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Flux Control of Cytochrome *c* Oxidase in Human Skeletal Muscle*

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In the present work, by titrating cytochrome *c* oxidase (COX) with the specific inhibitor KCN, the flux control coefficient and the metabolic reserve capacity of COX have been determined in human saponin-permeabilized muscle fibers. In the presence of the substrates glutamate and malate, a 2.3 ± 0.2-fold excess capacity of COX was observed in ADP-stimulated human skeletal muscle fibers. This value was found to be dependent on the mitochondrial substrate supply. In the combined presence of glutamate, malate, and succinate, which supported an approximately 1.4-fold higher rate of respiration, only a 1.4 ± 0.2-fold excess capacity of COX was determined. In agreement with these findings, the flux control of COX increased, in the presence of the three substrates, from 0.27 ± 0.03 to 0.36 ± 0.08. These results indicate a tight *in vivo* control of respiration by COX in human skeletal muscle. This tight control may have significant implications for mitochondrial myopathies. In support of this conclusion, the analysis of skeletal muscle fibers from two patients with chronic progressive external ophthalmoplegia, which carried deletions in 11 and 49% of their mitochondrial DNA, revealed a substantially lowered reserve capacity and increased flux control coefficient of COX, indicating severe rate limitations of oxidative phosphorylation by this enzyme.

Mitochondrial myopathies are a heterogeneous group of metabolic muscle disorders characterized by abnormalities of mitochondrial oxidative phosphorylation (1). Typical histopathological hallmarks are the presence of "ragged red fibers," indicating mitochondrial proliferation and of COX[1]-negative fibers pointing to a COX deficiency (2). Although, in many forms of these metabolic myopathies, various mtDNA mutations have been shown to be the cause of the diseases (1), the metabolic consequences of the mitochondrial defects for the skeletal muscle are still poorly understood. One problem is the frequent co-existence of mutant and wild-type mtDNAs in the same fiber (3). Additionally, studies with isolated mitochondria have suggested the presence of a large excess of various enzymes of the mitochondrial respiratory chain. As a consequence, the individual enzymes of the oxidative phosphorylation pathway can be inhibited up to 80% without significant changes in the overall flux, thus creating threshold effects (4, 5). This situation is predicted from the application to oxidative phosphorylation in isolated mitochondria of the metabolic control analysis (6), a theory that allows a quantification of control of flux exerted by the individual enzymes of a metabolic pathway (7, 8). However, the examination of thresholds of cytochrome *c* oxidase in intact cells (9, 10) has raised the critical issue of how accurately the data obtained with isolated mitochondria reflect the *in vivo* situation. Important complications of the analysis on isolated mitochondria are the possible loss of essential metabolites during the organelle isolation and the disruption of the normal interactions of mitochondria with the cytoskeleton and other organelles, as well as of the possible regulation of enzyme activities by cytoplasmic effectors (11–15). In this report we have therefore used saponin-permeabilized muscle fibers to determine the reserve capacity of COX in human skeletal muscle. Muscle fibers treated with low concentrations of saponin have been shown to be a suitable model for studying oxidative phosphorylation in skeletal muscle under conditions reflecting more closely the *in vivo* situation than isolated mitochondria (12, 16). Our data are consistent with previous findings on cultured human cells and support the conclusion that there is in human skeletal muscle only a low excess capacity of COX, with significant implications for mitochondrial myopathies.

EXPERIMENTAL PROCEDURES

Human Muscle Samples—Skeletal biopsy specimens were obtained from two patients (ages 40 and 48) with the clinical picture of chronic progressive external ophthalmoplegia, who underwent a diagnostic biopsy. Skeletal muscle samples from diagnostic biopsies of 10 patients (ages 32–63) with questionable abnormalities in electromyography, but no biopsy evidence for a manifest myopathy, were used as controls. All patients gave written informed consent prior to biopsy.

Genetic Analysis—Total DNA was isolated from 10 to 40 mg of liquid nitrogen-frozen muscle samples by standard methods, and Southern blots were performed with 1 μg of DNA digested by *Pvu*II as described previously (17).

Solutions—The "relaxing solution" contained 10 mM calcium EGTA buffer, free concentration of calcium 0.1 μM, 20 mM imidazole, 20 mM taurine, 49 mM potassium MES, 3 mM KH_2PO_4, 9.5 mM $MgCl_2$, 5 mM ATP, and 15 mM phosphocreatine, pH 7.1. The measurements were performed in a medium consisting of 110 mM mannitol, 60 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM Na_2EDTA, and 60 mM Tris-HCl, pH 7.4.

Preparation of Muscle Fibers—About 50 mg of biopsy tissue (*Musculus vastus lateralis*) were used for isolation of saponin-permeabilized fibers. Bundles of muscle fibers containing usually 2–4 single fibers were isolated by mechanical dissection. The saponin treatment was performed by incubation of the fiber bundles in "relaxing solution" containing 50 μg/ml saponin, as described in Ref. 16.

Respiration—The respiration of muscle fibers was measured at 25 °C using an Oroboros high resolution oxygraph (Anton Paar, Graz, Aus-

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[1] The abbreviations used are: COX, cytochrome *c* oxidase; mtDNA, mitochondrial DNA; TMPD, N,N,N′,N′-tetrametyl-*p*-phenylenediamine; COX_{Rmax}, reserve capacity of cytochrome *c* oxidase; MES, 4-morpholineethanesulfonic acid.

tria) in the measurement medium containing 2 mM ADP and 10 mM glutamate + 5 mM malate as mitochondrial substrates, or, additionally 10 mM succinate, as described previously (18). The KCN titration of COX activity of permeabilized fibers was performed in the same oxygraphic chamber in the presence of 500 μM TMPD, 1 mM ascorbate, 2 mM ADP, and 0.2 μM antimycin A. In control experiments, it was shown that the ADP-stimulated respiration of the saponin-permeabilized fibers with all three respiratory substrate combinations could not be further stimulated by the uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (10 μM) or cytochrome *c* (8 μM), indicating, respectively, a maximal respiratory rate and a preserved integrity of the mitochondrial outer membrane.

Enzyme Activities—The activity of COX was measured spectrophotometrically in 100 mM phosphate buffer, pH 7.4, in the presence of 0.1% laurylmaltoside and 200 μM ferrocytochrome *c*. With this high cytochrome *c* concentration, we utilized for the analysis a dual wavelength photometer (Aminco DW 2000, SLM Instruments, Rochester, NY) and measured in the β-band of ferrocytochrome *c* at 515–535 nm (19).

Determination of the Flux Control Coefficient—Using specific noncompetitive inhibitors, the flux control coefficient C_i of an enzyme i can be determined experimentally (18) according to the following equation.

$$C_i = -(dJ/J)/(dp_i/I_{max}) \quad \text{(Eq. 1)}$$

where J is the flux through the pathway, p_i is the concentration of a specific inhibitor of enzyme i, and I_{max} is the maximal amount of inhibitor binding sites. The flux control coefficients of cytochrome *c* oxidase were determined from cyanide titration curves using nonlinear regression analysis accounting for the dissociation equilibrium of the inhibitor as described in Ref. 20.

Determination of COX Reserve Capacity—Inhibition plots were constructed of relative ADP-stimulated oxygen consumption with the different combinations of substrates *versus* the percentage of decrease of isolated COX activity at the same KCN concentrations. The resulting curves can be usually divided into two descending phases. The slope of the first part depends on the flux control coefficient of COX, whereas the second descending portion represents the inhibition of the flux rate-limited by COX. The reserve capacity of COX (COX_{Rmax}) was determined from the intersection of the best fitting regression line through the data points of the second descending part of the inhibition plot with the ordinate axis (9, 21).

Cytochrome Spectra and Determination of Cytochrome aa_3 Content—The ultra-turrax homogenized muscle was centrifuged, and the pellet was washed in 100 mM phosphate buffer, pH 7.4, to remove the hemoglobin contamination. The dithionite reduced-oxidized spectra were determined using the Aminco DW 2000 dual wavelength photometer. The content of cytochrome aa_3 was determined at 605 nm, using $\epsilon = 12$ mM^{-1} cm^{-1} (22) and applying a base-line correction.

RESULTS

To determine the reserve capacity of COX in human skeletal muscle, titrations of the ADP-stimulated oxygen consumption of saponin-permeabilized muscle fibers with cyanide were performed. Fig. 1 shows the averages of a series of titration curves with the substrate combination glutamate/malate (*panel* A) and with the additional presence of succinate (*panel B*). The titration curve with glutamate/malate has clearly a sigmoidal shape (Fig. 1*A*), whereas in the presence of succinate (Fig. 1*B*), which causes an approximately 1.4-fold increase of the rate of respiration of fibers (Table I), the sigmoidal shape is less pronounced. This is an indication of a substantial rate limitation of the respiratory flux by COX in the additional presence of this complex II substrate. The averages of all experimental points were fitted to a theoretical model (20) allowing calculation of the flux control coefficient of cytochrome *c* oxidase (equal to 0.27 in the presence of glutamate/malate), whereas this value approached almost 0.36 in the additional presence of succinate. Fig. 1*C* shows the KCN titration curve of TMPD/ascorbate-dependent respiration in antimycin A-treated permeabilized fibers. This oxygen consumption can be attributed to the isolated COX activity, as revealed by the hyperbolic shape of the curve. The comparison of the inhibition of the enzymatic reaction with the degree of inhibition of the respiration flux at identical cyanide concentrations allows the construction of an inhibition

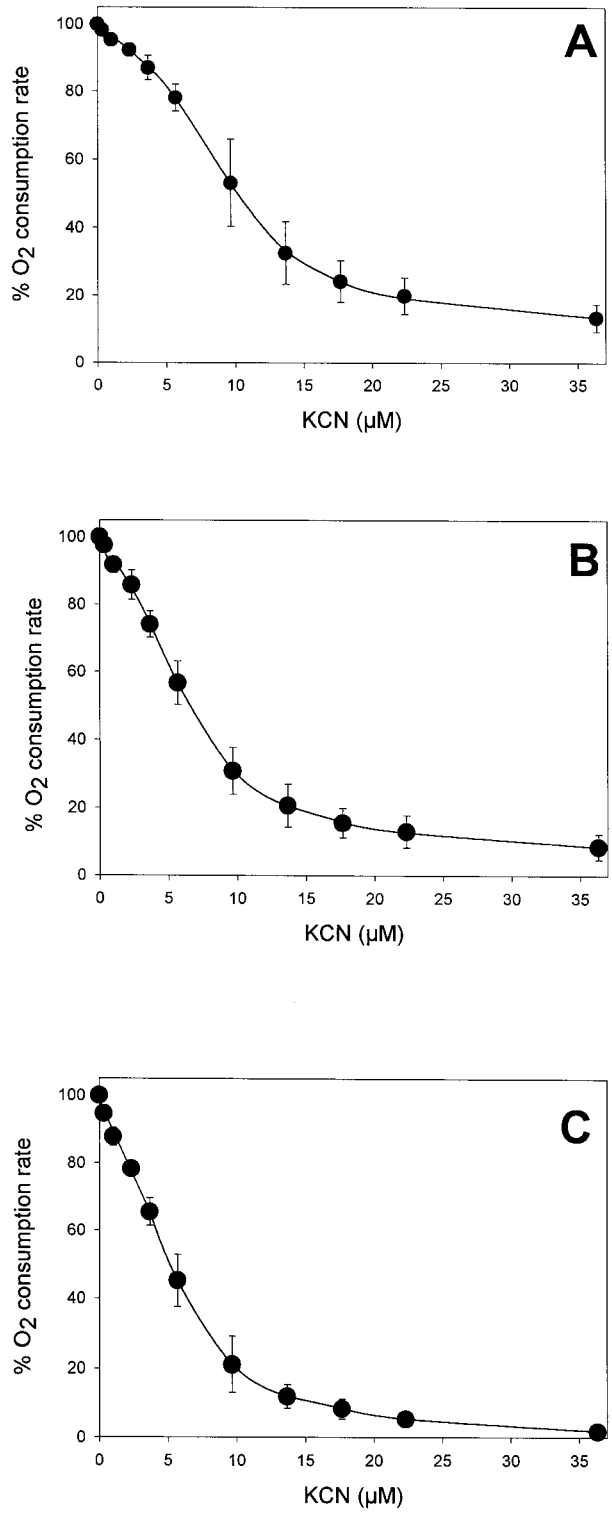

FIG. 1. **KCN titrations of ADP-stimulated oxygen consumption by saponin-permeabilized human muscle fibers.** *A*, substrates: 10 mM glutamate + 5 mM malate. The maximal rate in the presence of 2 mM ADP was 6.5 ± 1.8 nmol O_2/min/mg dry weight (n = 10). The data points are averages ± S.D. from titration experiments with muscle fibers from 10 control individuals. Parameters of the theoretical curve (*solid line*) fitted through the experimental points: C_i = 0.27 ± 0.03; I_{max} = 10.2 ± 0.6 μM; K_d = 0.5 ± 0.05 μM; J_i = 8.5 ± 1.4%. *B*, substrates: 10 mM succinate + 10 mM glutamate + 5 mM malate. The maximal rate in the presence of 2 mM ADP was 8.8 ± 2.5 nmol O_2/min/mg dry weight (n = 8). Parameters of the theoretical curve (*solid line*) fitted through the experimental points: C_i = 0.36 ± 0.08; I_{max} = 6.4 ± 0.8 μM; K_d = 0.6 ± 0.08 μM; J_i = 4.0 ± 1.6%. *C*, substrates: 500 μM TMPD + 1 mM ascorbate. The maximal rate in the presence of 2 mM ADP and 0.2 μM antimycin A was 13.1 ± 4.0 nmol O_2/min/mg dry weight (n = 8).

TABLE I
Maximal rates of respiration of saponin-permeabilized human skeletal muscle fibers, COX turnover rates, flux control coefficients, and reserve capacity of cytochrome c oxidase

Conditions	Controls (n = 10)	CPEO patient J. (11% deletion) (n = 4[a])	CPEO patient W. (49% deletion) (n = 2[a])
Glutamate/malate oxidation rate (nmol O_2/min/mg dry weight)	6.5 ± 1.8	6.7 ± 1.2	4.6 ± 1.6
COX turnover with glutamate/malate (nmol e^-/s/nmol aa_3)	9.2 ± 2.5[b]	9.8 ± 1.7[b]	8.5 ± 0.9[b]
Succinate/glutamate/malate oxidation rate (nmol O_2/min/mg dry weight)	8.8 ± 2.5	8.7 ± 0.6	7.1 ± 1.8
COX turnover with glutamate/malate/succinate (nmol e^-/s/nmol aa_3)	12.5 ± 3.5[b]	12.8 ± 0.9[b]	13.1 ± 1.0[b]
TMPD + ascorbate oxidation rate (nmol O_2/min/mg dry weight)	13.1 ± 4.0	10.7 ± 2.6	7.5 ± 0.6
Maximal COX turnover (nmol e^-/s/nmol aa_3) (based on TMPD/ascorbate rate)	18.6 ± 5.6[b]	15.7 ± 3.8[b]	13.9 ± 1.1[b]
Maximal COX turnover (nmol e^-/s/nmol aa_3) (based on enzyme activity)	14.2 ± 6.4	16.4	19.7
Citrate synthase (units/g wet weight)	12.2 ± 2.5	17.4	21.9
aa_3 (nmol/g wet weight)	6.8 ± 2.1	6.6	4.4
COX (units/g wet weight)	5.8 ± 0.9	6.5	5.2
Ci (glu/mal)	0.27 ± 0.03	0.27	0.53
Ci (succ/glu/mal)	0.36 ± 0.08	0.49	0.63
COX_{Rmax} (glu/mal)	2.3 ± 0.2	1.8	1.4
COX_{Rmax} (succ/glu/mal)	1.4 ± 0.2	1.2	1.3

[a] Average of individual determinations ± S.D.
[b] A wet weight/dry weight ratio of 6.9 (12) was used for the calculations.

plot (6, 9) for COX. The inhibition plots obtained for the substrate combinations glutamate/malate and glutamate/malate/succinate are presented in Fig. 2 (*A* and *B*, respectively). The averaged experimental points were fitted to a theoretical curve describing the inhibition kinetics of an irreversible inhibitor (*solid line*; cf. Ref. 20). The intersection of the best fitting regression line of the second descending portion of the plot with the ordinate axis marked the COX_{Rmax} value. Using this procedure for human skeletal muscle fibers in the presence of glutamate/malate, an approximately 2.3-fold reserve capacity of COX was determined (Fig. 2*A*). In the additional presence of succinate, the reserve capacity was only about 1.4-fold (Fig. 2*B*). Similar results were obtained if the TMPD + ascorbate oxygen consumption rates were compared with the respiratory rates obtained with the two substrate combinations (Table I). It is interesting noting that the maximal COX activities (expressed as turnover numbers) measured polarographically by using ascorbate (1 mM) and TMPD (0.5 mM) as artificial reductants for the endogenous cytochrome *c* pool in permeabilized fibers are also similar to those obtained by spectrophotometric measurements on fiber homogenates in presence of 0.1% laurylmaltoside and an excess (200 μM) of ferrocytochrome *c*. Using this procedure, with glutamate/malate a COX excess capacity of 2.0-fold, and, with glutamate/malate/succinate, an excess capacity of 1.5-fold was obtained. This is a clear indication that both the COX reserve capacity and flux control coefficient critically depend on the substrate supply and, consequently, on the overall flux rate (9).

Because mitochondrial myopathies are known to cause changes in the composition of enzymes of the mitochondrial respiratory chain (1), they should in turn affect the flux control coefficients (18) as well as the reserve capacities (9, 10) of these enzymes. To investigate which changes of the metabolic control by COX occur in these diseases, we investigated the respiration of saponin-permeabilized muscle samples from two patients with chronic progressive external ophthalmoplegia. As shown in the Southern blots of *Pvu*II-cleaved DNA in Fig. 3*A*, both patients carried large scale mtDNA deletions in skeletal muscle. In Fig. 3*B*, the cytochrome spectra of the skeletal muscle samples from both patients are shown. It can be seen that the cytochrome aa_3 content (605 nm peak) decreased with the increasing proportion of deleted mtDNA, whereas the content of cytochromes $c + c_1$ and b determined at 553 and 563 nm, respectively, remained almost unchanged. This is an indication of a change in the composition of the respiratory chain. The inhibition plots of COX obtained with saponin-permeabilized muscle fibers from patient W. respiring in the presence of glutamate/malate or glutamate/malate/succinate are shown in Fig. 4 (*A* or *B*, respectively). In agreement with the lower activity of COX in the skeletal muscle of this patient, the COX reserve capacity decreased with both substrate combinations used. The results of the various analyses performed with the muscle samples from both patients are summarized in Table I. In the sample carrying a higher proportion of deleted mtDNA, we observed a lower COX activity (see also Ref. 23) and a lower cytochrome aa_3 content. This accounts for the lower values of COX reserve capacity and for the increase in flux control coefficient of COX in the presence of both substrate combinations.

DISCUSSION

In the present work, for the first time the control of respiration by COX in human skeletal muscle has been measured in saponin-permeabilized muscle fibers, and the results obtained fully confirm the tight control observed earlier for intact human cells (9, 10). Previous studies with isolated mitochondria (6) had reported high threshold values for COX in skeletal muscle, which appeared to be in accordance with data showing that the molar ratio of COX and NADH:CoQ oxidoreductase is about 6–7 (24, 25). In agreement with this apparent large "excess" capacity of COX for skeletal muscle mitochondria, COX inhibition threshold values of about 70% (or even over 80%) were observed (5, 6). On the basis of these results, it was concluded that COX defects in skeletal muscle should lead to a phenotypic expression only if more than about 70% of the enzyme activity is lost (5, 6). However, this prediction has not been supported by findings on copper-deficient mottled brindled mice, in which COX activity deficits of about 50% already seriously affected the muscle energy metabolism (26, 27) and by quantitative studies on patients with mitochondrial myopathies carrying mtDNA deletions or point mutations, which

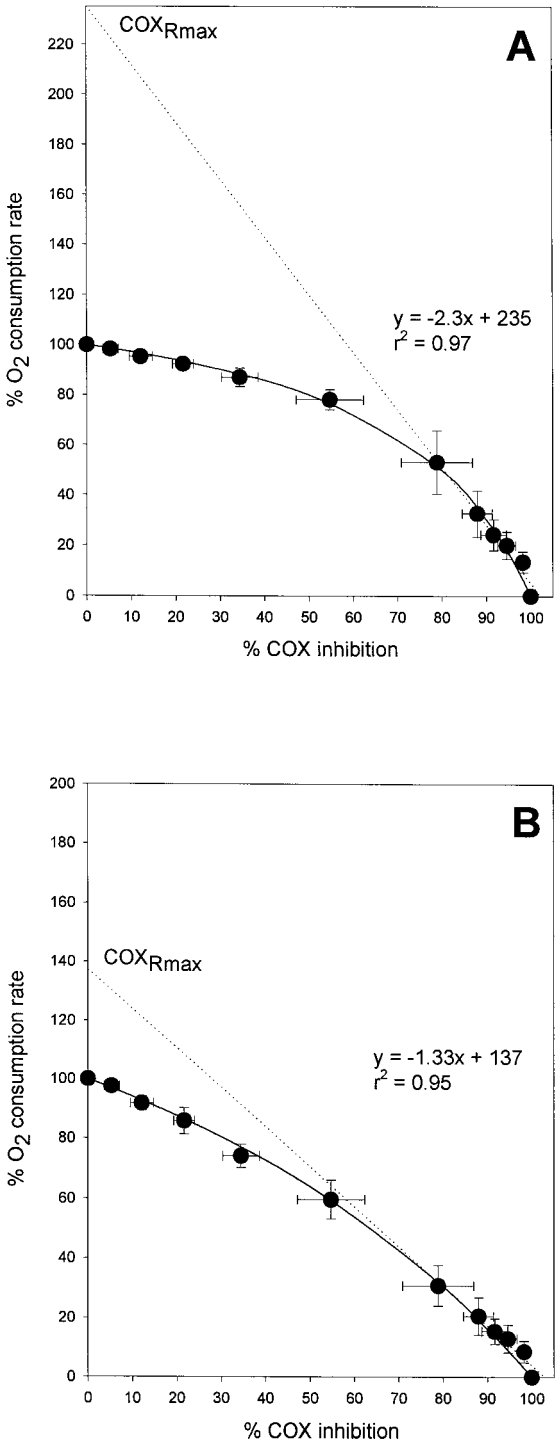

FIG. 2. **Inhibition plots of relative ADP-stimulated oxygen consumption of saponin-permeabilized muscle fibers *versus* percentage of decrease of isolated COX activity at the same KCN concentrations.** *A*, substrates: 10 mM glutamate + 5 mM malate (n = 10). *B*, substrates: 10 mM succinate + 10 mM glutamate + 5 mM malate (n = 8). COX_{Rmax} was determined from the intersection of the best fitting regression line through the data points of the second descending part of the inhibition plot (*dotted line*) with the ordinate axis. The parameters of this best fit regression line are given in the figure.

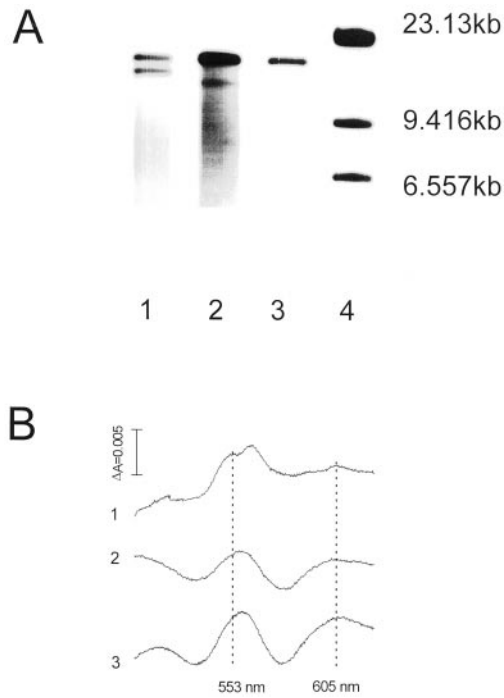

FIG. 3. **Southern blots of *Pvu*II-digested mtDNA (*A*) and cytochrome spectra (*B*) from skeletal muscle samples of two patients with chronic progressive external ophthalmoplegia and one control.** *A*, Southern blots of mtDNA from muscle sample of patient W. harboring a 1.5-kilobase deletion in 49% of its mtDNA (*lane 1*), muscle sample of patient J. harboring a 2.7-kilobase deletion in 11% of its mtDNA (*lane 2*), control muscle sample (*lane 3*), and molecular mass markers (*lane 4*, digoxigenin-labeled *Hin*dIII-cleaved λDNA). *B*, dithionite reduced-oxidized difference spectra of skeletal muscle homogenates from patient W. (*spectrum 1*), patient J. (*spectrum 2*), and a control muscle sample (*spectrum 3*). The spectra were recorded between 500 and 650 nm. The individual samples contained 39.2, 24.5, or 28.2 mg of wet weight/ml skeletal muscle homogenates, respectively (see "Experimental Procedures"). The *vertical dotted lines* indicate the wavelengths of cytochrome $c + c_1$ absorption (553 nm) and cytochrome aa_3 absorption (605 nm). *kb*, kilobases.

have indicated low threshold values for mtDNA mutations (23, 28). Moreover, the examination of COX thresholds in intact human cells (9, 10) has raised the critical issue of how accurately the data obtained with isolated mitochondria reflect the *in vivo* situation. To investigate the metabolic control of respiration by COX in human skeletal muscle, we have therefore used saponin-permeabilized muscle fibers. In the contest of a tight control by COX, as revealed in the present work, it would not have anymore functional meaning to measure inhibition threshold values, because significant decreases for the respiratory fluxes are obtained even at low degrees of inhibition of the isolated COX.

Our results for the flux control coefficient of COX obtained with saponin-permeabilized muscle fibers and the substrate combination glutamate/malate (0.27 ± 0.03) are higher than the values previously reported for isolated rat skeletal muscle mitochondria with the substrates pyruvate/malate (0.2 ± 0.04) (6, 29). This discrepancy could be related either to problems resulting from nonrigorous mitochondria isolation conditions (efflux of NAD^+, etc.), which might affect the maximal electron flow through complex I, or to species differences. On the other hand, our results show clearly that the flux control coefficient and the reserve capacity of COX in human skeletal muscle critically depend on the substrate supply and, consequently, on the overall respiratory rate. In the presence of both the complex I and II substrates (glutamate/malate and succinate, respectively), we observed an approximately 1.4-fold higher respiration rate than with glutamate/malate alone. Under these conditions of increased electron flow through the respiratory chain, the COX activity exceeds the capacity of the respiratory chain only by about 40%. This is in agreement with the high flux control coefficient of COX (0.36) observed with the substrates glutamate/malate/succinate. Because the process of substrate supply to skeletal muscle mitochondria *in vivo* is

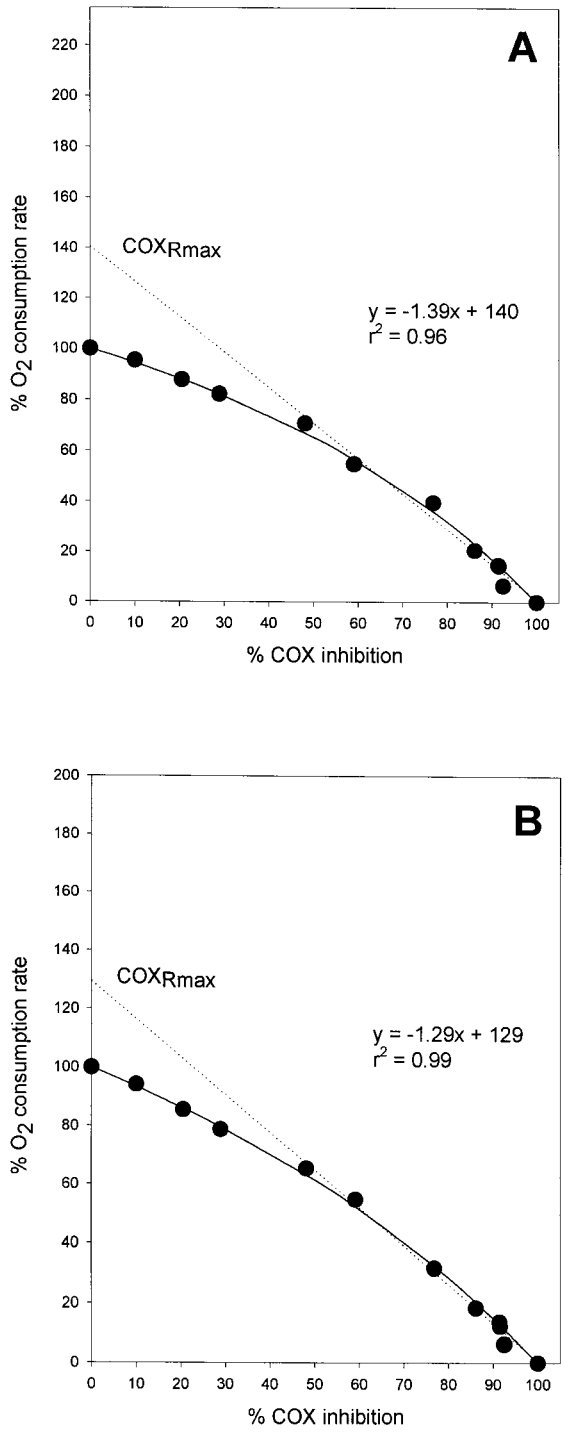

FIG. 4. **Inhibition plots of relative ADP-stimulated oxygen consumption of saponin-permeabilized muscle fibers of patient W. *versus* present decrease of isolated COX activity at the same KCN concentrations.** *A*, substrates: 10 mM glutamate + 5 mM malate (averaged data points from two independent titration experiments). *B*, substrates: 10 mM succinate + 10 mM glutamate + 5 mM malate (averaged data points from two independent titration experiments). COX_{Rmax} was determined from the intersection of the best fitting regression line through the data points of the second descending part of the inhibition plot (*dotted line*) with the ordinate axis. The parameters of this best fit regression line are given in the figure.

rather complex (oxidative degradation of pyruvate originating from glycogen breakdown and glycolysis of fatty acids and amino acids), at least under certain circumstances, high flux rates through the respiratory chain can be reached (30). At these high flux rates there is obviously a tight control of respiration flux by COX. The present results are completely in agreement with the data obtained using intact cultured human cells respiring with endogenous substrates (9, 10). In addition, there are in skeletal muscle low levels of oxygen, which are known to increase the flux control coefficient of COX (31).

In additional studies, we have investigated the effect of mtDNA mutations on the flux control and reserve capacity of COX. To detect the influence of low amounts of mutant DNA, we have chosen patients carrying deletions in less than 50% of the skeletal muscle mtDNA population. In agreement with our results indicating a tight control of high respiration flux by COX in control human skeletal muscle, we have observed significant effects on the flux control coefficient and the COX reserve capacity in samples harboring only ~10–50% deleted mtDNA, which caused effects on COX activity and heme aa_3 content. This finding can explain why a pathological phenotype can occur in individuals carrying a low proportion of mutant mtDNA in their susceptible tissues.

In conclusion, we report here a low reserve capacity and high flux control coefficient of COX in saponin-permeabilized human skeletal muscle fibers at high rates of mitochondrial respiration by COX. These findings can explain the defects in energy metabolism in patients with mitochondrial myopathies carrying mutations in a low proportion of the muscle mtDNA population.

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