exclusively in mature brown adipocytes. These forms, UCP2 and UCP3, are both expressed in BAT and in various other tissues, such as white adipose tissue, skeletal muscle, and heart (1, 16, 17, 19, 41). These, and perhaps other yet to be cloned UCPs, have been proposed to mediate the mitochondrial proton leak (17, 18, 24).

Brand and colleagues (3–9) have shown that the proton leak in tissues other than BAT contributes significantly to cellular energy expenditure. As studied in intact hepatocytes, thymocytes, and in mitochondria from a variety of tissue types, the proton leak has been estimated to account for ~25–35% of total cellular oxygen consumption rate or 35–45% of mitochondrial oxygen consumption rate (3, 4). Brand et al. (3) have estimated that up to 38% of resting metabolic rate in the rat may be caused overall by the leak.

To assess the metabolic significance and control of proton leak in BAT mitochondria from Ucp1-deficient mice and controls, we have used top-down metabolic control analysis (11, 22) and its extension, top-down elasticity analysis (5, 8, 28). Previous applications of these approaches include those used to characterize thyroid hormone regulation of mitochondrial proton leak and oxidative phosphorylation in liver mitochondria and hepatocytes (23, 25–27) and to investigate the sites of action of fatty acids in hepatocytes (37) and of glucagon (9) in liver mitochondria.

In the original paper describing the characteristics of the Ucp1-deficient mice, it was shown that Ucp2 mRNA is increased fivefold in BAT compared with the message level in BAT of control mice (16). No significant changes in Ucp2 expression were detected in any of the other tissues studied, including epididymal and inguinal white fat, liver, and muscle. Subsequent analyses show that there were no significant changes in the levels of expression of Ucp3 mRNA in any of the tissues studied (Kozak, unpublished results). If UCP2 is functionally analogous to UCP1, as well as structurally homologous, one might predict that the fivefold elevation in BAT

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UCP2 mRNA would provide a mechanism for maintaining body temperature when mice were exposed to the cold. That this protection did not occur suggests that UCP2 and UCP3 cannot compensate for UCP1 with respect to the role of the latter in the regulation of body temperature on an acute exposure of mice to the cold. Accordingly, a role for either UCP2 or UCP3 in the regulation of either body temperature or body weight has not been established. With this in mind, we have investigated the characteristics of the proton leak and respiration in mitochondria isolated from Ucp1-deficient and control mice during its modulation by GDP. We wished to ascertain whether the residual UCPs in Ucp1-deficient mice are subjected to regulation by GDP and control of mitochondrial proton leak in mitochondria in the incubation chamber were maintained at 37°C and magnetically stirred. Each rate was assessed by incubating enough mitochondria in 1.0 ml of suspension medium (120 mM KCl, 20 mM sucrose, 3 mM HEPES, 2 mM KH2PO4, 2 mM MgCl2, 2 mM EGTA, 0.5% BSA; pH 7.2) to give ~1.0 mg mitochondrial protein/ml in the electrode chamber. All respiration rates were determined simultaneously and in parallel with measurements of proton motive force (Δp). State 3 respiration rate was defined as the oxygen consumption rate in the presence of 10 mM succinate, 0.75 U/ml hexokinase, and 10 mM ADP. Oxidation of any endogenous substrate during incubations was inhibited with 5 μM rotenone. State 4 oxygen consumption was determined in the presence of maximal amounts of the ATP synthase inhibitor oligomycin (6 μg/ml). It was confirmed that ATP synthase was completely inhibited in each experimental condition by additional oligomycin, which caused no further inhibition of oxygen consumption and no further increases in Δp.

Measurement of mitochondrial Δp. The Δp was determined using a methyltriphenylphosphonium (TPMP+)‐sensitive electrode that was constructed using the methods of Kamo et al. (31). The pH component of the electrochemical gradient converted to voltage units by incubating mitochondria in the presence of 80 ng/ml of nigericin. The outputs from the TPMP+ electrode and the oxygen electrode were transferred to two voltmeters, the reference sockets of which were connected together; data were then fed in a data analysis software package that allows real-time monitoring and recording on a personal computer.

The calibration of the TPMP+‐sensitive electrode, determination of mitochondrial matrix volumes, and calculation of Δp from TPMP+ electrode data were carried out as described by Brand (6). The Δp is calculated using the Nernst equation as

\[
\Delta p = 61.5 \cdot \log \left( \frac{a_m \cdot TPMP_m}{TPMP_e} \right)
\]

TPMP+/TPMP+ represents the ratio of the accumulation of the cation inside to that which is external to the mitochondria. The nonspecific binding of TPMP+ in mitochondria is reflected in am. The latter indicates the proportion of probe that is free (i.e., not bound). Because TPMP+ is a hydrophobic probe and is able to cross membranes, one drawback is that it is prone to nonspecific binding. The am was determined using the method that adjusts the TPMP+ accumulation ratio to the accumulation ratio for 86Rb, a K+ congener that does not bind, over a range of membrane potentials and ignores any effect of matrix volume on the relative binding of TPMP+ (6). Correction factors were 0.25 and 0.20, respectively, for Ucp1-deficient mice and controls. These mean values were based on duplicate determinations conducted over a range of membrane potentials in BAT mitochondria isolated and pooled from 12 Ucp1-deficient and 12 control mice. Average mitochondrial matrix volumes, determined by the exclusion of radiolabeled [14C]sucrose in relation to the distribution of H2O (6), were 0.85 μl/mg (+/− 0.15; SE, n = 3) mitochondrial protein in Ucp1-deficient mice and 0.45 μl/mg (+/− 0.20; SE, n = 3) mitochondrial protein in controls. These values were based on triplicate determinations completed on BAT mitochondria isolated and pooled from seven Ucp1-deficient and seven control mice.

Application of top-down elasticity analysis and top-down control analysis. To quantitatively determine the effects of knocking out UCP1 on oxidative phosphorylation processes in BAT mitochondria, we used metabolic control analysis and top-down elasticity analysis, as described by Brand and co-workers (5, 8, 28).

**MATERIALS AND METHODS**

Treatment of animals. Male 6-month-old Ucp1-deficient (−/−; Ucp1<sup>−/−</sup>) and male heterozygous controls (+/− of a hybrid C57BL/6j and 129/SvPas genetic background (I)) were obtained from the research colonies of Leslie P. Kazak. The mice were group housed (3/cage), given free access to Purina 5001 chow (4.5% fat by weight) and water, and kept at 23°C with light from 0700 to 1900. Mice used in this study were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council).

Isolation of mitochondria. Mitochondria were isolated from interscapular BAT depots of 12 Ucp1-deficient and 12 control mice. Mitochondria were isolated from interscapular BAT depots of 12 Ucp1-deficient and 12 control mice. Mice were killed by decapitation before removal of BAT. BAT was dissected free of other adhering tissues and homogenized in 3.5 ml of buffer containing 250 mM sucrose, 1 mM HEPES, and 0.2 mM EDTA (pH 7.2 with KOH) using a glass-Teflon Potter-Elvehjem tissue grinder. Fractionation of the homogenate was carried out by spinning at 1,500 × g for 14 min at 4°C to obtain a mitochondrial pellet. The pellet was resuspended (on ice) in 175 μl of a suspension medium containing 120 mM KCl, 20 mM sucrose, 3 mM HEPES, 2 mM MgCl2, 2 mM EGTA, and 0.5% BSA (pH 7.2 with KOH). Resuspension was carried out in both the presence and absence of 1 mM GDP. Stock 9% BSA was defatted by the method of Chen (13) and dialyzed against 153 mM NaCl and 11 mM KCl. Protein concentration of the mitochondrial suspension was assayed by the biuret method (21) using BSA as the reference standard.

Measurement of oxygen consumption. The respiration rate of BAT mitochondria was measured using a Hansatech (Norfolk, UK) Clark-type oxygen electrode. Suspensions of mitochondria in the incubation chamber were maintained at 37°C and magnetically stirred. Each rate was assessed by incubating enough mitochondria in 1.0 ml of suspension medium (120 mM KCl, 20 mM sucrose, 3 mM HEPES, 2 mM KH2PO4, 2 mM MgCl2, 2 mM EGTA, 0.5% BSA; pH 7.2) to give ~1.0 mg mitochondrial protein/ml in the electrode chamber. All respiration rates were determined simultaneously and in parallel with measurements of proton motive force (Δp). State 3 respiration rate was defined as the oxygen consumption rate in the presence of 10 mM succinate, 0.75 U/ml hexokinase, and 10 mM ADP. Oxidation of any endogenous substrate during incubations was inhibited with 5 μM rotenone. State 4 oxygen consumption was determined in the presence of maximal amounts of the ATP synthase inhibitor oligomycin (6 μg/ml). It was confirmed that ATP synthase was completely inhibited in each experimental condition by additional oligomycin, which caused no further inhibition of oxygen consumption and no further increases in Δp.
Briefly, while metabolic control analysis allows the identification and quantitative description of the important sites of control within metabolic pathways, top-down elasticity analysis renders additional data describing pathway regulation. Elasticity analysis can be valuable in identifying the sites of metabolic effects in pathways of the insertion of a transgene, of gene knockouts, or of the treatment with hormones or drugs. Some of the useful parameters determined in an elasticity analysis include “elasticity coefficients,” “flux control coefficients,” and “concentration control coefficients.” An elasticity coefficient (often referred to simply as an “elasticity”) describes the responsiveness of a branch of a metabolic pathway to changes in the amount of an intermediate in that pathway. If the elasticity for that branch differs between the transgenic and the control preparations being compared, then one site of action of the drug is located within the reactions circumscribed by that branch. Other parameters include flux control and concentration control coefficients, which describe, respectively, the relative proportion of control by branches of the pathway over the flux rate of the pathway and over the amounts of intermediates within the pathway. Several useful reviews on both approaches have been published recently (e.g., see Refs. 7 and 28).

The oxidative phosphorylation system can be defined as the tripartite system shown in Fig. 1. We have determined the overall elasticities to changes in Δp of the reactions that produce Δp (i.e., substrate transport, the tricarboxylic acid cycle, and the electron transport chain, referred to herein simply as substrate oxidation reactions) and two blocks of reactions that consume it (i.e., ATP synthesis and consumption reactions and the mitochondrial proton leak reactions). The kinetic response of the Δp producers to Δp was measured by titrating the Δp consumers with oligomycin (1–6 μg/mg protein). The kinetic response of the leak to Δp was assessed by completely inhibiting proton return through ATP synthase by use of oligomycin (6 μg/mg protein) and titrating the substrate oxidation block of reactions with malonate (0.20–2.0 mM), a competitive inhibitor of complex II of the respiratory chain. The elasticity of the phosphorylating subsystem to Δp was measured by titrating Δp producers with malonate alone (0.2–2.0 mM). All titrations were performed both in the presence and in the absence of 1 mM GDP.

Statistical analysis. Data were analyzed using Student’s t-tests or ANOVA, which was followed by Tukey’s post hoc tests. Linear regression lines were compared by analysis of covariance using Prism 2 for Windows. A P value of <0.05 was considered statistically significant. Unless otherwise stated, results are presented as means and SE.

Materials. Oligomycin, malonate, valinomycin, BSA (fraction V), TPMP, succinate, nigericin, and rotenone were from Sigma. TPMP bromide was from Aldrich. [3H]O2, [86Rb]Cl, [14C]sucrose, and [3H]TPMP bromide were from NEN. Water-insoluble compounds were dissolved in DMSO.

RESULTS

Overall kinetics of the three blocks of reactions comprised by the oxidative phosphorylation system. As described in the introduction, the approach that we have used to study metabolic differences in BAT mitochondria isolated from Ucp1-deficient and control mice is referred to as top-down elasticity analysis. In Figs. 2–5, the overall responsiveness, or elasticity to changes in Δp for the three blocks of reactions of the oxidative phosphorylation (as depicted in Fig. 1), are shown. The graphs in Fig. 2 show the overall kinetics of the leak, substrate oxidation reactions, and phosphorylation plus leak reactions. Results from BAT mitochondria of Ucp1-deficient mice are shown in Fig. 2A, whereas those of control mitochondria are shown in Fig. 2B. Mitochondria were incubated in the absence of GDP but in the presence of 0.5% BSA to limit fatty acid-activated uncoupling and extend the time over which the mitochondria respired. Fatty acids are known to activate uncoupling through UCP1 (20, 32, 33) and may activate acutely other UCPs. In Fig. 2, A and B, the furthermost point on the right shows the state 3 respiration rate and Δp values; comparison of values for UCP1-deficient and control mitochondria shows that the presence of UCP1 in control mitochondria causes an ~70% increase in “state 3” respiration rate. It should also be noted that this state 3 rate in control mitochondria, in the absence of GDP, is higher than that which is achieved in its presence. This results presumably from the situation where UCP1 is not inhibited by GDP, protons leak into the matrix through it, and the chain responds by oxidizing substrates at a higher rate to sustain Δp.

State 4 (i.e., nonphosphorylating) respiration (highest Δp values and located at the top of the proton leak curves) is ~100% greater in mitochondria from control BAT than in those from UCP1-deficient mitochondria; again, this is due to the activity of UCP1 in control mitochondria. The respiratory control ratios (i.e., state 3/state 4 respiration values) were 2.0 and 1.6 for control and UCP1-deficient mitochondria, respectively. Overall kinetics of the mitochondrial proton leak and their sensitivity to the purine nucleotide GDP. The data in Fig. 3 clearly show that state 4 (maximal leak-dependent) oxygen consumption in BAT mitochondria from UCP1-deficient mice is insensitive to GDP (Fig. 3A), whereas the leak in BAT mitochondria from control mice is inhibited by the presence of GDP (Fig. 3B). Technical details are provided in the legend to Fig. 3. The data presented also allow the comparison of the overall kinetics of the leak reactions over a range of mitochondrial membrane potentials between UCP1-deficient and control BAT mitochondria.

Fig. 1. Oxidative phosphorylation system in brown adipose tissue (BAT) mitochondria. The intermediate within the system, protonotive force (Δp), is produced by the substrate oxidation subsystem, which includes the tricarboxylic acid cycle, oxidative reactions, and the electron transport chain. The Δp is consumed by the proton leak and phosphorylating subsystems. The proton leak subsystem consists of the leak of protons through uncoupling proteins (UCP) and any cation cycles across the mitochondrial inner membrane. In UCP1-deficient BAT mitochondria the UCPs include UCP2 and UCP3. In BAT mitochondria from control mice, the UCPs include UCP1, UCP2, and UCP3. The phosphorylation subsystem includes Δp-dependent ATP synthesis and all cellular ATP-consuming reactions.
kinetics of the leak in UCP1-deficient mitochondria (Fig. 3A) in the presence and absence of 1 mM GDP are virtually identical. These data were unexpected; 1 mM GDP is well known to inhibit the activity of UCP1 in BAT mitochondria (15, 36). Because a fivefold increase in mRNA for Ucp2 in BAT was observed in Ucp1-deficient mice compared with heterozygous controls, it was hypothesized that an increased activity of UCP2 protein compensated to some degree for the loss of UCP1 and this, in turn, contributed to the lean phenotype. These data show that, over a wide range of $\Delta p$ values, there is no increase in the oxygen used to balance the leak of protons back into the matrix. This is evident when the oxygen used to balance the leak is compared over a range of $\Delta p$ between controls in the presence of GDP (Fig. 3B) and UCP1-deficient mitochondria in the presence or absence of GDP (Fig. 3A); oxygen consumption is roughly equal at any given $\Delta p$. 

Fig. 2. Overall kinetic response of the substrate oxidation, proton leak, and phosphorylating subsystems to $\Delta p$ in isolated BAT mitochondria from Ucp1-deficient (A) and control (B) mice in the absence of 1 mM GDP but in the presence of 0.5% defatted BSA. The kinetic response of the substrate oxidation subsystem (○) to $\Delta p$ is obtained by titration of state 3 respiration (furthest point on the right) with increasing amounts of oligomycin to inhibit the $\Delta p$ consumer, ATP synthase. The kinetic response of the proton leak pathway (□) was determined via titration of state 4 respiration (point with the highest $\Delta p$) with malonate to inhibit the $\Delta p$ producers; because ATP synthesis is completely inhibited with oligomycin, the only other route of proton return in mitochondria is via proton leak reactions. The kinetic response of the phosphorylation subsystem (△) was obtained by titration of state 3 respiration with malonate; because a small amount of proton leak remains at these $\Delta p$ values, the values plotted must be corrected for this to show the kinetics of the phosphorylating system alone. These corrected values are plotted in Fig. 5. Results show that, compared with controls, state 3 respiration is −70% greater, and state 4 respiration is −100% greater in control than in UCP1-deficient mitochondria. The overall kinetics of these three blocks of reactions are further analyzed in Figs. 3–5. Results are from 12 Ucp1-deficient and 13 control mice.
Fig. 4. Effect of 1 mM GDP on the kinetic response of the substrate oxidation subsystem of reactions to $\Delta p$ in BAT mitochondria from Ucp1-deficient (A) and control (B) mice. Open symbols, absence of GDP; filled symbols, presence of GDP. Lines were fitted by linear regression to the oligomycin titration points, showing no difference in the kinetics ($P > 0.05$ by analysis of covariance) of the substrate oxidation subsystem between UCP1-deficient mitochondria in the presence or absence of GDP (A). These results show that, at any given value of $\Delta p$, the amount of oxygen used to balance the activity of the substrate oxidation reactions is not significantly affected by the presence of GDP. However, as shown in B, the same analysis conducted on BAT mitochondria from controls indicates a significant difference in the kinetics ($P < 0.002$ by analysis of covariance) in the presence and absence of GDP. These results simply show that, when UCP1 is functional, the activity of the substrate oxidation reactions increases (i.e., increasing the activity of the electron transport chain proton pumps) to fuel this leak and maintain $\Delta p$ in a normal range. Results are from 12 Ucp1-deficient and 13 control mice.

Fig. 5. Effect of 1 mM GDP on the kinetic response of the phosphorylating subsystem to $\Delta p$ in BAT mitochondria from Ucp1-deficient (A) and control (B) mice. Open symbols, absence of GDP; filled symbols, presence of GDP. Lines were fitted by linear regression to the malonate titration points showing no difference in the kinetics ($P > 0.05$ by analysis of covariance) of the phosphorylating subsystem between UCP1-deficient mitochondria in the presence or absence of GDP (A) but a significant difference in the kinetics ($P < 0.02$ by analysis of covariance) of the phosphorylating subsystem between control mitochondria in the presence or absence of GDP (B). Results are from 7 Ucp1-deficient and 8 control mice. Importantly, these results show that the kinetics of ATP turnover reactions are affected significantly by the absence of UCP1 activity. When GDP is absent, the amount of oxygen used (at similar values of $\Delta p$) to support phosphorylation reactions is much lower than it is when GDP is present. For example, if oxygen consumption rates at 100 mV are compared, it is clear that, in the absence of GDP, the oxygen used to support these reactions is $\sim$14 nmol O$_2$·mg$^{-1}$·min$^{-1}$, whereas, in the presence of GDP, it is $\sim$24 nmol O$_2$·mg$^{-1}$·min$^{-1}$. This shows that, when GDP is present, a greater proportion (and total amount) of oxygen is used in the synthesis of ATP than when GDP is absent and UCP1 is active.
The overall kinetics of substrate oxidation and phosphorylation reactions in the presence and absence of GDP. The results in Figs. 4 and 5 show the overall kinetics of substrate oxidation and phosphorylation reactions, respectively. In Fig. 4 the kinetics of the substrate oxidation reactions in the presence of GDP are compared with those in the absence of GDP. In UCP1-deficient mitochondria, there is no difference between the kinetics of the substrate oxidation system in the presence and absence of GDP (P > 0.05 by analysis of covariance). These results show that, at any given value of Δp, the amount of oxygen used to balance the activity of the substrate oxidation reactions is not significantly affected by the presence of GDP. In mitochondria from control mice, however, the presence of GDP had a marked effect on the kinetics of substrate oxidation reactions (P < 0.002). These results show simply that, when UCP1 is functional, the activity of the substrate oxidation reactions increases to fuel this leak and to maintain a relatively normal Δp (i.e., by increasing the activity of electron transport chain proton pumps).

The graphs shown in Fig. 5 depict the kinetics of the phosphorylation reactions. As indicated in the legend to Fig. 2, and as described previously (22), to obtain the kinetics of the phosphorylation reactions alone (i.e., in the absence of proton leak reactions), the oxygen used to support leak reactions at each mean data point must be subtracted. The data from mitochondria of Ucp1-deficient mice shown in Fig. 5A indicate that there is no significant effect of GDP on the kinetics of the phosphorylation reactions. At any given value of Δp, the amount of oxygen used to support ATP turnover reactions is not significantly different in the presence and absence of GDP. In control mitochondria, however, a significant difference between the kinetics of the phosphorylating system in the presence of GDP compared with its absence. When GDP is present, the amount of oxygen used (at similar Δp values) to balance phosphorylation reactions is much greater than it is when GDP is absent (e.g., compare rates at a Δp value of 100 mV). This is intuitive; when GDP is present and UCP1 is inhibited, the proton gradient is used to fuel the activity of ATP synthase; in its absence the gradient can be rapidly dissipated through the UCP1-mediated leak.

Application of top-down metabolic control analysis. As well as being useful in the identification of differences in the activities of blocks of reactions in a metabolic pathway, top-down elasticity analyses provide all of the data needed for a top-down control analysis of the pathway (11, 22). The latter provides extensive data describing the distribution of control by the blocks of reactions over the overall flux through the system (e.g., mitochondrial oxygen consumption, as in the present study) and over each of the other blocks of reactions in the pathway or system being studied. The elasticity coefficients, describing the responsiveness of the three blocks of reactions to Δp in state 3 and state 4 respiration, are shown in Table 1.

Flux control coefficients and concentration control coefficients of the three blocks of reactions over the rate of each of the subsystems are shown in Tables 2–5. The concentration control coefficients of the three subsystems over the intermediate in the system, Δp, are given in Table 5. The flux control coefficients in Table 2 show that the greatest proportion of control over state 3 mitochondrial oxygen consumption (equivalent to flux through the substrate oxidation subsystem) is by the substrate oxidation reactions themselves, regardless of the presence or absence of 1 mM GDP. This is similar to the high degree of control over state 3 respiration in liver mitochondria (e.g., see Ref. 11). The data in Table 2 also show that the presence of GDP in the incubation medium has effects on the distribution of control over

<table>
<thead>
<tr>
<th>State 3</th>
<th>State 4</th>
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<tbody>
<tr>
<td>With GDP</td>
<td>Without GDP</td>
</tr>
<tr>
<td>Δp</td>
<td>3.65</td>
</tr>
<tr>
<td>Δp</td>
<td>5.2</td>
</tr>
<tr>
<td>Δp</td>
<td>4</td>
</tr>
<tr>
<td>Δp</td>
<td>2.52</td>
</tr>
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Elasticity coefficient (e) is defined as the fractional change in flux caused by a change in protonmotive force (Δp). Results were calculated from the mean values of Δp and mitochondrial oxygen consumption rates at state 3 and state 4 values from metabolic titrations described in Fig. 2. As there is no ATP synthesis occurring in state 4, there are no elasticity or control coefficients (Tables 2–5) for this subsystem. UCP, uncoupling protein; BAT, brown adipose tissue; subscripts S, P, and L, substrate oxidation, phosphorylating, and proton leak subsystems, respectively.

Table 2. Flux control coefficients over mitochondrial oxygen consumption in BAT mitochondria from Ucp1-deficient and control mice in the presence and absence of 1 mM GDP

<table>
<thead>
<tr>
<th>Js</th>
<th>With GDP</th>
<th>Without GDP</th>
</tr>
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<tbody>
<tr>
<td>Cs</td>
<td>0.56</td>
<td>0.70</td>
</tr>
<tr>
<td>Cp</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>Cl</td>
<td>0.19</td>
<td>0.04</td>
</tr>
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UCP1 deficient

Control

UCP1 deficient

Control
used to support the phosphorylating reactions.

Control by the three blocks of reactions over the oxygen increased control by the leak.

in control by the phosphorylation reactions, and in control by the substrate oxidation reactions, no change 

GDP is different; there is a decrease in the proportion of 

in the control by the phosphorylation and leak reactions, and slight decreases 

BAT mitochondria. For example, in control mitochondria 

Flux control coefficients over protein leak in 

Table 4. Flux control coefficients over phosphorylation reactions in BAT mitochondria from Ucp1-deficient and control mice in the presence and absence of 1 mM GDP

<table>
<thead>
<tr>
<th>State 3</th>
<th>State 4</th>
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<tbody>
<tr>
<td>(J_p)</td>
<td>With GDP</td>
</tr>
<tr>
<td>UCP1 deficient</td>
<td></td>
</tr>
<tr>
<td>(C_S)</td>
<td>0.63</td>
</tr>
<tr>
<td>(C_P)</td>
<td>0.63</td>
</tr>
<tr>
<td>(C_L)</td>
<td>0.72</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>(C_S)</td>
<td>0.72</td>
</tr>
<tr>
<td>(C_P)</td>
<td>0.36</td>
</tr>
<tr>
<td>(C_L)</td>
<td>0.08</td>
</tr>
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</table>

Table 5. Concentration control coefficients over \(\Delta p\) in BAT mitochondria from Ucp1-deficient and control mice in the presence and absence of 1 mM GDP

<table>
<thead>
<tr>
<th>State 3</th>
<th>State 4</th>
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<tbody>
<tr>
<td>(\Delta p)</td>
<td>With GDP</td>
</tr>
<tr>
<td>UCP1 deficient</td>
<td></td>
</tr>
<tr>
<td>(C_S)</td>
<td>0.12</td>
</tr>
<tr>
<td>(C_P)</td>
<td>-0.07</td>
</tr>
<tr>
<td>(C_L)</td>
<td>-0.05</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>(C_S)</td>
<td>0.28</td>
</tr>
<tr>
<td>(C_P)</td>
<td>-0.25</td>
</tr>
<tr>
<td>(C_L)</td>
<td>-0.03</td>
</tr>
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</table>

Concentration control coefficients over \(\Delta p\) by the substrate oxidation, phosphorylating, and proton leak subsystems. Results were calculated from the mean values of \(\Delta p\) and mitochondrial oxygen consumption rates at state 3 and state 4 values from the metabolic titrations shown in Fig. 2.

Table 3. Flux control coefficients over phosphorylation and proton leak subsystems over the proton leak system flux. Results were calculated from the mean values of \(\Delta p\) and mitochondrial oxygen consumption rates at state 3 and state 4 values from the metabolic titrations shown in Fig. 2. \(J_p\), rate of oxygen required to pump protons out of the mitochondrial matrix at a rate equal to the rate of their return through the ATP synthase.

There are no data for state 4. Flux control coefficients of substrate oxidation and phosphorylating and proton leak subsystems over the phosphorylating system flux. Results were calculated from the mean values of \(\Delta p\) and mitochondrial oxygen consumption rates at state 3 and state 4 values from the metabolic titrations shown in Fig. 2. Flux control coefficients of substrate oxidation and phosphorylation reactions is greater than in the absence of GDP; the amount of control by leak remains approximately the same.

The data in Table 3 describe the distribution of control by the three blocks of reactions over the oxygen used to support the phosphorylating reactions in state 3. As no ATP synthesis occurs during state 4 respiration, there are no data for state 4 in Table 3. The control over phosphorylating reactions in state 3 is held to a large extent by the substrate oxidation reactions; however, the degree of control by the phosphorylating reactions is also relatively high. These data also show that the shifts in the control in the presence of GDP compared with its absence are similar to those described for the control over mitochondrial respiration (Table 2). The flux control coefficients shown in Table 4 describe the distribution of control between the three blocks of reactions over the oxygen used to support the mitochondrial proton leak. In state 3, the control over the small rate of proton leak is shared in most instances roughly equally between the three blocks of reactions. In control mitochondria in the presence of GDP, the control by substrate oxidation and phosphorylation reactions is greater than in the absence of GDP; the amount of control by leak remains approximately the same. In contrast, in UCP1-deficient mitochondria in the presence of GDP, the control by substrate oxidation and phosphorylation reactions is lower than in the absence of GDP; the amount of control by leak again remains approximately the same.

Flux control coefficients of substrate oxidation and phosphorylating and proton leak subsystems over the proton leak system flux. Results were calculated from the mean values of \(\Delta p\) and mitochondrial oxygen consumption rates at state 3 and state 4 values from the metabolic titrations shown in Fig. 2. \(J_L\), rate of oxygen required to pump protons out of the mitochondrial matrix at a rate equal to the rate of their return through the proton leak.
between the producers and the consumers of ΔΨ (i.e., the substrate oxidation and proton leak subsystems). These results are thus similar to results from intact hepatocytes of hypothyroid, euthyroid, and hyperthyroid rats (12, 26, 27).

**DISCUSSION**

The overall purpose of this study was to analyze the metabolic control and characteristics of proton leak in mitochondria from BAT of Ucp1-deficient mice and of heterozygous controls. The proton leak that remains in these mitochondria may be mediated through the activity of UCP2 and/or UCP3. The levels of the proteins themselves have not as yet been quantified, as antibodies that are unequivocally specific for one or the other are not available. An important finding of this study is that the leak in these mitochondria is insensitive to GDP at concentrations and incubation conditions that cause leak in mitochondria from control mice to be cut in half (Fig. 3, A and B). The flux control coefficients show that the control over mitochondrial oxygen consumption and proton leak reactions is affected strongly by the presence of 1 mM GDP in control mitochondria, whereas in UCP1-deficient mitochondria there is little effect of the purine nucleotide. These findings are discussed below, after a short discussion of some of the characteristics of UCP2 and UCP3.

The first UCP1 homologue, UCP2, was reported in March 1997 (17). This protein has 59% amino acid identity to UCP1. Several protein motifs known to be important in UCP1 function are conserved in UCP2. Similar to UCP1, there are three mitochondrial carrier motifs, consistent with ion transport activity. The putative purine nucleotide-binding sequences are also conserved. The amino acid sequences of mouse and human UCP2 are 95% identical; UCP1 is only 79% identical between mouse and human forms. Northern blot analyses for Ucp2 show widespread expression (17, 19). The highest levels are found in white adipose tissue in both mice and humans (19). It is also highly expressed in muscle in humans and in BAT in mice; this may reflect the relative importance of these thermogenic tissues (19).

The evidence that UCP2 is, in fact, capable of uncoupling oxidative phosphorylation includes that yeast transfected with UCP2 grow more slowly than controls [as reported earlier for UCP1 (2, 17)]. Second, mitochondrial membrane potential, as estimated by flow cytometry, is lowered in yeast transfected with either UCP1 or UCP2 (17). Third, there are data that suggest that the expression of UCP2 confers some protection from the development of obesity in mice. Fleury et al. (17) found higher levels of Ucp2 mRNA in white adipose tissue of the obesity-resistant mouse strain, A/J, than the obesity-prone strain, C57BL/6J. Subsequently, Surwit et al. (40) showed that, in the obesity-resistant strains A/J and C57BL/KsJ, Ucp2 expression in white fat increases roughly twofold in response to 2 wk of a high-fat diet, whereas no diet effect was observed in C57BL/6 mice. In BAT, only the expression of Ucp1 was increased by high-fat feeding. Overall, these authors conclude that the consumption of a high-fat diet selectively regulates Ucp2 expression in white fat and Ucp1 expression in brown fat and that resistance to obesity is correlated with this early selective induction of Ucp1 and Ucp2 and is not associated with changes in expression of Ucp3. Insofar as there is increased expression of Ucp2 (16), but not of Ucp3 in tissues of the Ucp1-deficient mouse, our results support their findings. The fact that we do not observe any increase in leak-dependent oxygen consumption (either in the presence or absence of GDP) in BAT mitochondria from Ucp1-deficient mice shows that the relative levels of Ucp2 mRNA do not correspond to any differences in uncoupling.

Two groups reported the cloning of a third UCP, UCP3 (1, 41). One group screened a human skeletal muscle cDNA library and isolated UCP3L and UCP3S, as well as UCP2 (1). They found that UCP3 is highly skeletal muscle specific and is 57 and 73% identical to UCP1 and UCP2, respectively, at the amino acid level. The potential purine nucleotide-binding domain is found in the long form, UCP3L, but not in the short, UCP3S. The other group (41) showed that human UCP3 is 71% identical to human UCP2 and 57% identical to human UCP1. Human UCP3 is expressed abundantly and preferentially in skeletal muscle; in rodents, expression is in skeletal muscle and BAT. As these tissues are important sites of energy expenditure, UCP3 may be an important thermogenic mediator (41).

At this point, it is not understood how the activities of UCP2 and UCP3 are controlled. Sequences consistent with purine nucleotide binding appear to be conserved in UCP2 and in the UCP3L (1, 17). However, after it was discovered almost 20 years ago that purine nucleotides bind and inhibit UCP1 (15, 29), GDP binding was assessed in mitochondria from a variety of tissues and found to be low to negligible. As a result, purine nucleotide binding has been a key factor in identifying UCP1 activity. In addition, the loose coupling observed in skeletal muscle mitochondria is insensitive to GDP, unlike that in BAT mitochondria (Monemjou and Harper, unpublished observation and Ref. 14). Gimeno et al. (19) hypothesize on the control of UCP2: because a single amino acid mutation in the inhibitory nucleotide-binding site of UCP1 (268Phe to Tyr) creates a UCP that has higher uncoupling activity (2) and because both mouse and human UCP2 naturally contain Tyr at the equivalent position (270Tyr), UCP2 may be less susceptible to inhibitory effects of purine nucleotides. This hypothesis is congruent with our findings showing that the mitochondrial proton leak remaining in BAT mitochondria of Ucp1-deficient mice is GDP insensitive under our incubation conditions. In addition, results from metabolic control analyses of oxidative phosphorylation show that proton leak in liver, kidney, and skeletal muscle may be controlled independently of ATP turnover. Control over leak by phosphorylation reactions is very low (<10%); ~80% of control lies in the leak mechanism itself (3, 26). However, it has recently been shown using rhodamine-123 uptake in rat liver nonparenchymal cells, which express UCP2, that GDP raises relative membrane potential (34). Moreover, in a recent study, Simonyan and Skulachev (39) found that,
in oligomycin-treated heart mitochondria of cold-exposed rats, the addition of GDP caused an increase in membrane potential; this effect was not observed in mitochondria from room temperature-acclimated rats. Cold exposure caused an increase in palmitate-induced mitochondrial uncoupling, and induction was GDP insensitive. Our measurements were made in the presence of albumin and may not allow for the detection of any fatty acid-induced UCP-mediated leak. The latter possibility requires examination.

Our findings also suggest that adaptive thermogenesis may occur in other tissues of the mouse, allowing the mice to maintain a normal resting metabolic rate, feed efficiency, and phenotype as lean controls during the feeding of a high-fat diet (16). The obvious tissue to analyze in relation to this hypothesis is skeletal muscle, and this is one aspect of our current investigations.

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REFERENCES


