Physiological diversity of mitochondrial oxidative phosphorylation


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Submitted 20 April 2006; accepted in final form 7 June 2006

Benard, G., B. Faustin, E. Passerieux, A. Galinier, C. Rocher, N. Bellance, J.-P. Delage, L. Casteilla, T. Letellier, and R. Rossignol. Physiological diversity of mitochondrial oxidative phosphorylation. Am J Physiol Cell Physiol 291: C1172–C1182, 2006.—To investigate the physiological diversity in the regulation and control of mitochondrial oxidative phosphorylation, we determined the composition and functional features of the respiratory chain in muscle, heart, liver, kidney, and brain. First, we observed important variations in mitochondrial content and infrastructure via electron micrographs of different tissue sections. Analyses of respiratory chain enzyme content by Western blot also showed large differences between tissues, in good correlation with the expression level of mitochondrial transcription factor A and the activity of citrate synthase. On the isolated mitochondria, we observed a conserved molar ratio between the respiratory chain complexes and a variable stoichiometry for coenzyme Q and cytochrome c, with typical values of [1–1.5]:[30–135]:[3]:[9–35]:[6.5–7.5] for complex I:coenzyme Q:complex III:cytochrome c:complex IV in the different tissues. The functional analysis revealed important differences in maximal velocities of respiratory chain complexes, with higher values in heart. However, calculation of the catalytic constants showed that brain contained the more active enzyme complexes. Hence, our study demonstrates that, in tissues, oxidative phosphorylation capacity is highly variable and diverse, as determined by different combinations of 1) the mitochondrial content, 2) the amount of respiratory chain complexes, and 3) their intrinsic activity. In all tissues, there was a large excess of enzyme capacity and intermediate substrate concentration, compared with what is required for state 3 respiration. To conclude, we submitted our data to a principal component analysis that revealed three groups of tissues: muscle and heart, brain, and liver and kidney.

ESSENTIAL MECHANISMS OF ENERGY production, signalization, biosyntheses, and apoptosis are contained within mitochondria. Hence, their orchestration and respective control must play a determinant role in cell physiology. Moreover, the differences observed between organs that present with highly specialized functions and variable energy demands, as well as preferences in the type of energy substrates (27, 34), are likely to have shaped the mitochondrial system in a tissue-specific manner. It is estimated from different studies performed in human heart (45), mouse (30), and rat (10) that mitochondria contain ~600–700 identified proteins. However, a striking diversity in their expression levels was reported across tissues, suggesting a large regulatory and composition diversity of the mitochondrial proteome (10, 30). This could explain, in part, the morphological (6, 13, 49) and functional differences (3) observed in mitochondria isolated from different tissues. Such differences in organellar composition could also contribute to the observed tissue-specific control of mitochondrial respiration (37). In a previous study performed on mitochondria isolated from five rat tissues, our group (36) showed that the control of respiration was mostly carried out by the respiratory chain in muscle and heart (highest control coefficient values), in contrast to liver, kidney, and brain where it was more supported by the phosphorylating system. These differences in the control of oxidative phosphorylation might reflect physiological variations in the OXPHOS machinery, which could allow the efficiency and the rate of ATP production to adapt to the tissue-specific energy demand. Therefore, to understand what determines the control of mitochondrial respiration, an integrated analysis of the functional and compositional features of the OXPHOS system in different tissues is required. According to the metabolic control theory (21, 24), the importance of the control of a given isolated step on the global flux depends on several parameters, including the absolute content of enzyme, its intrinsic kinetic parameters and associated regulations, the architecture of the metabolic network, the concentration of intermediate substrates, and the respective steady state (9). The mitochondrial respiratory chain consists of four enzyme complexes (complexes I–IV) and two mobile carriers [coenzyme Q (CoQ) and cytochrome c] along which the electrons liberated by the oxidation of NADH and FADH2 are passed and ultimately transferred to molecular oxygen (39). This respiratory process generates the electrochemical gradient of protons used by the F1–F0 ATP synthase (i.e., complex V) to phosphorylate ADP and produce ATP (29).

Here, we performed a global comparative study of oxidative phosphorylation on tissue lysates and mitochondria isolated from rat skeletal muscle, heart, liver, kidney, and brain under similar experimental conditions. For this, we used a wide array of biochemical techniques, including polarography, enzymology, Western blot, cytochrome spectroscopy, and HPLC coupled to electrochemical measurements (HPLC-EC). In this manner, we measured the relative and absolute content of mitochondrial respiratory chain complexes II, III, and IV, their kinetics, and stoichiometry with respective substrate. We also analyzed the global functioning of the respiratory chain by measuring the redox status of cytochrome c and CoQ9 at state 3, and the respiratory control ratio (RCR). Mitochondrial structure and content were also determined in the different tissues. Our results show that, in tissues, oxidative phosphorylation capacity is highly variable and diverse, as determined by different combinations of 1) the mitochondrial content, 2) the amount of...
respiratory chain complexes, and 3) their intrinsic activity. We discuss these differences in regard to the variability of OXPHOS control and the dramatic tissue specificity of mitochondrial diseases (43, 50).

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals used for mitochondrial preparations and enzymological and polarographic studies were purchased from Sigma. Chemicals for Western blot experiments were purchased from Bio-Rad. Chemicals for Western blot experiments were purchased from Bio-Rad. Chemicals for Western blot experiments were purchased from Bio-Rad. Chemicals for Western blot experiments were purchased from Bio-Rad.

Animals. Male Wistar rats weighing 180–200 g, having free access to water and standard laboratory diet, were used for this study. The animals were killed by cervical shock and decapitation. All of the procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals (Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects). Our experimental protocol was performed in accordance with the guidelines of the French National Institute for Science and Medical Research (INSERM).

Preparation of rat muscle mitochondria. Rat muscle mitochondria were isolated by differential centrifugation. Muscle from two hind legs were collected in isolation medium I (in mM: 210 mannitol, 70 sucrose, 50 Tris-HCl, pH 7.4, and 10 K+ EDTA) and digested by trypsin (0.5 mg/g of muscle) for 30 min. The reaction was stopped by addition of trypsin inhibitor (soybean 3:1 inhibitor to trypsin). The homogenate was centrifuged at 1,000 g for 5 min. The supernatant was strained through gauze and centrifuged at 7,000 g for 10 min. The resulting pellet was resuspended in ice-cold isolation medium II (in mM: 225 mannitol, 75 sucrose, 10 Tris-HCl, pH 7.4, and 0.1 K+ EDTA), and a new series of centrifugations (1,000 and 7,000 g) was performed. The last mitochondrial pellet was resuspended into a minimum volume of isolation medium II to obtain a mitochondrial concentration between 50 and 80 mg/ml. Protein concentration was measured by the Biuret method using BSA as standard.

Preparation of rat liver and kidney mitochondria. Liver was collected in isolation medium A (in mM: 250 sucrose, 10 Tris-HCl, pH 7.6, and 1 K+ EDTA) and homogenized. The homogenate was centrifuged at 1,000 g for 5 min. The supernatant was strained through gauze and centrifuged at 7,000 g for 10 min. The resulting pellet was resuspended in ice-cold isolation medium B (in mM: 250 sucrose, 10 Tris-HCl, pH 7.6, and 0.1 K+ EDTA), and a new series of centrifugations (1,000 and 7,000 g) was performed. The last mitochondrial pellet was resuspended in a minimum volume of isolation medium B to obtain a mitochondrial concentration of between 50 and 70 mg/ml.

Preparation of rat brain mitochondria. Brain mitochondria were isolated from whole brain. Rats were killed by decapitation without stunning, and the brains were removed and homogenized in isolation buffer (in mM: 250 sucrose, 10 Tris-HCl, pH 7.4, and 0.5 K+ EDTA). The homogenate was centrifuged at 1,000 g for 5 min. The supernatant was strained through gauze and centrifuged at 7,000 g for 10 min. The resulting pellet was resuspended in ice-cold isolation buffer, and a new series of centrifugations (1,000 and 7,000 g) was performed. The crude mitochondrial pellet was resuspended in a final volume of 10 ml in 3% Ficoll medium (3% Ficoll, 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 0.5 mM K+ EDTA). This suspension was carefully layered onto 20 ml of 6% Ficoll medium (6% Ficoll, 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 0.5 mM K+ EDTA) and centrifuged for 30 min at 11,500 g. The mitochondrial pellet was resuspended in isolation medium and centrifuged for 10 min at 12,500 g. The mitochondria were made up to a concentration of ~50 mg protein/ml in the isolation buffer.

Transmission electron microscopy. To allow comparisons of the mitochondrial organization in muscle, heart, liver, kidney, and brain, we prepared sections from tissues obtained from the same animal. These were also submitted to the same procedure of fixation and coloration. First, the tissues were dissected immediately after death and fixed by immersion in solution A (2.5% glutaraldehyde, 4% paraformaldehyde, 4% saccharose, and 2% polyvinylpyrrolidone in 0.1 M cacodylate buffer). These were sectioned in 40-μm3 blocks, immersed for 1 h in fixative solution A, and rinsed with water. The blocks were recut into smaller samples of ~12 mm3 and immersed in a second fixative (solution B: 2% osmic acid in 0.1 M cacodylate buffer) for 1 h. After dehydration, the blocks were embedded in epoxy (epon) resin and cut into longitudinal or transversal sections 0.1 μm thick. The different sections were stained with a solution of uranyl acetate and lead citrate. Sections were observed on a Philips CM10 microscope. Morphometric analysis was performed by randomly analyzing selected tissue sections of the different tissues (n ≥ 5) obtained from three different animals.

Western blotting. Samples were diluted into SDS-PAGE tricine sample buffer (Bio-Rad) containing 2% β-mercaptoethanol by incubation for 30 min at 37°C, and separated on a 10–22% SDS polyacrylamide gradient mini-gel (Bio-Rad) at 150 V. Proteins were transferred electrophoretically to 0.45-μm polyvinylidene difluoride membranes for 2 h at 100 mA in CAPS buffer (3.3 g CAPS, 1.5 liters of 10% methanol, pH 11) on ice. Membranes were blocked overnight in 5% milk-PBS + 0.02% azide and incubated for 3 h with primary antibodies purchased from Mitosciences. The antiporin antibody was purchased from Calbiochem. After three washes with PBS-0.05% Tween 20, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad) diluted in 5% milk-PBS. This secondary antibody was detected using enhanced chemiluminescent Plus reagent (Amersham). The signal was quantified by densitometric analysis with the use of Image J (National Institutes of Health) software.

Respiration measurements. Mitochondrial oxygen consumption was monitored at 30°C in a 1-ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Oxy 1, Hansatech) in respiration buffer [75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris phosphate, 10 mM Tris-HCl, pH 7.4, 50 μM EDTA, plus respiratory substrates (10 mM pyruvate in presence of 10 mM malate)]. The mitochondrial concentration used for respiration measurements was 1 mg/ml, and state 3 was obtained by the addition of 2 mM ADP. Respiration rates were expressed in nanograms atom O per minute per milligram of protein. The OCR is defined as the ratio of state 3 (in the presence of ADP) to state 4 (in the absence of ADP) respiratory rate. The uncoupling ratio was defined as the ratio of the uncoupled respiratory rate (measured in presence of 1–10 μM carbonyl cyanide 3-chlorophenyl hydrazone) to state 3.

Enzymatic determination. Assays of all respiratory chain enzyme activities were carried out spectrophotometrically at 30°C using a double-wavelength Xenius spectrophotometer from SAFAS (Monaco) and standardized reproducible methods as described previously (1). All activities were expressed in nanomoles per minute per milligram. The kinetic parameters (Vmax and Km) of complexes III and IV were obtained by fitting the experimental curve V = f(S), with the Michaelis-Henri equation, V = (Vmax × [S])/(Km + [S]), where [S] is substrate concentration and V is velocity, using Kaleida Graph 3.0.2 (Abelbeck software). Activity measurements were performed for complex III using 10 μg/ml of mitochondrial protein (muscle and liver) with a ubiquinone concentration ranging from 0 to 200 μM. For complex IV, we used 10 μg/ml of mitochondrial protein (muscle and liver) and a reduced cytochrome c concentration that also ranged from 0 to 200 μM.

Complex II (succinate dehydrogenase). The assay was performed by following the decrease in absorbance at 600 nm resulting from the reduction of 2,6-dichlorophenolindol-phenol in 1 ml of medium containing 60 mM KH2PO4 (pH 7.4), 3 mM KCN, 20 μM/l rotenone, 20 mM succinate, and 10 μg mitochondrial protein. The reaction was initiated by the addition of 1.3 mM phenazine methasulfate and 0.18 mM 2,6-dichlorophenol sodium salt hydrate. The extinction coefficient used for DCIP was 21 M−1·cm−1.
Complex III (ubiquinol cytochrome c reductase). The oxidation of 6.5 mM decylubiquinol by complex III was determined by using cytochrome c (III) as an electron acceptor. The assay was carried out in basic medium supplemented with 2.5 mg/ml BSA, 15 μM cytochrome c (III), and 5 μg/ml rotenone. The reaction was started with 10 μg of mitochondrial protein, and the enzyme activity was measured at 550 nm. The extinction coefficient used for cytochrome c was 18.5 mM⁻¹cm⁻¹.

Complex IV (cytochrome c-oxidase). Two methods were used for determining cytochrome c-oxidase activity. Initially, cytochrome c-oxidase activity was determined spectrophotometrically with cytochrome c (II) as substrate. The oxidation of cytochrome c was monitored at 550 nm at 30°C. The extinction coefficient used for cytochrome c was 18.5 mM⁻¹cm⁻¹. In the second method, we monitored cytochrome c-oxidase activity by inhibiting the rest of the respiratory chain with rotenone and antimycin, using 3 mM ascorbate and 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine, as an electron donor system.

Citrate synthase. The reduction of 5,5-dithiobis(2-nitrobenzoic acid) by citrate synthase at 412 nm (extinction coefficient of 13.6 mM⁻¹cm⁻¹) was followed in a coupled reaction with coenzyme A and oxaloacetate. A reaction mixture of 0.2 M Tris-HCl, pH 8.0, 0.1 mM acetyl-coenzyme A, 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid), and 5–20 μg of muscle or brain mitochondrial protein was incubated at 30°C for 5 min. The reaction was initiated by the addition of 0.5 mM oxaloacetate, and the absorbance change was monitored for 5 min.

Determination of cytochrome b, h, bII, bL, cL, c, c1, and aa3 absolute content by spectrophotometry. Mitochondria isolated from muscle and liver were used at 1 mg/ml, in 1 ml of the respiratory buffer described above. Individual fully reduced (with excess sodium dithionite) or fully oxidized (with excess ferricyanide) absorbance spectra were recorded between 500 and 650 nm on a SAFAS Genius double-wavelength spectrophotometer (SAFAS, Monaco). The concentration of cytochrome aa3 ([aa3]) contained in the heme of cytochrome c-oxidase (complex IV) was determined from the difference spectrum [reduced – oxidized (red-ox)] at the maximum absorption value of 605 nm (A_red-ox 605), normalized by the absorbance of the isobestic point at 630 nm (A_red-ox 630). Values were calculated by the Beer-Lambert law (see Eq. 1), with an extinction coefficient ε_red-ox aa3 of 24,000 M⁻¹cm⁻¹ (40) and a cuvette length of 1 cm

\[ [aa3] = (A_{\text{red-ox 605}} - A_{\text{red-ox 630}}) / \varepsilon_{\text{red-ox aa3}} \] (1)

The absolute content of c-type cytochromes (c + c1) was determined from the same difference spectrum [reduced – oxidized] at the maximum absorption value of 550 nm (A_red-ox 550), normalized by the absorbance of the isobestic point at 535 nm (A_red-ox 535). The concentration of cytochrome c + c1 was determined according to the Beer-Lambert law (see Eq. 2), with an extinction coefficient ε_red-ox c + c1 of 18,500 M⁻¹cm⁻¹ (26) and a cuvette length of 1 cm

\[ [c + c1] = (A_{\text{red-ox 550}} - A_{\text{red-ox 535}}) / \varepsilon_{\text{red-ox c + c1}} \] (2)

The total content of cytochrome c was then calculated from this total content in cytochrome c + c1 by subtracting the contribution of c1, which was determined by considering its molar equivalence of 1:1, with cytochrome b (22). This content in cytochrome b was determined experimentally, using a detergent-based partial solubilization of mitochondrial proteins described in Ref. 40, which leads to the physical separation of complex II and complex III. In this manner, it is possible to determine the part of the absorbance of the different b-type cytochromes from the total (bH + bL + bII), calculated from the maximum absorption peak at 562 nm (A_red-ox 562), normalized by the absorbance of the isobestic point at 575 nm (A_red-ox 575). The total extinction coefficient of bH + bL + bII (ε_red-ox bH+bL+bII) was equal to 56,100 M⁻¹cm⁻¹ and that of bII (ε_red-ox II) was 26,000 M⁻¹cm⁻¹ (40). The cuvette length was 1 cm. In this manner, the concentration of cytochrome c1 was calculated from that of bII according to Eq. 3:

\[ [c1] = 0.5 \left[ \frac{A_{562-575}(1 - R)}{\varepsilon_{bH,bL} \times 1} \right] \] (3)

\[ [c] = [c + c1] - [c1] \] (4)

with R = bH/bH_tot (ratio of the absorbance of cytochrome bH to the total absorbance of the b-type cytochromes at 562 nm). The total content of cytochrome c was then obtained from c + c1 (see Eq. 4) by subtracting the amount of c1 (see Eq. 3), determined in the same volume of 1 ml.

Determination of steady-state cytochrome c redox status by spectrophotometry. To determine the concentration of reduced cytochrome c during state 3, we performed the above detailed analysis of cytochrome spectra on mitochondria taken from muscle or liver mitochondria respiring as described above in Respiration measurements. After the addition of substrates pyruvate and malate, state 3 respiration was initiated by the addition of 2 mM ADP. Absorbance spectra were recorded individually every 40 s in the presence of different concentrations of myxothiazol or KCN. Steady state was typically reached within 1 min after the addition of ADP, as observed by polarography on the same mitochondrial samples.

Determination of CoQ absolute content and steady-state redox status by HPLC-EC measurement. The absolute CoQ content (oxidized + reduced form) was measured by HPLC-EC as described in Ref. 12. Determination of the concentration of reduced CoQ during state 3 was performed on mitochondria respiring at steady state as described for cytochrome c in Respiration measurements above. During each steady-state assay, respiration samples were withdrawn from the cuvette and immediately stored at −80°C.

Statistical analysis. All of the data presented in this study correspond to the mean value of n experiments ± SD, with n ≥ 3. Comparison of the data obtained from isolated muscle and liver mitochondria were performed with Student’s t-test, using Excel software (Microsoft). Two sets of data were considered as statistically different when P < 0.05.

Principal component analysis. The principal component analysis (PCA) method is concerned with interpreting the variance-covariance structure through a few linear combinations of the original variables. Its general objectives are dimensionality reduction and ease of interpretation. PCA is performed according to the usual method with Statbox version 3.2 (Grimmersoft) as described in Ref. 8. If we restrict ourselves to the first two principal components, the results may be represented graphically by a biplot.

RESULTS

Mitochondrial content in the different tissues. We evaluated the global mitochondrial content in tissues by measuring the activity of citrate synthase in tissue lysates. We obtained values of 11,155 ± 576 nmol·min⁻¹·mg⁻¹ in heart, compared with 7,415 ± 1,230 in muscle, 3,798 ± 521 in brain, 1,516 ± 71 in kidney, and 736 ± 66 in liver. As another method to evaluate the mitochondrial content in tissues, we determined the amount of respiratory chain complexes I and III per milligram of tissue proteins by Western blot. We also measured the expression level of the mitochondrial transcription factor A (mtTFA). An example of Western blot is shown in Fig. 1A, inset. The corresponding results of the densitometric analysis are given in the histogram of Fig. 1A. It can be seen that heart contains the highest content of complex III core 2 per microgram of tissue proteins, compared with muscle, brain, liver, and kidney, respectively. A very similar hierarchy between tissues can be observed for the expression level of the complex I 20-kDa subunit, as well as for mtTFA (Fig. 1A). Comparison of the tissue respiratory chain content determined by Western blot
Mitochondrial structure in the different tissues. We analyzed several electron micrographs of various tissue sections, including slow-type and rapid-type skeletal muscle, heart, liver, kidney, and brain, all observed at the same magnification (Fig. 2). A large diversity in mitochondrial shape and organization according to tissue was observed, with an important variability of organelar section profile, intracellular localization, and heterogeneity. First, we compared two types of skeletal muscle, the soleus (Fig. 2A) vs. the peroneus digiti quarti (Fig. 2B). Accordingly, the former is a “slow type” and contained more mitochondria than the latter, which is a “rapid type.” In these two types of muscle, the mitochondria appeared to be contained within membranous compartments all along the myofibers and looked highly compacted. In heart (Fig. 2C), mitochondria were more numerous and presented with a larger section. In liver (Fig. 2D), they were difficult to distinguish, as they presented with a less clear envelope and fewer cristae. They also looked more perinuclear and were closely intricated with the Golgi apparatus. In kidney (Fig. 2E), there was a large number of mitochondria essentially located in the interdigitations of the tubules, where the ion-pumping activity is very active. In brain (Fig. 2F), mitochondrial sections were more dispersed throughout the cytosol, with an important variability in section profiles. The subsequent observation of mitochondrial internal organization revealed differences between tissues concerning the number of cristae and matrix density. For instance, there was more cristae per surface unit of mitochondrial section in heart (124 ± 32) than in muscle (107 ± 11), brain (97 ± 13), kidney (46 ± 6), and liver (26 ± 4). Measurement of the matrix density, expressed as the ratio of matrix vs. cytosolic densitometry, showed a higher value in heart (1.91 ± 0.42) than in brain (1.35 ± 0.32), soleus muscle (1.30 ± 0.17), kidney (1.23 ± 0.29), and liver (1.04 ± 0.30).

Variable enrichment in respiratory chain proteins on mitochondria isolated from different tissues. To extend the above detailed analysis at the organellar level, we isolated the mitochondria from rat muscle, heart, liver, kidney, and brain. First, we looked at the relative expression level of each respiratory chain complex I-V by Western blot using a set of seven monoclonal antibodies. This was performed on 5 μg of mitochondrial proteins under nonsaturating conditions, as shown in Fig. 3. To evaluate the respiratory chain content per mitochondrial compartment, we normalized their expression level to the porin band density (Fig. 3). It can be seen that mitochondria isolated from heart and muscle contained more respiratory chain complexes (normalized to porin) than shown in liver, kidney, and brain. Similar differences were observed for the other complexes subunits (Fig. 3), with heart and muscle consistently presenting with the highest values, compared with brain, liver, or kidney (P > 0.05).

Absolute content and molar ratio of the respiratory chain complexes in mitochondria isolated from different tissues. To perform an absolute determination of the respiratory chain content in mitochondria isolated from the different tissues, we calculated their actual number of moles per milligram of mitochondrial proteins, from the absorbance of cytochrome bH, bH, or bL, and aA3, respectively. An example of difference absorbance spectra is given in Fig. 4. It can be seen that the different cytochromes of type b (bH, bL, and bO) formed a large peak at 562 nm. For this reason, we performed a physical separation of complex II and complex III that allowed us to...
determine the individual parts of cytochrome $b_H + b_L$ and $b_{II}$ in this peak (see histogram in Fig. 4). Calculation of the absolute content of these different cytochromes (Table 1) indicated a higher content (factor of 2–3) in heart and muscle than in liver, kidney, and brain. In contrast, the molar ratio between these different cytochromes was conserved between tissues. We chose to set the value of complex III content at 3 for an easier comparison to the results of the literature. In this manner, we obtained a conserved ratio ~1:3:7 between complexes II, III, and IV in the five tissues (Table 1).

Intermediate substrates of the respiratory chain: absolute content and stoichiometry with respective enzyme complexes in different tissues.

The total content in cytochrome $c$ was determined by spectrophotometry on mitochondria isolated from muscle, heart, liver, kidney, and brain (see spectra in Fig. 4). The results, expressed in picomoles of cytochrome per milligram of mitochondrial proteins, are given in Table 1. They show large differences between tissues, with higher values in heart and muscle than in liver, kidney, and brain. The total content of CoQ$_9$ was determined by HPLC-EC on the same mitochondrial preparations (Table 1). It showed a large variability between tissues, with high values in muscle and liver compared with values in kidney, heart, and brain. From these values and the absolute content in respiratory chain complexes determined above, we calculated the stoichiometry between complex III and CoQ$_9$ as well as complex IV and cytochrome
Table 1. Absolute amounts of the different respiratory chain cytochromes in mitochondria isolated from different tissues

<table>
<thead>
<tr>
<th></th>
<th>Muscle</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
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</thead>
<tbody>
<tr>
<td>Total content in cytochrome b₅, pmol/mg protein</td>
<td>32±5</td>
<td>60±9</td>
<td>16±4</td>
<td>21±6</td>
<td>17±9</td>
</tr>
<tr>
<td>Total content in cytochrome b₆, pmol/mg</td>
<td>121±29</td>
<td>124±16</td>
<td>43±4</td>
<td>51±5</td>
<td>53±16</td>
</tr>
<tr>
<td>Total content in cytochrome a + a₅, pmol/mg protein</td>
<td>277±15</td>
<td>320±25</td>
<td>93±8</td>
<td>119±14</td>
<td>125±21</td>
</tr>
<tr>
<td>Total content in CoQ₉, pmol/mg</td>
<td>2,348±104</td>
<td>975±95</td>
<td>1,292±97</td>
<td>1,291±78</td>
<td>967±123</td>
</tr>
<tr>
<td>Total content in cytochrome c, pmol/mg</td>
<td>489±25</td>
<td>509±21</td>
<td>129±12</td>
<td>307±35</td>
<td>548±47</td>
</tr>
<tr>
<td>Molar ratio CII:CoQ₉:CIII:cytochrome c:CIV</td>
<td>0.89:58.2:3:11.2:6.9</td>
<td>1.45:23.6:3:11.9:7.5</td>
<td>1.1:13.4:3:9.0:6.5</td>
<td>1.26:73.2:3:18.2:7.1</td>
<td>1.0:57.9:3:34.8:7.5</td>
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Values are means ± SD. CoQ₉, coenzyme Q; CII, complex II; CIII, complex III; CIV, complex IV.

Table 2. Respiratory chain complexes activity in mitochondria isolated from different tissues

<table>
<thead>
<tr>
<th></th>
<th>Muscle</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal activity of CS, nmol·min⁻¹·mg protein⁻¹</td>
<td>1,089±115</td>
<td>1,175±206</td>
<td>334±98</td>
<td>195±67</td>
<td>478±128</td>
</tr>
<tr>
<td>Maximal activity of complex III, nmol·min⁻¹·mg protein⁻¹</td>
<td>771±136</td>
<td>645±112</td>
<td>294±89</td>
<td>277±97</td>
<td>709±223</td>
</tr>
<tr>
<td>Maximal activity of Complex IV, nmol·min⁻¹·mg protein⁻¹</td>
<td>2,310±307</td>
<td>3,488±651</td>
<td>1,213±117</td>
<td>766±143</td>
<td>2,154±446</td>
</tr>
<tr>
<td>K₅₀ for complex III, µM</td>
<td>9.9±0.7</td>
<td>11.2±1.3</td>
<td>15.8±0.9</td>
<td>42.7±2.6</td>
<td>23.4±2.1</td>
</tr>
<tr>
<td>K₅₀ for complex IV, µM</td>
<td>28.2±2.7</td>
<td>27.2±1.8</td>
<td>27.9±3.9</td>
<td>19.2±2.3</td>
<td>11.3±0.9</td>
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Values are means ± SD. CS, citrate synthase.
substrate delivery. Accordingly, the different tissues present with large differences in the composition of the OXPHOS machinery and the organization of mitochondria, which could reflect their variable physiological activity. In addition, mitochondria also participate in fundamental cell signaling and apoptotic processes, the regulation of which may also lead to changes in organelar content, composition, and functionality. Recently, different studies have evidenced important differences in the mitochondrial proteome between tissues, but their functional implication was not assessed. Here, we have analyzed the diversity of mitochondrial oxidative phosphorylation between tissues and its consequences for the control of energy production. For this, we determined the compositional and functional features of the respiratory chain on mitochondria isolated from rat muscle, heart, liver, kidney, and brain.

First, we looked at the mitochondrial content in the different tissues. This simple question raised numerous issues concerning the accuracy of the different methods of quantification generally employed. For instance, the classic measurement of mitochondrial section area on electron micrographs is limited by the very complex three-dimensional organization of mitochondria as a global network (16, 31, 51), the morphometric parameters of which also vary widely on energy status (17–19, 23, 35). Accordingly, in our study, there was an important variability of mitochondrial morphology between tissues: in heart and skeletal muscle, they presented essentially with a regular quasi-crystalline organization (46), whereas in liver and kidney they looked more irregular and less compacted by tissue architecture. In brain, the mitochondria looked more like a collection of small and numerous ovoid sections. Moreover, we observed a strong heterogeneity of mitochondrial intracellular distribution within tissues, so that the number of organelar profiles varies dramatically with the localization of the section. For instance, in brain, there are fewer mitochondria around the nucleus, in the basal corpus, whereas they are omnipresent along the axone, reaching a centimeter range in length. Likewise, in kidney, there are more mitochondria in the tubule (microvilitosities) than in the basal corpus. In the other tissues, mitochondria present with a less heterogeneous organization. The internal organization of mitochondria, i.e., the form of the cristae, is also irregular and variable on physiological conditions (28). Accordingly, in our study, in tissue sections viewed by transmission electron microscopy, we observed important differences in the internal organization of the mitochondria. For instance, there was a variable number of cristae per organellar section, along with differences in their arrangement. The heart presented with the highest number of cristae per surface of mitochondrial section vs. the other tissues (factor of 1.3–4.8). Moreover, we observed variations in the density of the mitochondrial matrix, compared with the cytosol, with again a high value in heart vs. that shown in the other tissues (factor of 1.4–1.9). A high number of cristae, as observed in heart, could indicate a higher mitochondrial content in respiratory chain complexes, in agreement with studies showing a preferential localization of these proteins in the crystal membranes (11, 14). Accordingly, heart mitochondria also present with the darker matrix, which could indicate a higher state of respiration in vivo (17–19). Another method of mitochondrial quantification frequently used is the determination of mitochondrial-to-tissue protein ratio. However, in our

**Table 3. Functional parameters of the respiratory chain at state 3**

<table>
<thead>
<tr>
<th></th>
<th>Muscle</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 respiratory rate, ng atom O/min$^{-1}$/mg$^{-1}$</td>
<td>184±10</td>
<td>229±21</td>
<td>149±17</td>
<td>156±21.3</td>
<td>176±11</td>
</tr>
<tr>
<td>Respiratory chain ratio</td>
<td>4.6±1.2</td>
<td>5.0±1.5</td>
<td>4.1±1.9</td>
<td>3.7±0.9</td>
<td>2.6±1.4</td>
</tr>
<tr>
<td>Reduced [CoQ] at state 3, % of total</td>
<td>2.0±0.7</td>
<td>1.9±0.4</td>
<td>6.4±14.7</td>
<td>55.6±12.9</td>
<td>2.2±0.8</td>
</tr>
<tr>
<td>Reduced [cytochrome c] at state 3, % of total</td>
<td>63±5.1</td>
<td>69±7.6</td>
<td>43±4.5</td>
<td>37±4.1</td>
<td>39±6.8</td>
</tr>
</tbody>
</table>

Values are means ± SD. Brackets indicate concentration.

Fig. 5. Principal component analysis of the main mitochondrial features in different tissues: biplot representation of the PCA results for muscle, heart, liver, and kidney. The variables correspond to the mean value of the following parameters obtained in the different tissues: CIII content, CIV content, CIII apparent (app) $K_m$, CIV apparent $K_m$, coenzyme Q$_9$ (CoQ9) content, cytochrome $c$ (cyt $c$) content, state 3 reduction level of CoQ9, state 3 reduction level of cytochrome $c$, biochemical threshold value of CIII (taken from Ref. 37), and biochemical threshold value of CIV (taken from Ref. 37). CS, citrate synthase.
conditions, it could not be used to compare accurately the mitochondrial content of the different tissues, given the important variability in the yield of the different methods of organelar isolation from the various tissues.

To avoid these errors, we determined the content of mitochondria on total lysates from the different tissues, by measuring 1) the citrate synthase activity and 2) the expression level of respiratory chain complexes I and III, as well as mtTFA. The results show that heart contains more mitochondria than muscle, liver, brain, and kidney. This is in good correlation with the expression level of mtTFA, suggesting the importance of that protein in determining the tissue OXPHOS capacity. We also observed a good correlation between citrate synthase activity and complex III or complex I content in tissues, confirming its utilization as a marker of the respiratory chain content in tissues. This also suggests that the citrate synthase expression level could be coordinated with that of the respiratory chain complexes.

In the second part of our study, we questioned the possible qualitative differences in mitochondria isolated from the different tissues, regardless of their variable tissue content, as discussed above. The analysis of the relative expression level of seven proteins of the respiratory chain by Western blot indicates a higher content of complex I, II, III, IV, and V in heart- and muscle-isolated mitochondria than in liver, kidney, and brain. For instance, the complex III core 2 subunit was more expressed in heart-isolated mitochondria (set as 100%) than in muscle (86 ± 17%), kidney (39 ± 9%), liver (34 ± 06%), and brain (25 ± 07%). This is also true for the other proteins analyzed, suggesting a diversity in intramitochondrial respiratory chain content between tissues. We also determined the absolute content in cytochromes b, c, c1, and a3, as well as the content in CoQ9 on the isolated mitochondria. The results show that the highest absolute contents in respiratory chain complexes are in muscle and heart mitochondria, compared with liver, kidney, and brain (factor of 3). However, the molar ratio between complex II, III, and IV was conserved, with boundary values of [0.89 –1.45]:[3]:[6.5–7.1]. We chose to set the complex III content at 3 to allow comparison with values in the literature. Indeed, other authors have obtained similar values for the molar ratio between respiratory chain complexes determined on bovine heart using a variety of techniques (4, 20, 40). Our results also support the general idea that fixed values between the different complexes could constitute a strong argument for the “supercomplexes hypothesis” (41). In our study, we precise this organization by determining the actual stoichiometry between respiratory chain complexes and respective substrates. We observe a large variability between tissues, with a higher coenzyme Q9-to-complex III ratio in liver, than in kidney, muscle, brain, and heart. This is in agreement with values (ranging between 1 and 32) obtained previously in bovine heart (4). The cytochrome c-to-complex IV ratio is higher in brain than in kidney, muscle, heart, and liver. Again, this is in agreement with values obtained in bovine heart (4). Hence, the total content in these two intermediate substrates is not correlated with the expression levels of the different respiratory chain complexes determined in tissues. These different stoichiometries could reflect the adaptability of the tissues to a variable energy demand. However, the values that we obtained might not correspond to the actual stoichiometries, as recent studies also suggest the existence of different physical pools of cytochrome c (32, 42, 48) and CoQ (2) that could play different functions in the cell.

The functional analysis revealed a large diversity in the $V_{\text{max-app}}$ values obtained for complexes II, III, and IV, with muscle and heart generally presenting with the highest values. Such differences in $V_{\text{max}}$ could be explained by a variable content in respiratory chain complexes per milligram of mitochondrial proteins and/or a difference in the $k_{\text{cat}}$. To take into account the content in respiratory chain complexes, we normalized these $V_{\text{max-app}}$ values to those of citrate synthase. The results show that, after normalization by citrate synthase activity, the highest velocities of complexes II, III, IV are observed in the brain, kidney, and liver, compared with muscle and heart (factor of 2 between brain and heart). To look more closely at the differences between respiratory chain complexes in tissues, we calculated the $k_{\text{cat}}$ values by dividing $V_{\text{max-app}}$ by the molar content in enzyme complex. Again, we observe a higher $k_{\text{cat}}$ for brain than for the other tissues (factor of 1.4–2.6 for complex IV and 2.1–2.6 for complex III). The $k_{\text{cat}}$ values that we obtained are similar to those obtained on pure complex IV (47) and present with comparable variations between the type of tissue.

In the last part of our analysis, we looked at the differences in the integrated function of the respiratory chain between the different tissues. First, we verified the integrity of our mitochondrial preparations, as a prerequisite for the analysis of the respiratory rate. We considered the following indexes: state 3 value of respiration and OCR, as OCR alone is not a good indicator, given its important variability on several parameters, even on intact mitochondria (44). The analysis of the respiratory flux values measured at state 3 revealed an apparent discrepancy. Indeed, although skeletal muscle- and heart-isolated mitochondria present with higher respiratory chain enzyme contents, along with higher $V_{\text{max}}$ values, the state 3 respiratory rate is not different. This could be explained by the fact that most of the control of mitochondrial respiration is supported, at state 3, by the pyruvate carrier (36). However, the situation must be very different in vivo as the various tissues consume different physiological energy substrates, with important preferences in their utilization (27). The steady-state analysis of mitochondrial respiration also reveals large differences between the maximal respiratory chain complexes activity and the flux value, i.e., their velocity at state 3. This indicates again that, in our conditions, the rate of mitochondrial respiration is not tightly controlled by the sole content in OXPHOS complexes, nor their maximal velocities. For instance, the maximal activity of complex IV exceeds by a factor of 15 the activity measured during state 3 respiration in heart-isolated mitochondria. This factor is equal to 12.6, 12.2, 8.1, and 4.9 in muscle, brain, liver, and kidney, respectively. For complex III, this excess capacity ranges from 1.8 to 4.2 in the different tissues. The observation that complex IV does not function at the maximal velocity during state 3 respiration led to the definition of the “excess capacity” (15). This excess could be utilized, at least in part, to accommodate the flux to an increase in energy demand, so that tissues with a high excess capacity could adapt more easily to large-scale variations in energy demand. Interestingly, our observations allow us to extend this notion of excess capacity to the intermediary substrates of the respiratory chain. Indeed, the comparison of the amount of reduced CoQ9 determined during state 3, compared with the total content,
reveals that only \( \sim 2\% \) is reduced during steady-state 3 mitochondrial respiration in heart, muscle, and brain. Conversely, this proportion is equal to \( \sim 60\% \) in liver and kidney, using the same substrate combination (pyruvate-malate). For cytochrome \( c \), the fraction used at state 3 is \( \sim 65\% \) of the total in heart and muscle, compared with \( 40\% \) in liver, kidney, and brain. Therefore, our results indicate that not all the substrate is engaged (reduced) during mitochondrial respiration. This raises again the problem of a possible existence of different physical and functional pools of mitochondrial CoQ and cytochrome \( c \), as well as their possible compartmentation. This could also be explained by the equilibrium constant or bypass reactions, such as superoxide production from the reduced cytochrome \( c \). Hence, our work generalizes the existence of an excess of both enzyme capacities and substrates concentrations in mitochondria from different tissues. This could be of particular importance for the control of mitochondrial energy production, as well as the physiological adaptation to a sudden change in energy demand or substrate delivery. Such differences could also participate to the observed tissue specificity of mitochondrial diseases. In particular, the excess capacity could be utilized for the compensation of pathological defects in respiratory chain activity and determine in part the biochemical threshold value (37).

To conclude, we aimed to compare the different tissues, while taking into account the numerous data obtained in our study. For this, we performed a PCA that revealed three groups of tissues: 1) heart and muscle (slow type), 2) liver and kidney, and 3) brain. They present with important differences at the level of respiratory chain content, composition, activity, and flux response. Interestingly, each group represents organs of the same embryonic origin, which could suggest that OXPHOS features could be set up early in the tissue development period, or closely related to the type of tissue function. The first group, skeletal muscle and heart, presents with the highest OXPHOS capacity and a low resistance against the occurrence of respiratory chain perturbation, as illustrated by lower threshold values and high control coefficients. Conversely, the second group, liver and kidney, is characterized by a lower OXPHOS capacity and a lower sensitivity to OXPHOS defects. The third group, which contains solely the brain, is between the first and second group regarding the OXPHOS capacity and flux response. More generally, our study demonstrates that oxidative phosphorylation capacity is highly variable and diverse in tissues. It appears to be determined by different combinations of the mitochondrial content, the amount of respiratory chain complexes, and their intrinsic activity (see Fig. 6). This underlines the complexity of the regulation of mitochondrial energy production, which can occur at the level of 1) organellar biogenesis, 2) mitochondrial or nuclear DNA transcription, and 3) enzyme regulation. Different signaling pathways have been described for the modulation of each of those determinants, but their overall control and orchestration are presently not understood. The present observations of a different dosage of mitochondrial content, respiratory chain expression levels, and enzyme complex intrinsic activity in the different tissues also suggest that mitochondrial metabolism is tailored to meet organ-specific features. For instance, a larger mitochondrial compartment per surface of tissue could allow better access to oxygen more efficiently and could allow delivery of ATP throughout the cytosolic compartment. The mitochondrial content and architecture could also be related to some tissue-specific adaptative needs, such as mechanosensing in skeletal muscle (33). Moreover, the different options of a higher content in enzyme complexes (i.e., heart) than more active ones (i.e., brain) could determine differences in tissue sensitivity to changes in energy demand, controlled by the concentration of available substrates. Accordingly, determination of \( K_m \) for
cytochrome c for complex IV revealed a twice lower value in brain (11.3 ± 0.9 μM) than in heart (28.2 ± 2.7 μM). Hence, the role of energy demand and the type of substrate utilized for energy production could play an important role in defining the mitochondrial compositional features and steady-state functioning characteristics. Accordingly, our group (35) demonstrated previously on living human cells that a change in the type of energy substrate was accompanied by a rapid modulation of the expression of mitochondrial proteins, as well as organelar structural parameters. Our group (7) also showed that physical exercise could modify the content in mitochondria and the amount of respiratory chain complexes in skeletal muscle. Thus, to further detail the diversity in the OXPHOS system between tissues, it will be necessary to consider the numerous regulations that can occur at the level of the respiratory chain or at the level of the mitochondrial network morphofunctional characteristics. For instance, recent evidence suggests that changes in mitochondrial activity can trigger morphological adaptations of the mitochondrial network (23, 35), and clinical studies further indicate that molecular defects affecting its dynamics lead to pathology (38). This suggests that a link between energy status and organelar network configuration must exist. Indeed, mitochondrial overall configuration is controlled by a balance between fusion and fission events, mediated by specific proteins (5, 25) that could participate in the modulation of energy production. Accordingly, the different tissues present with variable expression levels of the fusion and fission proteins, possibly related to specific energy needs. Also, the in-depth study of the other mitochondrial functions related to the OXPHOS system will contribute to understand what regulates mitochondrial structural and functional features in tissues. Our results have implications for the understanding of mitochondrial physiopathology. Indeed, the clinical manifestation of respiratory chain disorders typically present with a tissue specificity, characterized by the fact that a given pathological mutation can affect the different tissues to a variable extent (37). Our data suggest that mutations affecting either the amount of active complexes (i.e., Surf 1) or their catalytic activity (i.e., point mutations) will lead to different degrees of energy defect according to the tissue considered.

ACKNOWLEDGMENTS

The authors thank INSERM, Université Victor Segalen Bordeaux 2, Association Française contre les Myopathies (AFM), Association contre les Maladies Mitochondriales (Ammi), Ligue contre le Cancer, and Région Aquitaine for financial support. We are also grateful to Jean-Pierre Mazat for discussion. We also thank Devin Oglesbee for text corrections.

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