Increase of Flux Control of Cytochrome c Oxidase in Copper-deficient Mottled Brindled Mice*

(Received for publication, April 18, 1995, and in revised form, October 6, 1995)

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The brindled mottled mouse (Mobr), an animal model of the Menkes' copper deficiency syndrome, was used for the investigation of changes in respiratory flux control associated with cytochrome c oxidase deficiency in muscle. Enzymatic analysis of cardiac and skeletal muscles showed an approximately 2-fold decrease in cytochrome c oxidase activity of brindled mutants in both types of muscles as compared with controls. The activities of NADH-cytochrome c oxidoreductase (respiratory chain segment I–III) and succinate-cytochrome c oxidoreductase (segment II–III) were normal. Assessment of mitochondrial respiratory function was performed using chemically skinned musculus quadriceps or heart muscle fibers isolated from control and brindled mottled mice. In skeletal muscle, there was no difference found in maximal rates of respiration. In the Mobr hearts, this parameter was slightly lower than control. Alternately, the determination of flux control coefficients of cytochrome c oxidase performed by a step by step inhibition of respiration with increasing concentrations of azide or cyanide revealed significantly sharper inhibition curves for brindled mice than for control, indicating more than 2-fold elevated flux control coefficients of cytochrome c oxidase. This investigation proved essential in characterizing the metabolic effect of a cytochrome c oxidase deficiency. We conclude, therefore, that application of metabolic control analysis can be a valuable approach to study defects of mitochondrial oxidative phosphorylation.

The brindled mouse is a variant of the X-linked mottled mutants (Mo⁺) with severe copper deficiency and is considered to be an animal model of Menkes' syndrome (Menkes' kinky hair disease) (1–5). The copper homeostasis disorders in humans, in mottled mice, or in copper-deficient rats are all associated with distinct mitochondrial alteration in various tissues. The brain and other regions of the central nervous system are particularly affected (6–7). Among mitochondrial abnormalities are various ultrastructural and biochemical changes, such as the well documented depressed activity of cytochrome c oxidase (a mitochondrial cuproenzyme) (7–10). Thus, it was suggested that the lower level of energy metabolism caused by the decrease in both copper concentration and cytochrome c oxidase activity may be responsible for brain degeneration associated with the Menkes' disease (6, 11). However, there is little information available concerning mitochondrial function or cytochrome c oxidase activity in cardiac and skeletal muscles of animals with copper deficiency.

Measurements of maximal rates of mitochondrial respiration are often used for the functional determination of the different mitochondrial defects (12–14). It has been shown that assessment of respiratory activities of saponin-skinned muscle fibers can be especially applicable for the study of small human biopsy specimens (15–17). However, in cases where the defect is located at a non-rate-limiting step of oxidative phosphorylation, simply determining the maximal rate of respiration will not reveal the mitochondrial defect. Alterations in individual respiratory complexes, for example in cytochrome c oxidase activity, can therefore be hard to find or even missed. Recently it was suggested that metabolic control analysis of oxidative phosphorylation can be a successful approach for quantifying the enzymatic defect in certain mitochondrial diseases (18–20). However, direct evidence is still absent, and acceptable pathological models were not used.

In this work we applied metabolic control analysis (21, 22) to study the consequences of cytochrome c oxidase deficiency in Mobr mice. For this we used incremental inhibition of the mitochondrial respiration with increasing concentration of azide and cyanide. Due to the limited amount of tissue, and in order to avoid possible artifacts of the preparation, we used chemically skinned muscle fibers, which allowed us to investigate mitochondrial functions in situ, without isolation of mitochondria (15, 17, 23).

EXPERIMENTAL PROCEDURES

Animals—Five mottled brindled mutant male mice (Mo⁺) obtained from Genetic Division MRC Radiobiology Unit (Chilton, Didcot Oxon OX11 ORD, United Kingdom) and five normal controls were used to study mitochondrial and enzymatic properties of cardiac and skeletal (quadriceps) muscles. The mice were not exercised. Animals were euthanized by cervical dislocation, hearts and m. quadriceps were rapidly removed, washed, and placed in ice-cold Krebs solution. 50–100 mg of each tissue were frozen in liquid nitrogen for enzyme assays, and the remaining tissue was used for respiration experiments. The histochemical analysis of the used m. quadriceps portions revealed 40 ± 3% slow oxidative fibers (succinate dehydrogenase staining). No difference between Mo⁺ quads and control quadriceps muscles using myosin ATPase and succinate dehydrogenase staining was detected.

Isolation of Skinned Fibers—The bundles of muscle fibers were isolated from heart (left ventricle) or skeletal muscle (m. quadriceps) of control and Mo⁺ mice. Saponin-skinned fibers were prepared by incu-
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TABLE I

| Enzyme Activities of Cardiac Homogenates from Control and Brindled Mottled Mice |
|-----------|------------|-----------|
| Enzyme activities (in units/g, wet weight) and the cytochrome $a_3$ content (in nmol/g, wet weight) were measured as described under "Experimental Procedures." LDH, lactate dehydrogenase; AK, adenylate kinase; CPK, creatine kinase; ASAT, aspartate aminotransferase; CS, citrate synthase; COX, cytochrome c oxidase; NADH-cyt c, NADH-cytochrome c oxidoreductase; Succ-cyt c, succinate-cytochrome c oxidoreductase; $a_3$, cytochrome $a_3$ NS, not significant.

| Enzyme | Control mice | Brindled mice | Significance |
|--------|--------------|---------------|--------------|
| LDH    | 106 ± 6      | 110 ± 6       | NS (n = 11)  |
| AK     | 318 ± 9      | 273 ± 10      | p < 0.01 (n = 10) |
| CPK    | 802 ± 30     | 732 ± 32      | NS (n = 10)  |
| ASAT   | 302 ± 6      | 317 ± 11      | NS (n = 11)  |
| CS     | 216 ± 6      | 237 ± 6       | p < 0.05 (n = 12) |
| COX    | 38 ± 2       | 20 ± 2        | p < 0.001 (n = 11) |
| NADH-cyt c | 17 ± 1   | 20 ± 2        | NS (n = 10)  |
| Succ-cyt c | 10.5 ± 1.4 | 12.2 ± 0.6    | NS (n = 10)  |
| $a_3$  | 44 ± 6       | 31 ± 1        | p < 0.001 (n = 5) |

Next, we determined the maximal respiration activities of chemically skinned muscle fibers. Saponin is frequently used as a skinning agent to ensure mitochondrial intactness (15, 17). In addition to this we adapted a procedure of plasma membrane permeabilization with glycerol as a chemical skinning agent previously used for mechanical experiments only (26). As shown in Table III and Fig. 1 the mitochondrial function in these fibers is similarly preserved as reported for saponin-skinned fibers (15, 17). When comparing the maximal rates of mitochondrial respiration in skinned m. quadriceps fibers isolated from normal and brindled mice (Table II) almost no difference was observed. The same result was obtained using saponin-skinned fibers and glycerol-skinned fibers with two different substrate combinations: glutamate + malate, and glutamate + malate and succinate. In each case the maximal rate of respiration was achieved by the addition of 1 mM ADP. On the other hand, skinned fibers isolated from the cardiac muscle of copper-deficient animals demonstrated slightly lower respiratory parameters than control (Table III).

To determine the flux control coefficients for cytochrome c oxidase we used titrations of the rate of respiration of skinned fibers with the specific inhibitors of this enzyme: sodium azide (28) and potassium cyanide (29). To do this, the maximal rate of respiration (in the presence of 1 mM ADP) was inhibited by incrementally increasing sodium azide (0–800 μM) or potassium cyanide (0–60 μM) concentrations. The inhibition of respiration of skinned cardiac fibers from control (open circles) and Mo17 mice (filled circles) by sodium azide is shown in Fig. 2 (upper part). It can be seen that the shapes of the curves are different for control and brindled mice. The inhibition curve for brindled mutants is characterized by a sharper slope and, therefore, higher sensitivity to azide. A similar effect was found for skinned fibers isolated from m. quadriceps of control and brindled mice (Fig. 2, lower part; compare open and filled
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Maximal rates of respiration of cardiac and quadriceps skinned fibers isolated from normal and brindled mottled mice

The rates of respiration are expressed in ng atoms of oxygen/min/mg, dry weight, at 25°C. NS, not significant.

| Tissue          | Saponin-skinned fibers | Glycerol-skinned fibers |
|-----------------|------------------------|------------------------|
| Heart           |                        |                        |
| Control mice    | 67.6 ± 0.9             | 50.5 ± 6.6             |
| Brindled mice   | 39.4 ± 3.0             | 43.2 ± 0.9             |
| Significance    | p < 0.05 (n = 3)       | NS (n = 3)             |
| Quadriceps      |                        |                        |
| Control mice    | 16.8 ± 0.6             | 12.1 ± 1.0             |
| Brindled mice   | 17.7 ± 0.6             | 12.3 ± 1.1             |
| Significance    | NS (n = 3)             | NS (n = 5)             |

To define the flux control coefficient (C_i) using this non-competitive inhibitor, the following equation can be used.

\[ C_i = -\frac{dJ}{dI/K_d} \]  

(Eq. 1)

where \( J \) is the respiration flux, \( dJ \) is the decrement of respiration flux caused by the increment of inhibitor addition \( dI \), and \( K_d \) is the dissociation constant for sodium azide. To exclude possible changes of the \( K_d \) value under the conditions of copper deficiency it was necessary to determine the inhibition curves of cytochrome c oxidase activity by azide. The dependence of cytochrome c oxidase activity of heart muscle homogenates from control and Mo^0 mice on azide concentration is shown in Fig. 3. In the inset the data are transformed using the Dickson linearization. Both curves were within experimental error similar with the same \( K_d \) value for azide (85.5 ± 5.1 \( \mu \)M and 83.9 ± 9.2 \( \mu \)M for control and brindled mice, respectively). Interestingly, in the m. quadriceps homogenates we determined \( K_d \) values of 73.5 ± 6.9 \( \mu \)M and 107.3 ± 6.7 \( \mu \)M for control and brindled mice, respectively. Thus, it was possible to calculate the flux control coefficients using Equation 1. As shown in Table IV, both heart and quadriceps muscles of Mo^0 mice have significantly higher values of flux coefficients of cytochrome c oxidase.

To prove the result of these titration experiments, another specific inhibitor of cytochrome c oxidase, potassium cyanide, was applied. The inhibition curves of oxygen consumption of skinned muscle fibers with KCN are shown on Fig. 4. Once again, it can be seen that KCN titrations for control and brindled mice are strikingly different for both types of skinned fibers from heart and m. quadriceps with less pronounced sigmoidal behavior in copper-deficient mutants. The control coefficients obtained from KCN titrations are summarized in Table IV. In this case of irreversible enzyme inhibition the flux control coefficients were calculated using the following equation.

\[ C_i = -\frac{dJ}{dI/I_{\text{max}}} \]  

(Eq. 2)

where \( I_{\text{max}} \) is the maximal amount of inhibitor. To avoid overestimation of flux control coefficients, we performed nonlinear regression analysis of the entire inhibitor titration curves (27). Interestingly, the values determined by titrations with cyanide are substantially smaller than the values with azide. Nevertheless, as seen in Table IV, the values of flux control coefficients obtained from copper-deficient brindled mice are approximately 2-fold elevated, compared with the control values for both cardiac and skeletal muscles.

To examine the cause for the reduced cytochrome c oxidase activities in copper-deficient mice, we determined the cytochrome content in Triton X-100 solubilized membrane pellets of homogenates from heart and skeletal muscles from difference spectra in the \( \alpha \)-band of cytochromes. Reduced minus oxidized spectra of these membrane pellets are shown in Fig. 5, A and B. It was found that the cytochrome aa_3 content is in cardiac

![Image](http://www.jsb.org/Downloaded from http://www.jsb.org)
homogenates of Mobr mice about 1.4-fold lower in comparison with control homogenates (cf. quantitative data in Table I), whereas no significant difference was observed for homogenates from skeletal muscle (cf. quantitative data in Table II).

DISCUSSION

In the present study mottled brindled copper-deficient mice were used as a model of the more common cytochrome c oxidase deficiency (30–35). Previous findings provided direct evidence for decreased activities of copper-containing enzymes (particularly of cytochrome c oxidase), being most probably responsible for mitochondrial abnormalities and brain degeneration associated with Menkes' disease (11). Histochemical investigations of Menkes' mutants showed elevated copper concentration in organelle-free cytoplasm as compared with nuclei, mitochondria, or lysosomes, suggesting the disturbed copper transport from the cytosol to the organelles in the cell (36). Using 31P NMR spectroscopy, it was shown that in the brain the observed decreased energy metabolism (decline in ATP content, PCR:Cr ratio, and mitochondrial respiration) can be a pathophysiological mechanism of disturbed nervous function in copper deficiency and Menkes' diseases (37).

In our study an approximately 2-fold lower cytochrome c oxidase activity was measured as described under “Experimental Procedures.” Inset, Dickson plot of azide inhibition of cytochrome c oxidase activity.

**TABLE IV**

Flux control coefficients of cytochrome c oxidase on maximal mitochondrial respiration of glycerol-skinned fibers from control and brindled mice

| Tissue          | Azide titration | Cyanide titration |
|-----------------|-----------------|-------------------|
| Heart           |                 |                   |
| Control mice    | 0.31 ± 0.06     | 0.13 ± 0.03       |
| Brindled mice   | 0.59 ± 0.2      | 0.38 ± 0.12       |
| Significance    | p < 0.05 (n = 5)| p < 0.05 (n = 4)  |
| Quadriceps      |                 |                   |
| Control mice    | 0.33 ± 0.1      | 0.12 ± 0.024      |
| Brindled mice   | 0.71 ± 0.29     | 0.35 ± 0.18       |
| Significance    | p < 0.05 (n = 6)| p < 0.05 (n = 4)  |

**FIG. 3.** Inhibition of cytochrome c oxidase activity of cardiac homogenates by azide. The homogenate (50 mg, wet weight/ml) was obtained from hearts of control mice (open circles) and of Mobr mice (filled circles). The cytochrome c oxidase activity was measured as described under “Experimental Procedures.” Inset, Dickson plot of azide inhibition of cytochrome c oxidase activity.

**FIG. 4.** Inhibition of mitochondrial respiration of glycerol-skinned fibers by cyanide. Upper part, skinned fibers (1.5–2 mg, dry weight) of control (open circles) and brindled (filled circles) mice heart. The respiration was measured in the presence of 10 mM glutamate, 5 mM malate, 10 mM succinate, and 1 mM ADP. Curves with the following parameters (cf. Ref. 27) were fitted to the titration points. Open circles: flux control coefficient (C_i) = 0.11; maximal amount of inhibitor (I_max) = 5.8 μM; dissociation constant of the inhibitor (K_i) = 0.55 μM; initial rate of respiration (J) = 57.1 ng atoms of oxygen/min/mg dry weight. Filled circles: C_i = 0.54; I_max = 5.6 μM; K_i = 0.42 μM; J = 53 ng atoms of oxygen/min/mg, dry weight. Lower part, skinned fibers (3–3.5 mg, dry weight) from m. quadriceps of control (open circles) and brindled (filled circles) mice. The respiration was measured as described above. Curves with the following parameters were fitted to the titration points. Open circles: C_i = 0.09; I_max = 7.1 μM; K_i = 0.35 μM; J = 16.1 ng atoms of oxygen/min/mg dry weight. Filled circles: C_i = 0.38; I_max = 8.9 μM; K_i = 0.9 μM; J = 15.4 ng atoms of oxygen/min/mg, dry weight.
oxidase activity is seen in cardiac and skeletal muscles of brindled mice (Tables I and II). As has been pointed out (38), this decline cannot be related exclusively to the role of copper as a metal center of cytochrome c oxidase, but also may be due to a decreased synthesis of nuclear encoded subunits. Reduced expression of cytochrome c oxidase subunits in the cerebellum, spinal cord, and other regions of central nervous system in Menkes’ disease was shown using specific antibodies against subunits II and IV of cytochrome oxidase (10). This reflects also a decrease of synthesis of mtDNA-encoded subunits. As has been shown in our previous study, this approach has a number of advantages (15, 17). Selective permeabilization of plasma membrane essentially results in unobstructed access to the mitochondria in skinned fibers for substrates or ADP. In this way, the mitochondrial parameters can be estimated inside thin muscle fibers. It allows one to investigate the total mitochondrial population, in situ, without isolation of these organelles and using very small samples of tissue.

The significantly lower values of flux control coefficients for cytochrome oxidase observed with KCN titration as compared with azide titration can be explained by the more specific inhibition of cytochrome c oxidase by cyanide. Due to the fact that in some conditions azide may also inhibit ATP splitting and ATP synthesis activities of mitochondrial ATPase (44), the application of this inhibitor may have resulted in an overestimation of the value of flux control coefficient for cytochrome c oxidase (Table IV).

FIG. 5. Difference spectra of cytochromes in homogenates of control and Mo br mice. The membrane fractions of homogenates from heart (containing 50 mg, wet weight, of tissue/ml) and m. quadriceps (containing 100 mg, wet weight, tissue/ml) were prepared as described under "Experimental Procedures." Upper spectrum of each part, dithionite reduced minus oxidized difference spectrum of membrane fraction from controls; lower spectrum of each part, dithionite reduced minus oxidized difference spectrum of membrane fraction from Mo br mice.

ACKNOWLEDGMENT—The excellent technical assistance of K. Kaiser is gratefully acknowledged.

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J. Biol. Chem. 1996, 271:283-288.
doi: 10.1074/jbc.271.1.283

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