

Spectrophotometric Assay for Complex I of the Respiratory Chain in Tissue Samples and Cultured Fibroblasts

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Background: A reliable and sensitive complex I assay is an essential tool for the diagnosis of mitochondrial disorders, but current spectrophotometric assays suffer from low sensitivity, low specificity, or both. This deficiency is mainly due to the poor solubility of coenzyme-Q analogs and reaction mixture turbidity caused by the relatively high concentrations of tissue extract that are often required to measure complex I.

Methods: We developed a new spectrophotometric assay to measure complex I in mitochondrial fractions and applied it to muscle and cultured fibroblasts. The method is based on measuring 2,6-dichloroindophenol reduction by electrons accepted from decylubiquinol, reduced after oxidation of NADH by complex I. The assay thus is designed to avoid nonspecific NADH oxidation because electrons produced in these reactions are not accepted by decylubiquinone, resulting in high rotenone sensitivity.

Results: The assay was linear with time and amount of mitochondria. The K_m values for NADH and 2,6-dichloroindophenol in muscle mitochondria were 0.04 and 0.017 mmol/L, respectively. The highest complex I activities were measured with 0.07 mmol/L decylubiquinone and 3.5 g/L bovine serum albumin. The latter was an essential component of the reaction mixture, increasing the solubility of decylubiquinone and rotenone. In patients with previously diagnosed complex I deficiencies,

the new assay detected the complex I deficiencies in both muscle and fibroblasts.

Conclusions: This spectrophotometric assay is reproducible, sensitive, and specific for complex I activity because of its high rotenone sensitivity, and it can be applied successfully to the diagnosis of complex I deficiencies.

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Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the first complex of the oxidative phosphorylation system. It is the entry point for electrons into the respiratory chain by oxidation of NADH and transport of electrons to coenzyme-Q₁₀. Complex I also has proton-transporting activity over the inner mitochondrial membrane to the intermembrane space. With a relative molecular mass of ~980 000, it is the largest complex of the respiratory chain. Complex I consists of 45 subunits (identified so far), forming a characteristic L-shaped configuration (1). The hydrophilic peripheral arm stretches out into the mitochondrial matrix and catalyzes the NADH oxidation and electron transport. The hydrophobic membrane arm is embedded in the inner mitochondrial membrane and contains the proton-transport activity. A deficiency of complex I is probably the most frequently encountered cause of mitochondrial disease, and mutations in several nuclear DNA-encoded and mitochondrial DNA-encoded subunits have been described to date (2). In addition, mutations in mitochondrial tRNAs, such as the m.3243A>G mutation in the mitochondrial tRNA^{LEU(UUR)}, usually result in complex I deficiency (2). The most commonly used technique for measuring complex I is a spectrophotometric assay measuring rotenone-sensitive NADH oxidation at 340 nm in tissue homogenate or mitochondria-enriched fractions from cultured fibroblasts (3, 4); however, the sensitivity and specificity of these assays are not optimal. One reason

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for this deficiency is the poor solubility of coenzyme-Q analogs. In addition, owing to the low sensitivity, relatively high concentrations of tissue extract are often required to detect complex I, resulting in turbidity of reaction mixtures. Here we describe a new sensitive and specific assay for complex I that is suitable for diagnostic purposes.

Materials and Methods

MATERIALS

Coenzyme Q₁, decylubiquinone, 2,6-dichloroindophenol (DCIP),¹ rotenone, and antimycin-A were obtained from Sigma. NADH and bovine serum albumin (BSA; fraction V, fatty acid free) were obtained from Roche. All other chemicals were of the highest purity commercially available. Spectrophotometric assays were performed on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer with an automatic water thermostatable 8-cell holder in disposable semimicro 10-mm acryl-cuvettes from Sarstedt.

PREPARATION OF MUSCLE-MITOCHONDRIA AND MITOCHONDRIA-ENRICHED FRACTIONS FROM CULTURED SKIN FIBROBLASTS

Human muscle tissue (musculus quadriceps or musculus semitendinosus) was homogenized as described (5). Fractions of 200 to 300 μ L of a frozen supernatant of muscle homogenate (centrifuged at 600g and 2 °C for 10 min) were thawed at 2–4 °C and centrifuged (10 min at 14 000g and 2 °C) in an Eppendorf 5402 centrifuge. We carefully removed the 14 000g supernatant and the fluffy layer with an Eppendorf pipettor. The mitochondrial pellet was resuspended in 150 μ L of 10 mmol/L Tris, pH 7.6, frozen in 25- μ L aliquots in liquid nitrogen, and kept at –80 °C.

We cultured human skin fibroblasts in M199 medium (Gibco) supplemented with 20% (vol/vol) fetal calf serum. Aliquots of 10 to 15 \times 10⁶ cells were washed with ice-cold phosphate-buffered saline, frozen in liquid nitrogen, and kept at –80 °C until use. For isolation of mitochondria-enriched fractions, the pellets were thawed at 2–4 °C and suspended in 2.9 mL ice-cold 10 mmol/L Tris, pH 7.6. We disrupted the cells mechanically with a 5-mL glass/Teflon Potter-Elvehjem homogenizer (clearance, 0.025 mm), 8 strokes at 1800 rpm in melting ice. After homogenization, we added 0.6 mL ice-cold 1.5 mol/L sucrose and centrifuged the homogenate (10 min at 600g and 2 °C) in a Sorval-RC2B centrifuge. The 600g supernatant was centrifuged again (10 min at 14 000g and 2 °C), and the resulting supernatant was carefully removed. The mitochondrial pellet was resuspended in 0.5 mL of 10 mmol/L Tris, pH 7.6, frozen in 50- μ L aliquots in liquid nitrogen, and kept at –80 °C.

COMPLEX I ASSAY

In the new complex I assay, DCIP is used as a terminal electron acceptor. Complex I oxidizes NADH, and the electrons produced reduce the artificial substrate decylubiquinone that subsequently delivers the electrons to DCIP. The reduction of DCIP can be followed spectrophotometrically at 600 nm. As the electrons produced by other NADH-dehydrogenases are not accepted by decylubiquinone (3), reduction of DCIP is almost completely caused by complex I activity, resulting in very high rotenone-sensitive activity.

We measured complex I spectrophotometrically at 600 nm in an incubation volume of 1.0 mL containing 25 mmol/L potassium phosphate, 3.5 g/L BSA, 60 μ mol/L DCIP, 70 μ mol/L decylubiquinone, 1.0 μ mol/L antimycin-A, and 0.2 mmol/L NADH, pH 7.8. Decylubiquinone and antimycin-A were dissolved in dimethyl sulfoxide (17.5 mmol/L and 1.0 mmol/L, respectively). We prepared a stock solution of 80 g/L BSA in 5 mmol/L potassium phosphate buffer, pH 7.4. Because BSA is a critical component of the complex I assay, we measured the concentration spectrophotometrically at 280 nm (A_{280} 1 g/L BSA = 0.667). The stock solution was diluted to 70 g/L and stored in 1-mL aliquots at –30 °C. Of this solution, 50 μ L was added to a final reaction volume of 1 mL. We preincubated an aliquot of 2.5 to 10 μ L mitochondrial suspension from muscle or 20 μ L mitochondria-enriched fraction from fibroblasts at 37 °C in 960 μ L incubation mixture without NADH. After 3 min, we added 20 μ L of 10 mmol/L NADH and measured the absorbance at 30-s intervals for 4 min at 37 °C. After 4 min, we added 1.0 μ L rotenone (1 mmol/L in dimethyl sulfoxide) and measured the absorbance again at 30-s intervals for 4 min.

Complex I activity was expressed as mU/U complex II, mU/U complex IV, or U/g protein, in which 1 U complex I activity equals 1 μ mol DCIP reduced per min. Fibroblasts from 6 patients and muscle tissue samples from 3 patients with a diagnosed complex I deficiency were used to measure complex I to demonstrate the applicability of the assay to the diagnosis of complex I deficiency. The controls and patients have been described (5).

COMPLEX II ASSAY

During isolation of mitochondria from frozen samples of muscle or fibroblasts, part of the citrate synthase leaks out of the mitochondria and is lost, so citrate synthase cannot be used as a mitochondrial marker enzyme. For that reason, we used complex II and complex IV as mitochondrial marker enzymes. We measured complex II spectrophotometrically at 600 nm as described, with some modifications (6). The 1.0-mL incubation volume contained 80 mmol/L potassium phosphate, 1 g/L BSA, 2 mmol/L EDTA, 0.2 mmol/L ATP, 10 mmol/L succinate, 0.3 mmol/L potassium cyanide (KCN), 80 μ mol/L DCIP, 50 μ mol/L decylubiquinone, 1 μ mol/L antimycin-A, and 3 μ mol/L rotenone, pH 7.8. We preincubated an aliquot of

¹ Nonstandard abbreviations: DCIP, 2,6-dichloroindophenol; BSA, bovine serum albumin; KCN, potassium cyanide.

5 μL mitochondrial suspension from muscle or 10 μL mitochondria-enriched fraction from fibroblasts at 37 °C in the incubation mixture without KCN and succinate. After 10 min, we added KCN and succinate to start the reaction and measured the absorbance at 1-min intervals for 5 min at 37 °C. Blanks were measured in the presence of 5 mmol/L malonate that was added before preincubation. Decylubiquinone (10 mmol/L) was dissolved in dimethyl sulfoxide. Antimycine-A (1 mmol/L) and rotenone (3 mmol/L) were dissolved in ethanol. For both assays, we used a molar absorptivity at 600 nm of 19.1 (mmol/L) $^{-1}$ cm $^{-1}$ for DCIP.

COMPLEX IV AND PROTEIN ASSAYS

We measured complex IV activity as described (7) and protein according to Lowry et al. (8).

Results

Lineweaver–Burk plots revealed K_m values of 0.04 mmol/L for NADH and 0.017 mmol/L for DCIP (data not shown). The pH optimum was 7.8 (data not shown). In a concentration series experiment with decylubiquinone, the highest complex I activity was at 0.07 mmol/L decylubiquinone (data not shown). We tested coenzyme Q_1 as an alternative ubiquinone analog and found that in the presence of 0.07 mmol/L, the activity was ~80% of that measured with decylubiquinone, with similar rotenone sensitivities. Therefore, we continued with decylubiquinone as electron acceptor for complex I in the reaction mixture. Although in most complex I assays Mg^{2+} and KCN are added, the latter to inhibit nonspecific NADH dehydrogenase activity (3), we observed no influence of Mg^{2+} and KCN in our assay (data not shown).

In most complex I assays, pretreatment of the mitochondria, such as repeated freezing and thawing or sonication, is necessary to disrupt the mitochondrial membrane. In our assay, a simple osmotic shock in 10 mmol/L Tris \cdot Cl, pH 7.6, followed by a single freeze-thaw cycle, was sufficient to measure optimal complex I activity. Repeated freezing and thawing did not improve this result, and sonication even decreased complex I activity (data not shown).

Complex I activity was linear for at least 4 min, with sample amounts varying between 0.25 μg protein (containing 0.9 mU complex IV) and 3 μg protein (11 mU complex IV; see Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol53/issue4>).

Because BSA is essential for measuring optimal complex I activity (3), we studied the effect of BSA on complex I activity in muscle mitochondria. We measured optimal complex I activity in the presence of BSA concentrations between 3.2 and 3.9 g/L, whereas the percentage rotenone sensitivity of complex I was near 100% at BSA concentrations between 2.1 and 4.9 g/L (Fig. 1). Similar results were obtained when decylubiquinone was re-

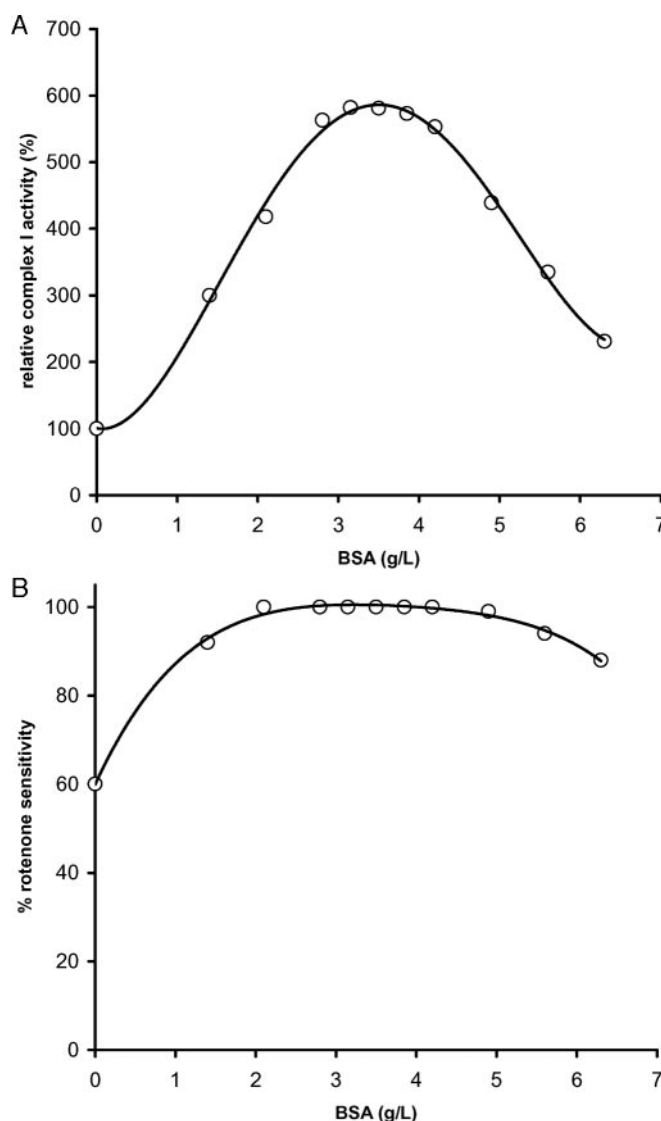


Fig. 1. (A), effect of BSA on relative rotenone-sensitive complex I activity in muscle mitochondrial suspensions.

Complex I activity measured in the absence of BSA was set to 100%. Mitochondrial protein content in each incubation was 0.3 g/L. Data are the mean values of 2 experiments performed with different mitochondrial preparations, and all incubations were performed in duplicate. (B), effect of BSA on the percentage of rotenone-sensitive activity measured in the complex I assay. Complete inhibition by rotenone corresponds to 100% rotenone sensitivity. The percentages calculated were derived from the incubations described in (A).

placed by coenzyme Q_1 in the reaction mixture (data not shown).

The IC_{50} for rotenone in muscle mitochondria, which could be measured only in the presence of BSA, was 13.5 nmol/L (mean of 2 results; range, 10–17 nmol/L; Fig. 2). The presence of BSA in the reaction mixture is required not only for rotenone sensitivity of the assay (probably by solubilizing rotenone), but also for solubilization of decylubiquinone, as illustrated by the fact that in the absence of BSA, we observed an orange/yellow layer on the surface of the reaction mixture after centrifugation at 14000g. By spectrophotometric analysis at 278 nm, we

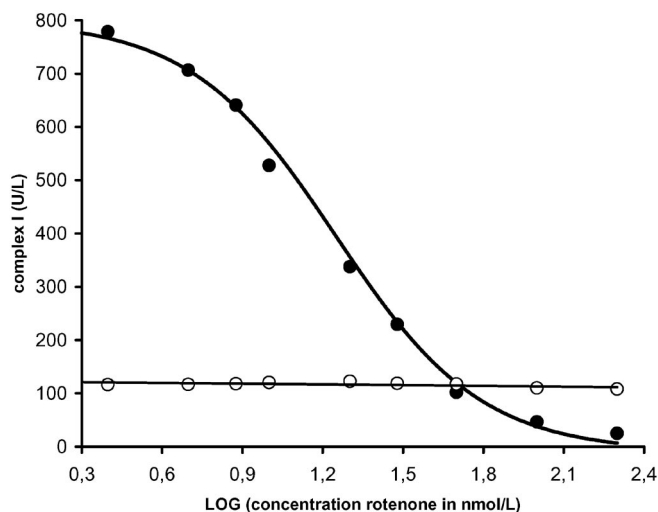


Fig. 2. Inhibition of complex I activity by rotenone measured in muscle mitochondria.

Complex I was measured in the absence (open circles) or presence (filled circles) of 3.5 g/L BSA and with the rotenone concentration interval as indicated. Rotenone-sensitive complex I activity (U/L mitochondrial suspension) is shown. Reaction mixtures contained 1.3 mg/L protein. Using nonlinear regression analysis, the mean (SD) IC_{50} for rotenone was 13.5 (3.5) nmol/L.

found that ~70% of the amount of decylubiquinone added to the mixture was present in this layer.

Intraassay imprecision (CV) was between 2% and 8%, and interassay imprecision was between 2% and 11% (Table 1).

To test the long-term reproducibility of the assay, complex I was measured repeatedly in 3 muscle mitochondrial samples over a period of 6 months. Mean (SD)

complex I activity in these samples was 607 (47) U/L ($n = 10$; CV 8%), 795 (30) U/L ($n = 6$; CV 4%), and 888 (49) U/L ($n = 6$; CV 5%).

To compare this method to the method described by Fischer et al. (3), we used the 2 methods to measure complex I in mitochondria from muscle and fibroblasts. The mean (SD) activity measured in muscle mitochondria using the method of Fischer et al. (3) was 31% (5%; $n = 7$; range, 26%–38%; paired t -test $P = 0.0005$) and in mitochondria from fibroblasts was 17% (3%; $n = 46$; range, 10%–26%; paired t -test $P = 7 \times 10^{-29}$) of the activities measured with our method, showing that the new method is 3-fold (for muscle) and more than 5-fold (for fibroblasts) more sensitive than the method of Fischer et al. (3).

We assessed the specificity of the complex I assay by measuring its rotenone sensitivity. The mean (SD) rotenone sensitivity in mitochondria from control muscle ($n = 17$) was 95% (5%), and in mitochondria from control fibroblasts ($n = 46$), it was 82% (9%).

We tested whether the new complex I assay could be applied to cruder muscle preparations than those used in the experiments described above. In 5 control muscle samples, the mean (SD) complex I activity in 600g supernatants was 46% (6%; range, 43%–51%) of that in equivalent amounts of mitochondrial fractions, and the rotenone sensitivity was 71% (14%; range, 48%–86%). Finally, we tested whether the new complex I assay could be applied to the diagnosis of complex I deficiencies in both muscle and fibroblasts. First, we measured control values for mitochondria from muscle and fibroblasts (Table 2).

Table 1. Intra- and interassay imprecision for complex I in mitochondrial fractions from muscle and cultured fibroblasts.^a

	Complex I (U/L)					
	Experiment 1	Experiment 2	Experiment 3	Mean	SD	CV, %
Intraassay imprecision						
Muscle mitochondria						
10 μ L	240	267	262	256	14	6
10 μ L, 1:1 dilution	248	234	276	253	21	8
10 μ L, 1:2 dilution	252	249	241	247	6	2
Fibroblast mitochondria						
20 μ L	117	131	124	124	7	6
20 μ L, 1:1 dilution	124	118	130	124	6	5
20 μ L, 1:2 dilution	132	129	123	128	5	4
Interassay imprecision						
Muscle mitochondria						
10 μ L	1	2	3			
10 μ L, 1:1 dilution	251	256	263	257	6	2
10 μ L, 1:1 dilution	280	252	280	271	16	6
10 μ L, 1:2 dilution	249	243	297	263	30	11
Fibroblast mitochondria						
20 μ L	124	131	110	122	11	9
20 μ L, 1:1 dilution	124	134	116	125	9	7
20 μ L, 1:2 dilution	129	147	123	133	13	9

^a Complex I activities are expressed as U/L undiluted sample. Intra- and interassay imprecision were determined with undiluted, 1:1 diluted, and 1:2 diluted mitochondrial fractions from muscle and cultured fibroblasts. Intraassay imprecision was determined by measuring complex I activities in 3-fold on the same day, and interassay imprecision was determined by measuring complex I activities on 3 different days. All incubations were performed in duplicate. The protein contents of the undiluted muscle and fibroblast mitochondrial fractions were 0.26 and 0.88 g/L, respectively.

Table 2. Control values for complex I in mitochondrial fractions from muscle and cultured fibroblasts.^a

	Complex I	
	mU/U CII	mU/U C IV
Muscle		
Mean (n = 17)	1140	343
SD	180	63
Observed range	783–1497	270–475
Mean (2 SD)	1140 (360)	343 (126)
Fibroblasts		
Mean (n = 46)	1161	1100
SD	237	245
Observed range	720–1708	678–1675
Mean (2 SD)	1161 (474)	1100 (490)

^a All incubations were performed in duplicate.

We examined fibroblasts from 6 patients carrying variations in different complex I genes and suffering from a previously established complex I deficiency (5). Using the new method, the enzyme deficiency could be confirmed in all 6 patients. In 3 of the patients, we measured complex I in muscle and also confirmed the deficiency in this tissue. The method showed lower results in all tested patients with complex I deficiencies than in any control subjects (Table 3).

Discussion

At present, the diagnosis of complex I deficiency is usually established using complex I assays that are based on the spectrophotometric measurement of rotenone-sensitive NADH oxidation in patient-derived tissue sam-

ples and cultured fibroblasts. In addition to complex I, muscle tissue and cultured fibroblasts contain several nonmitochondrial NADH-oxidizing dehydrogenases. Therefore, the use of tissue homogenates in complex I assays results in a relatively high rate of rotenone-insensitive NADH oxidation that interferes with the sensitivity of the complex I assay. Another disadvantage of measuring muscle homogenate is turbidity of the incubation-mixture, which interferes with the spectrophotometric assay. For these reasons, Brooks and Krähenbühl (9) developed a radiochemical assay for complex I in muscle by measuring ³H₂O production from [4B-³H]-NADH oxidation, based on the stereospecificity of complex I for the 4B hydrogen atom of NADH.

Our new assay uses no radioactivity, is suitable for the diagnostic analysis of complex I in fibroblasts and muscle tissue, and uses DCIP as a final electron acceptor. DCIP has a molar absorptivity that is ~3 times higher than that of NADH: the molar absorptivity at 600 nm of DCIP is 19.1 (mmol/L)⁻¹ cm⁻¹, whereas the molar absorptivity at 340 nm of NADH is 6.2 (mmol/L)⁻¹ cm⁻¹. Compared with the method described by Fischer et al. (3) that measures NADH oxidation, 3- to 5-fold more complex I activity is measured using our method.

DCIP has been used by others in a complex I assay (10), but that assay was not suitable for diagnostic purposes because of nonlinearity of the absorbance with time. In our assay, the addition of an optimal concentration of BSA to the reaction mixture, combined with the use of isolated mitochondrial preparations instead of crude sample homogenates, resulted in a complex I activity that was linear in time and had high rotenone sensitivity. BSA is

Table 3. Complex I in muscle and fibroblasts of 6 patients with a previously established complex I deficiency.

Patient ^a	Complex I by our method ^b		Complex I by the method of Fischer et al. (3), mU/U CS ^c
	mU/U CII	mU/U C IV	
Muscle			
1	154	48	14
3	510	144	16
5	465	157	24
Control mean (2 SD)	1140 (360)	343 (126)	85 (40)
Observed range	783–1497	270–475	53–163
n	17	17	43
Fibroblasts			
1 (NDUFS2)	255	179	29
2 (NDUFS4)	240	145	64
3 (NDUFS7)	484	459	65
5 (NDUFS7)	438	467	26
7 (NDUFV1)	426	416	85
8 (MT-ND2)	339	333	42
Control mean (2 SD)	1161 (474)	1100 (490)	188 (104)
Observed range	720–1708	678–1675	110–260
n	46	46	14

^a Patient numbering is the same as in Janssen et al. (5).

^b Complex I was measured in mitochondrial fractions.

^c Complex I was measured in 600g supernatants of muscle and mitochondrial fractions from fibroblasts.

essential, as it facilitates the solubilization of both rotenone and decylubiquinone. Rotenone and decylubiquinone are both hydrophobic and are practically insoluble in water; rotenone strongly and reversibly binds to BSA (11). Direct binding to BSA probably also plays a role in the solubilization of decylubiquinone, as it is known that BSA reversibly binds molecules with long alkyl chains (12).

The mean (SD) complex I activities measured in fibroblasts and expressed on complex IV activity were similar to those measured by Kramer et al. (4): 1100 (245; n = 46) vs 1200 (170; n = 15), respectively. The mean (SD) rotenone-sensitive activity in our assay was 82% (9%; n = 46) compared with 30% (range, 15%–50%) in the assay described by Kramer et al. (4). The mean (SD) rotenone sensitivity measured in digitonin- and Percoll-treated fibroblasts as described by Chretien et al. (13), 86 (19%; n = 22), was similar to our results. The radiochemical enzyme assay of Brooks and Krähenbühl (9) gave a slightly lower rotenone sensitivity of 60%–80%. As both Chretien et al. (13) and Brooks and Krähenbühl (9) measured complex I in crude lysates and expressed activities on protein base, it was not possible to directly compare the complex I activities measured by these 2 methods with our results.

The method we developed is also suitable for measurements of complex I in 600g supernatants from muscle. The sensitivity and specificity appeared to be lower than observed with mitochondrial fractions.

This complex I assay has clear advantages over the commonly used assays because it is nonradioactive; shows high sensitivity, precision, and rotenone sensitivity; and can be performed on a simple spectrophotometer. The method is applicable to the analysis of muscle samples and is also suitable for measuring complex I in cultured fibroblasts.

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References

1. Carroll J, Fearnley IM, Skehel JM, Shannon RJ, Hirst J, Walker JE. Bovine complex I is a complex of 45 different subunits. *J Biol Chem* 2006;281:32724–7.
2. Janssen RJ, Nijtmans LG, van den Heuvel LP, Smeitink JA. Mitochondrial complex I: structure, function and pathology. *J Inher Metab Dis* 2006;29:499–515.
3. Fischer JC, Ruitenbeek W, Trijbels JMF, Veerkamp JH, Stadhouders AM, Sengers RCA, et al. Estimation of NADH oxidation in human skeletal muscle mitochondria. *Clin Chim Acta* 1986;155:263–73.
4. Kramer KA, Oglesbee D, Hartman SJ, Huey J, Anderson B, Magera MJ, et al. Automated spectrophotometric analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts. *Clin Chem* 2005;51:2110–6.
5. Janssen AJM, Trijbels JMF, Sengers RCA, Wintjes LTM, Ruitenbeek W, Smeitink JAM, et al. Measurement of the energy-generating capacity of human muscle mitochondria: diagnostic procedure and application to human pathology. *Clin Chem* 2006;52:860–71.
6. Rustin P, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, et al. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 1994;228:35–51.
7. Cooperstein SJ, Lazarow A. A microspectrophotometric method for the determination of cytochrome oxidase. *J Biol Chem* 1951;189:665–70.
8. Lowry OH, Rosebrough NJ, Farr AL, Randell RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
9. Brooks H, Krähenbühl S. Development of a new assay for complex I of the respiratory chain. *Clin Chem* 2000;46:345–50.
10. Jewess PJ, Devonshire AL. Kinetic microplate-based assays for inhibitors of mitochondrial NADH:ubiquinone oxidoreductase (complex I) and succinate:cytochrome c oxidoreductase. *Anal Biochem* 1999;272:56–63.
11. Panov A, Dikalov S, Shalbuyeva N, Taylor G, Sheror T, Greenamyre JT. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. *J Biol Chem* 2005;280:42026–35.
12. Spector A, Fletcher J, Ashbrook J. Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. *Biochemistry* 1971;10:3229–32.
13. Chretien D, Bénit P, Chol M, Lebon S, Rötig A, Munnich A, et al. Assay of mitochondrial respiratory chain complex I in human lymphocytes and cultured skin fibroblasts. *Biochem Biophys Res Commun* 2003;301:222–4.