Optimization of respiratory chain enzymatic assays in muscle for the diagnosis of mitochondrial disorders.

Mitochondrion 11 (2011) 893–904

Contents lists available at SciVerse ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Optimization of respiratory chain enzymatic assays in muscle for the diagnosis of mitochondrial disorders

Marco Spinazzi [a,*], Alberto Casarin [b], Vanessa Pertegato [b], Mario Ermani [c], Leonardo Salviati [b], Corrado Angelini [a,d]

[a] Neuromuscular Laboratory, Department of Neurosciences, University of Padova, Via Orus 2 35129, Padova, Italy
[b] Human Genetics Unit, Department of Pediatrics, Via Giustiniani 3, 35128, Padova, Italy
[c] Clinica Neurologica I, Department of Neurosciences, University of Padova, Via Giustiniani 5, 35128, Padova, Italy
[d] IRCSS San Camillo, Via Alberoni 70, 30126, Venezia, Italy

ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 1 July 2011
Accepted 27 July 2011
Available online 9 August 2011

Keywords:
Diagnosis
Enzyme
Homogenization
Mitochondria
Muscle
Respiratory chain

ABSTRACT

The diagnosis of mitochondrial disorders is difficult due to clinical and genetic heterogeneity. Measurements of mitochondrial respiratory chain (RC) enzyme activities are essential for both clinical diagnoses and many basic research questions. Current protocols for RC analysis are not standardized, and so are prone to inter-laboratory variability, and also to biochemical interferences that lead to analytical discrepancies. Moreover, knowledge of the analytical performances of these assays, which is essential to draw meaningful conclusions from the results, is lacking. To understand this variability and to propose possible solutions, we systematically investigated the effect of different homogenization protocols and chemical conditions on RC assays using muscle homogenates. We developed optimized protocols and a novel complex III method with improved sensitivity, precision, and linearity. These methods can be reliably performed on minute muscle samples with a single-wavelength spectrophotometer. Moreover, we measured the variability of the proposed homogenization protocol and we provide a systematic evaluation of each assay's specificity, precision, and linearity. These data will be useful for quality control in both clinical and research laboratories.

1. Introduction

Mitochondrial disorders are a heterogeneous group of inherited metabolic diseases characterized by impaired function of the mitochondrial respiratory chain (RC). They can be caused by a large variety of mutations in either the mitochondrial or nuclear DNA, and can potentially affect every tissue in the organism. However, tissues with high metabolic rates such as the nervous system, skeletal muscles, and the heart are usually most severely affected (DiMauro and Schon, 2003).

Altogether, mitochondrial disorders are relatively common, with an estimated prevalence of 9.18 cases per 100,000 adults (Schaefer et al., 2008). Diagnosis is still a difficult task, due to the large number of nuclear genes involved and the heteroplasmy of mitochondrial DNA mutations in different tissues. Skeletal muscle is considered the most suitable tissue for the diagnosis of these disorders due to its availability and high metabolic rate. Morphological analyses by specific histochemical reactions and biochemical measures of RC enzyme activities are crucial for clinical diagnosis, clinical research, and many of the basic questions in cell biology.

Activities of RC complexes (I–IV) are assayed spectrophotometrically and the results are normalized to the total muscle protein content or to the activity of mitochondrial matrix enzyme citrate synthase. Despite the importance of biochemical measurements, there is still no consensus on the optimal conditions for these assays, nor a quality assurance scheme (Thorburn et al., 2004). Although most published protocols appear similar in principle, they have not been standardized and employ different muscle homogenization procedures, as well as different reaction conditions (Barrientos et al., 2009; Kirby et al., 2007; Rustin et al., 1994; Trounce et al., 1996). The lack of a uniform methodology has led to striking inconsistencies, as demonstrated by a recent multicenter study that compared the results of RC assays on the same muscle homogenate performed by several diagnostic laboratories specialized in mitochondrial disorders. There was considerable divergence of the results among different laboratories, with differences exceeding one order of magnitude (Gellerich et al., 2004). This issue has also been investigated by a French network of diagnostic laboratories for mitochondrial disorders, leading to the development of standardized assays that led to greater consistency

Abbreviations: BSA, defatted bovine serum albumin; CCA, sucrose buffer; ChP, Chappel-Perry buffer; CI, complex I; CI + III, complex I + III; CII, complex II; CII + III, complex II + III; CIII, complex III; CIV, complex IV; CV, coefficient of variation; KCl, KCl buffer; KP, potassium phosphate buffer; Mann, mannitol buffer; RC, respiratory chain.

* Corresponding author. Tel.: +39 49 8216143; fax: +39 49 8216163.
E-mail address: marco.spinazzi@unipd.it (M. Spinazzi).

1567-7249/$ – see front matter © 2011 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2011.07.006

(Medja et al., 2009). However, the performance of some assays (CIII, CI + III, and CII + III) was still unsatisfactory due to unreliability and the difficulty in obtaining linear kinetics and a systematic evaluation of their sensitivity, reliability, and linearity was not performed. Moreover, although the use of double-wavelength spectrophotometry has been suggested to be necessary (Trounce et al., 1996) or at least preferable to single-wavelength spectrophotometry for mitochondrial RC assays (Gellerich et al., 2004) no comparative study was performed between these two techniques.

The aim of our study was to measure the impact of different conditions on RC assays, both at the homogenization phase, and at the analytical phase (i.e. impact of different chemical conditions: and impact of single- vs double-wavelength spectrophotometry), in order to develop simple and reliable protocols for the standardized analysis of RC enzyme activities.

2. Material and methods

2.1. Sample preparation

In order to have a sufficient quantity of the same muscle tissue and to spare limited human samples, we used bovine quadriceps muscle obtained from a freshly slaughtered ox for the development of the protocols. Use of human muscle tissue from controls was limited to the validation of the assays and definition of preliminary reference values, as specified. The muscle was cut in fragments, flash-frozen in liquid nitrogen, and stored at −80 °C. For each experiment, small amounts of frozen muscle (about 30 mg) were dissected with a scalpel blade in small pieces and diluted in 19 volumes of ice-cold buffer of specified composition. Muscle tissue was homogenized in a glass–glass motor-driven 1 ml-tapered conical tissue grinder (Wheaton Science products) held in an ice bath, with a specified number of strokes and rotational speed. The homogenate was then centrifuged at 600×*g* for 10 min at 4 °C to remove nuclei and insoluble cellular debris sedimented in the pellet. The supernatant was kept on ice for the assays.

2.1.1. Homogenization buffers

We employed two main categories of homogenization buffers: 1) sugar-based iso-osmotic buffers including a sucrose based buffer (CCA) and its variations with the addition of different adjuvants such as heparin (CCA + Heparin) or BSA (CCA + BSA), and a mannitol based buffer; 2) ionic buffers including KCl buffer and Chappel-Perry buffer (ChP) with a relatively high ionic osmolarity, or a hypo-osmotic potassium phosphate buffer (KP). The full composition of the different homogenization buffers is indicated in Supplementary Table 1.

2.1.2. Protein quantification and Western-blot analysis

Protein concentrations were measured using the Bradford method. Protein samples (20 μg per well) were mixed with loading buffer 4x (Invitrogen), boiled for 5 min and separated on SDS-PAGE (4–12% NuPAGE bis–tris polyacrylamide denaturing gel, Invitrogen). Gels were stained with Coomassie Brilliant Blue. The assignment of muscle proteins was determined according to Salviati et al. (1982). Subsequently, proteins were transferred to a PVDF membrane (GE Healthcare). Membranes were blocked overnight in 2% milk in T-PBS 1X at 4 °C, then incubated at room temperature for 1 h with primary antibodies: mouse anti-complex I, 1:1000 (Molecular Probes); mouse anti-complex III, 1:1000 (Molecular Probes); mouse anti-COX1, 1:500 (Molecular Probes); mouse anti ATPase, 1:1000 (Mitosciences), mouse anti-cytochrome *c*, 1:1000 (BD Pharmingen); rabbit anti-citrate synthase 1:5000 (Abcam). Once washed in T-PBS 1X, membranes were incubated with secondary antibodies: IgG goat anti-mouse (Santa Cruz) conjugated with horseradish peroxidase at dilution of 1:10,000 for 1 h, or IgG goat anti-rabbit 1:5000, and then washed three times. The detection procedure was according to instructions from the manufacturers (GE Healthcare).

2.2. Enzyme assays

All assays were performed using a Beckman Coulter DU-800 or a Varian Cary 100 spectrophotometer at 37 °C in a final volume of 1 ml. Citrate synthase was assayed as previously described (Trounce et al., 1996).

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) activity was measured by recording the decrease in absorbance due to oxidation of NADH at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$). Muscle homogenate (30 μg of muscle protein) was assayed under five different reaction conditions (each component of the reaction was varied while the others remained constant): 1) different concentrations of Tris–HCl or potassium–phosphate buffer, 2) buffer pH of 7.5 or 8.0, 3) different concentrations of NADH, 4) different concentration of the electron acceptors $ubiquinone_1$ or decylubiquinone, and 5) different concentrations of the adjuvant bovine serum albumin (BSA). The reaction was started by the addition of the electron acceptor ($ubiquinone_1$ or decylubiquinone) and the decrease in absorbance was followed for 3 min. Specificity of complex I activity was measured by the percent inhibition after addition of the complex I inhibitor rotenone (10 μM).

Complex II (succinate dehydrogenase; EC 1.3.5.1) activity was measured by following the reduction of 2,6-dichlorophenolindophenol at 600 nm ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$). Muscle homogenate (25 μg of muscle protein) was assayed in 25 mM potassium phosphate (pH 7.5) under different conditions: 1) different concentrations of succinate, 2) use of decylubiquinone or phenazine methosulfate as electron acceptors (Hatefi and Stiggall, 1978), 3) different concentrations of decylubiquinone, 4) with or without the commonly used adjuvants BSA and ATP (Rustin et al., 1994). The reaction was started by the addition of decylubiquinone and the decrease in absorbance was followed for 3 min. Specificity of complex II activity was measured by the percent inhibition after addition of the specific complex II inhibitors malonate (5 mM) or 2-thenoyltrifluoroacetone (500 μM).

Complex III (ubiquinol cytochrome *c* oxidoreductase, EC 1.10.2.2) activity was measured by following the reduction of cytochrome *c* at 550 nm ($\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$). Muscle homogenate (6 μg of muscle protein) was assayed at pH 7.5 under six different conditions: 1) different concentrations of potassium phosphate and Tris–HCl buffers, 2) the presence or absence of the detergents Tween 20 or lauryl maltoside, 3) different concentrations of the substrate decylubiquinol, 4) different concentrations of cytochrome *c*, 5) different concentrations of BSA, and 6) using the CIV inhibitors potassium cyanide or sodium azide. The assay was started by the addition of decylubiquinol and the increase in absorbance was followed for 3 min. Specific complex III activity was calculated by subtracting enzymatic rates measured in parallel reactions with antimycin A (10 μg/ml). Decylubiquinol was prepared immediately before use as previously described (Trounce et al., 1996) with a slight modification; instead of using a fixed quantity of 5 μl HCl (1 M), we added small aliquots of HCl to the decylubiquinol preparation to reach a final pH between 2.0 and 3.0.

Complex IV (cytochrome *c* oxidase; EC 1.9.3.1) activity was measured by following the oxidation of reduced cytochrome *c* at 550 nm ($\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$). Muscle homogenate (1.5 μg of muscle protein) was assayed under four different conditions: 1) increasing concentrations of potassium phosphate buffer, pH 7.0 2) varying the pH of 50 mM potassium phosphate buffer, 3) different concentrations of reduced cytochrome *c*, and 4) different concentrations of three detergents, lauryl maltoside, Tween 20, and Tween 80. The reaction was started by the addition of muscle homogenate and the decrease in absorbance was followed for 3 min. Reduced cytochrome *c* was prepared immediately before use by adding a few grains of sodium dithionite (DiMauro et al., 1987) or was prepared before hand and stocked at −80 °C after reduction with ascorbate and separation by Sephadex G25 chromatography (Salviati et al., 2002). The specificity of complex IV activity was measured by the percent inhibition following addition of 500 μM potassium cyanide.

Complex I + III (NADH cytochrome *c* oxidoreductase) was assayed as previously described (Medja et al., 2009) with minor modifications. Muscle homogenate containing 30 μg muscle protein was preincubated in distilled water to induce osmotic shock (Chretien et al., 1990) and then assayed in 50 mM potassium phosphate (pH 7.5), 50 μm cytochrome *c*, 1 mg/ml BSA, and 300 μm KCN. The reaction was started by the addition of 200 μM NADH and the increase in absorbance was followed for 3 min. Specificity of complex I + III activity was measured by subtracting results obtained from parallel reactions with added rotenone (10 μM).

Complex II + III (succinate cytochrome *c* reductase) activity was measured by following the reduction of 50 μM cytochrome *c* at 550 nm ($\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$). Muscle homogenate (30 μg of muscle protein) was assayed in 25 mM potassium phosphate, pH 7.5, 300 μM KCN, different concentrations of succinate, and in the presence or absences of the commonly used adjuvants BSA, ATP, and magnesium (Kirby et al., 2007; Medja et al., 2009; Rustin et al., 1994). The reaction was started by the addition of cytochrome *c* and the increase in absorbance was followed for 3 min. Specificity of complex II + III activity was measured by the percent inhibition after addition of the inhibitors malonate (5 mM) or antimycin A (10 μg/ml).

2.3. Performance assessment of the enzymatic assays

To evaluate the analytical performances of each optimized protocol, we measured the coefficients of variation (CV), the specificity of enzyme activity, and the linearity. Here we defined both an intra-assay CV by repeated measures performed on the same day and an inter-assay CV by repeated measures performed on different days ($n = 10$). The specificity of the enzymatic activity was defined as the degree of inhibition by the appropriate enzymatic inhibitor ($n = 3 \pm SD$). The linearity of enzymatic reactions was defined by a novel parameter, the *linearity index,* as the ratio of the enzymatic rate observed within the first 60 s and the activity measured between 120 and 180 s ($n = 5 \pm SD$). This parameter can provide an estimate of the decay of the enzymatic rate calculated two minutes after the initiation of the reaction; values less than one indicate a reduced rate of reaction over time. The amount of homogenate included in the reaction was always well below the saturation threshold to avoid imprecision in the calculation of the initial reaction rate and to maximize linearity.

2.4. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis of group differences was performed using the Student's *t*-test or the ANOVA, when the independent groups were more than two. Post hoc analysis was performed using the Scheffé test. The Spearman Rho was used to check the linear relationship between two variables, when at least one was ordinal. The coefficient of variation was defined as the ratio between the standard deviation and the mean of repeated measurements of the same sample. The significance level was set at $p < 0.05$.

3. Results

3.1. Optimization of different muscle homogenization protocols for RC assays

3.1.1. The type of homogenization buffer affects the quantity and quality of supernatant's proteins

We analyzed the effect of different homogenization buffers on the protein composition of the supernatants obtained after centrifugation of the muscle extracts homogenized in different buffers.

Homogenization of muscle in ionic buffers with relatively high osmolarity (ChP and KCl) resulted in higher total muscle protein levels compared to either sugar-based buffers or low-osmolarity potassium phosphate buffer (Fig. 1A; $p < 0.0001$). This difference was explained by solubilization of myosin in the muscle supernatants induced by ChP and KCl buffers. As seen in Fig. 1C, the bands corresponding to myosin heavy chains are found only in the supernatants of samples processed with ChP or KCl, Interestingly, Western blot analysis revealed that with all buffers a considerable amount of RC proteins was found in the pellets after centrifugation (Fig. 1B) indicating that a portion of RC enzymes is lost to the biochemical analysis. However, when RC enzymatic activities were measured in muscular extracts before and after centrifugation, loss of absolute enzymatic activities did not exceed 10–20% after centrifugation for any respiratory chain complex (Supplementary Fig. 1), despite the reduction of about 50% of total muscular protein levels. Another difference concerns cytochrome *c*, that was found in the pellets only in the samples homogenized in mannitol buffer (Fig. 1B), whereas other buffers allowed virtually complete solubilization of this protein in the supernatants.

3.1.2. Impact of the homogenization buffer on the respiratory chain enzymatic activities

To compare the impact of different homogenization buffers on the function of RC enzymes, enzymatic activities were expressed as μmol/min/g of fresh tissue without normalization for total protein levels, because these values were differentially affected by individual homogenization buffers and therefore could not be used as normalizers. The protocols employed for this analysis are specified in Table 1.

Several differences between different buffers reached statistical significance; however these were not striking, with few exceptions. The use of mannitol buffer resulted in significantly low activities of most enzymes, including complexes I, II, and IV ($p < 0.05$ compared to all other buffers; Fig. 2A–B, D–E). The use of ChP buffer resulted in significantly higher CIV activities ($p < 0.01$ compared to all other buffers; Fig. 2D). The addition of the adjuvants BSA and heparin to the sucrose based-CCA buffer, had no remarkable effects. When activities are expressed normalized to protein levels, results are significantly lower for ionic buffers (i.e. complex I activities of CCA vs KCl/ChP: $p < 0.0006$), due to the higher values of total muscle proteins obtained with these buffers (Supplementary Fig. S2). Overall, the CCA buffer appeared a reasonable compromise of efficacy and simplicity, and it retained considerable enzymatic activities of the homogenates even after storage at low temperatures (−80 °C, data not shown). It was therefore chosen for subsequent experiments.

3.1.3. Effect of different homogenization procedures on the respiratory chain enzyme activities

In order to evaluate if different degrees of muscle homogenization might have an impact on the yield of respiratory chain enzyme activities, we measured the effects of different homogenization protocols, including different numbers of strokes (4, 8, 12, or 16 at 500 rpm) and low versus high rotational speed of the pestle (500 rpm vs 1600 rpm at 16 strokes). An increase in the number of strokes and in the rotational speed was associated with a significant linear increment of measured activities for all RC enzymes tested ($Rho = 0.7$, $p = 0.007$ for CI; $Rho = 0.76$, $p = 0.0013$, CII;, $Rho = 0.65$, $p = 0.011$ for CII + III; Supplementary Fig. 3A–C), except for CIV, where the enzymatic activities progressively increased in parallel with the number of strokes, but they were severely affected by a high rotational speed ($p < 0.012$ Supplementary Fig. 3D). Interestingly, total protein levels in homogenates were essentially constant for all the condition tested ($Rho = 0.21$, $p = 0.42$; Supplementary Fig. 3E).

3.1.4. Variability assessment of the implemented homogenization procedure

To estimate the magnitude of variability associated with the homogenization phase, we calculated the intra-day and inter-day

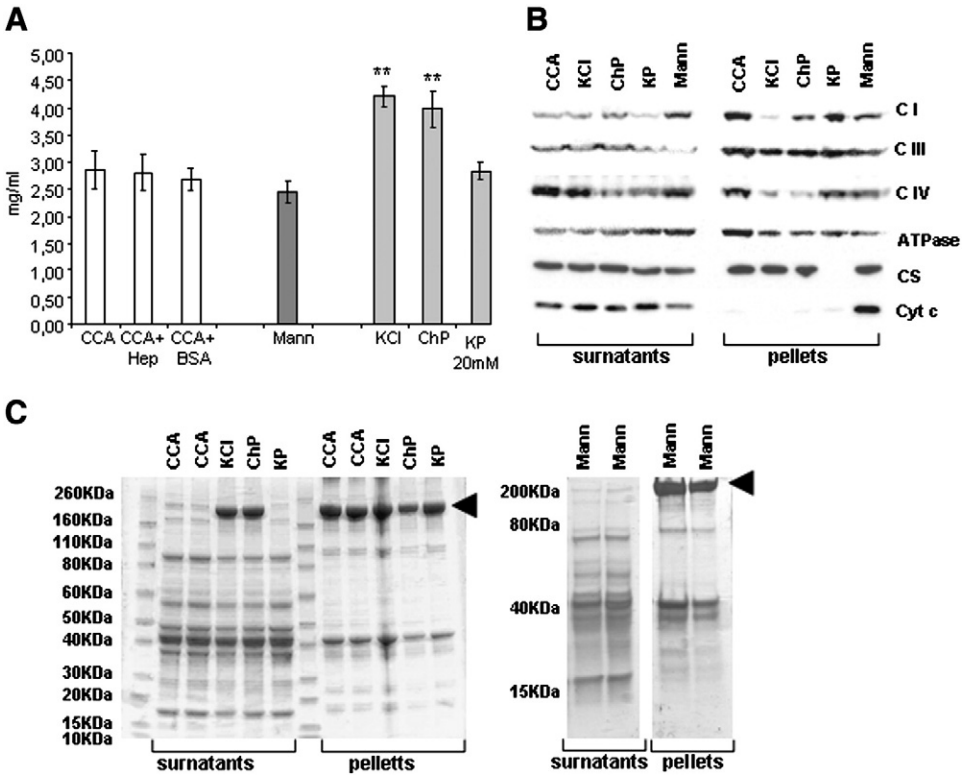

Fig. 1. Effect of different homogenization buffers on muscle homogenate protein quantity and composition. (A) Influence of different homogenization buffers on the total muscle protein quantity of muscle supernatants. Values are means ± SD of at least 6 measurements in at least 2 independent experiments. Statistical significance: ** = $p < 0.00005$ (B) Western-blot analysis of complex I (CI: 30 kDa subunit.), III (CIII: 48.5 kDa subunit.), IV (CIV: 57 kDa, COX I subunit.), ATPase (51 kDa subunit.), citrate synthase (51 kDa) and cytochrome *c* in supernatants and pellets obtained after homogenization in the specified buffers, followed by centrifugation at 600 ×*g*. (C) Representative PAGE of muscle supernatants and pellets obtained after homogenization in specific buffers followed by centrifugation at 600 ×*g*. Gels were stained with Coomassie blue. The arrowheads indicate the bands corresponding to myosin (molecular weight: 220 kDa).

coefficient of variations of respiratory chain enzyme activities (CI, CII, CII + III, CIV, and CS) of separated homogenates obtained from the same muscle, either in the same day and in different days (Table 2). The tested homogenization procedure employed the use of CCA buffer, 16 up-down strokes at a rotational speed of 500 rpm. The coefficients of variations for the different mitochondrial enzymes appeared similar both intra-day (range: 6.3–11.2%) and inter-day (range: 7–13.1%), and they did not change significantly when expressing the results normalized with total proteins or with citrate synthase activity (Table 2). Intra-assay and inter-assay coefficients of variation (n = 10) of the Bradford method used for total protein determination were 1.5% and 3.8%.

3.2. Optimization of biochemical conditions for spectrophotometric RC assays

To identify the optimal analytical conditions for each assay, we measured the effect of different reagents on enzymatic rates, and selected

Table 1
Conditions for spectrophotometric assays of respiratory chain enzymes and citrate synthase activities.

	CI	CII	CIII	CIV	CI + III	CII + III	CS
Buffer (mM)	KP (50)	KP (25)	KP (25)	KP (50)	KP (50)	KP (20)	Tris (100)
PH	7.5	7.50	7.50	7.00	7.50	7.50	8.00
Substrates	NADH 100 μM CoQ_1 65 μM	Succinate 20 mM DCPIP 80 μM Dub 50 μM	$DubH_2$ 100 μM Cyt *c* 75 μM	Cyt *c* H_2 60 μM	NADH 200 μM Cyt *c* 50 μM	Succinate 10 mM Cyt *c* 50 μM	DTNB 100 μM AcCoA 300 μM
Detergent	–	–	Tween 20 0.025%	LM 0.05%[a]	–		Triton X 0.1%
Specific inhibitor	Rotenone 10 μM	Malonate 5 mM TTFA 500 μM	Antimycin A 10 μg/ml	KCN 300 μM	Rotenone 10 μM	Malonate 5 mM	
Adjuvants/other reagents	BSA 3 mg/ml KCN 300 μM	BSA 1 mg/ml KCN 300 μM	KCN 500 μM	–	BSA 1 mg/ml KCN 300 μM	KCN 300 μM	
λ (nm)	340	600	550	550	550	550	412
Muscle proteins (μg)	7.5–90	8–130	1.5–18.0	0.5–4.0	7.5–90.0	7–60	5–80

KP: potassium phosphate buffer (concentration in mM); Tris: Tris buffer (concentration in mmol/l); CoQ_1 = ubiquinone$_1$; DCPIP = 2,6-dichlorophenolindophenol; Dub = decylubiquinone; $DubH_2$ = decylubiquinol; Cyt *c* = cytochrome *c*; Cyt cH_2 = reduced cytochrome *c*; DTNB = 5,5′-Dithiobis(2-nitrobenzoic acid); AcCoA = acetyl coenzyme A; LM = lauryl maltoside; KCN = potassium cyanide; BSA = bovine serum albumin; λ: selected wavelength for the assay; Muscle proteins: range of amount of skeletal muscle protein from muscle homogenates showing linear proportionality with enzymatic activity ($r^2 > 0.99$).

[a] Omitted when assaying human muscle tissues, in order to increase the linearity of the kinetics with time and extend muscle protein concentration range (1.5–24 μg).

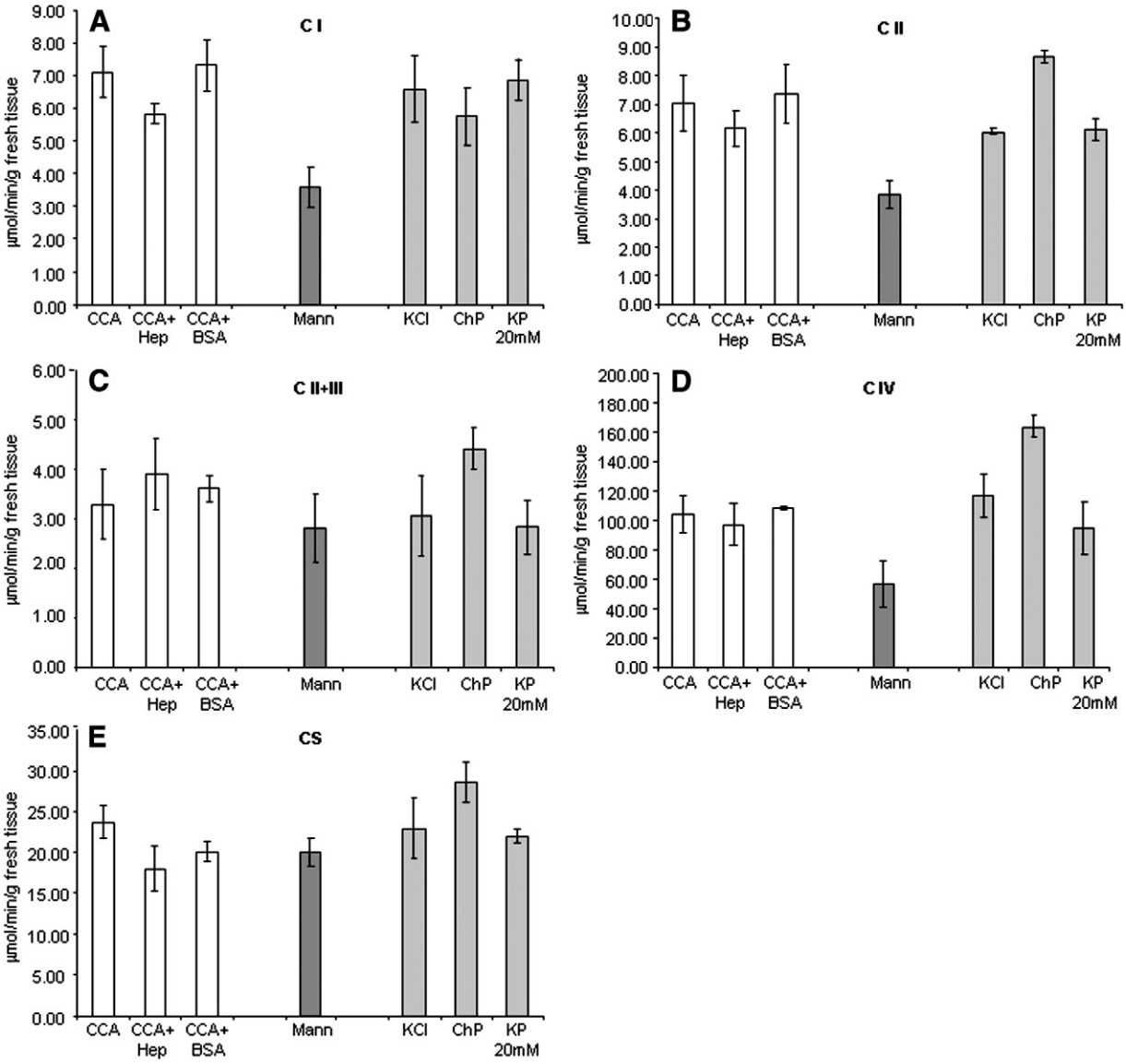

Fig. 2. Effect of different homogenization buffers on respiratory chain enzyme and citrate synthase activities, expressed as µmol/min/g fresh tissue. Values are means ± SD of at least 6 measurements in at least 2 independent experiments. (A) Complex I activities. (B) Complex II activities. (C) Complex II + III activities. (D) Complex IV activities. (E) Citrate synthase activities.

the conditions that yielded maximal specific enzymatic activities, the highest precision, and linearity with time and over a wide range of protein concentrations, all while maintaining procedural simplicity.

Table 2
Intra-day and inter-day coefficients of variations of respiratory chain enzymes extracted by separated homogenizations.

	CI	CII	CII + III	CIV	CS
CV intra-day (n = 10)	10.8% (12.7%)	9% (6.4%)	10.7% (7.9%)	11.2% (12.3%)	6.3%
CV inter-day (n = 10)	13.1% (11.1%)	7% (8.8%)	9.9% (9.1%)	8.3% (7.8%)	8.5%

CV intra-day: coefficient of variation intra-day, calculated as the ratio of standard deviation/mean of ten measurements of ten different homogenates from the same muscle processed on the same day; CV inter-day: coefficient of variation inter-day, calculated as the ratio of standard deviation/mean of ten different homogenates from the same muscle processed on different days. Activities are expressed both as nmol/min/mg total proteins and normalized for citrate synthase activity (in brackets).

3.2.1. Complex I (CI)

Published protocols propose the use of different pHs and concentrations of Tris–HCl or potassium phosphate (KP) in the CI assay (Bugiani et al., 2004; Gellerich et al., 2004; Rustin et al., 1994). We measured significantly higher enzymatic activities in potassium phosphate than in Tris–HCl at all concentrations tested (Fig. 3A), with optimal values between 50 and 250 mM. Changes in pH between 7.5 and 8.0 did not have a significant impact on the reaction. Next, we investigated the most suitable electron acceptor, ubiquinone$_1$ or decylubiquinone. In accordance with previous data (Estornell et al., 1993), we confirmed that ubiquinone$_1$ was preferable to decylubiquinone because decylubiquinone, despite higher initial rates, yielded invariably non-linear reactions (Fig. 3B) indicated by a low linearity index compared to ubiquinone$_1$ (linearity index: decylubiquinone 0.33 ± 0.05 vs ubiquinone$_1$ 0.9 ± 0.03; $n = 3$, $p < 0.0001$). This non-linearity, which was independent of decylubiquinone concentration and the amount of tissue added, led to considerable imprecision in the calculations of enzymatic rates. Ubiquinone$_1$ concentration was saturating at 50 µM (Fig. 3C), while concentrations above 100 µM

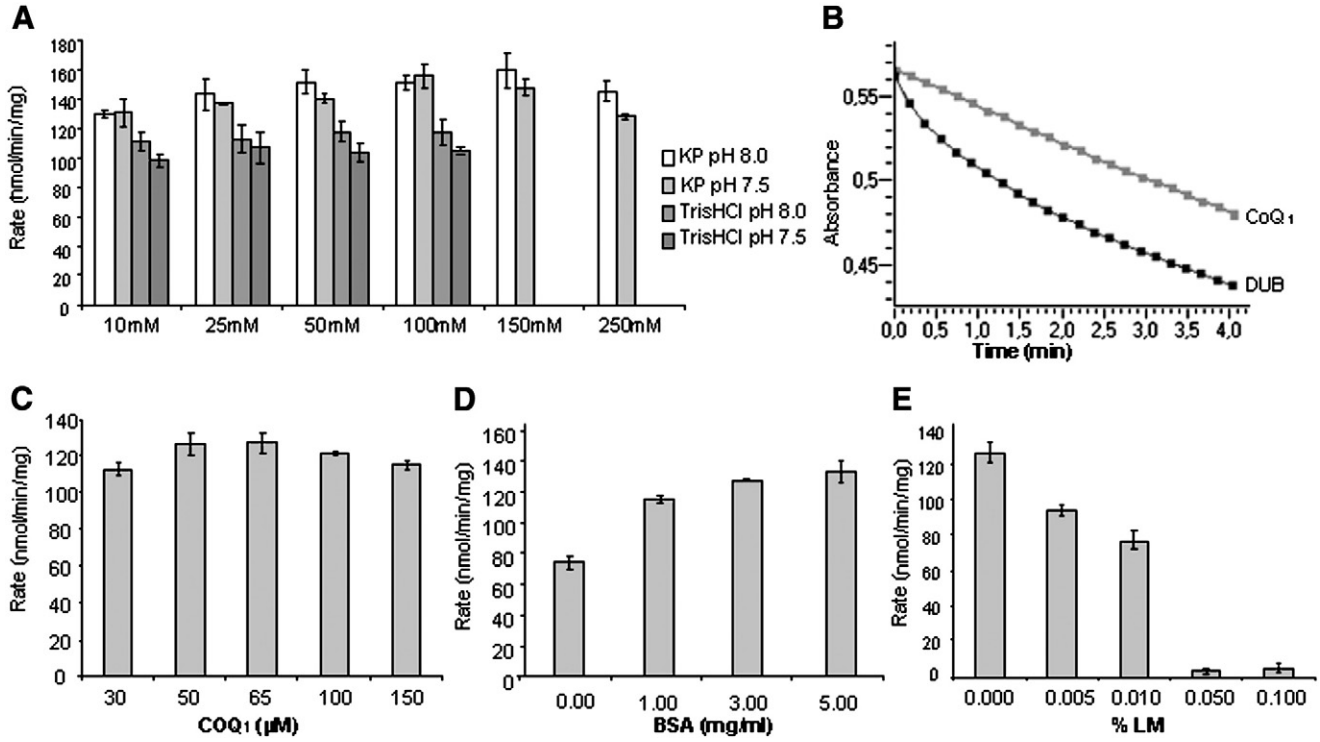

Fig. 3. Effect of different biochemical parameters on complex I specific activity, expressed in nmol/min/mg of protein from skeletal muscle homogenate. For each assay, about 30 μg of muscle proteins was used. Specific CI activities (represented in the graph bars) were calculated after addition of rotenone 10 μM and subtraction of the rotenone insensitive dA/dt. Values are means ± SD of 3 measurements. (A) Influence of the buffer type (potassium phosphate, KP or Tris–HCl), concentration, and pH on CI enzymatic activities. Reaction conditions included 3 mg/ml BSA, 300 μM potassium cyanide, 100 μM NADH, and 65 μM ubiquinone$_1$. (B) Representative CI kinetics in the same muscle homogenate using the two different acceptors, ubiquinone$_1$ (CoQ1 65 μM) and decylubiquinone (DUB 65 μM). Reaction conditions were as indicated in (A), except for the use of potassium phosphate buffer 50 mM, pH 8.0. (C) Saturation curve for ubiquinone$_1$. (D) Effect of different BSA concentrations on CI specific activity. (E) Effect of the detergent lauryl maltoside at different concentrations.

resulted in a mild enzymatic inhibition. The NADH concentration was saturating at 50 μM, but linearity was optimal using concentrations between 100 and 200 μM (data not shown). The use of BSA in the reaction buffer is still controversial (Gellerich et al., 2004; Long et al., 2009). We found that defatted BSA, at an optimal concentration between 3 and 5 mg/ml was necessary to measure maximum rotenone-sensitive complex I activity (73.5 ± 4 nmol/min/mg protein with no BSA vs. 127.3 ± 3 nmol/min/mg protein with 3 mg/ml BSA; $p<0.005$, Fig. 3D). Repeated freeze-thaw cycles in hypotonic buffer (25 mM potassium phosphate, pH 7.4) (Birch-Machin et al., 1994) or the use of the detergent saponin (Wibom et al., 2002) was reported to increase CI activity in isolated mitochondria by disrupting mitochondrial membranes. This did not apply to muscle homogenates from previously frozen muscle (Supplementary Fig. 4A–B), while the use of lauryl maltoside, a detergent used by some laboratories (Gellerich et al., 2004), dramatically inhibited complex I activity (Fig. 3E).

3.2.2. Complex II (CII)

In agreement with previous observations (Birch-Machin et al., 1994), preincubation of the sample with succinate for ten minutes was mandatory to fully activate CII, and the optimal concentration of succinate was 20 mM (Supplementary Fig. 5A–B). The use of phenazine methosulfate 1.5 mM or the ubiquinone analog decylubiquinone 50 μM as electron acceptors resulted in comparable enzymatic activities, although marginally higher when using phenazine methosulfate (172.2 nmol/min/mg ± 0.2 vs 180.1 ± 0.7). Decylubiquinone was chosen as electron acceptor, being considerably less subject to photo-oxidation than PMS, and its concentration was saturating at 50 μM (data not shown). The addition of BSA resulted in a slight increase in activity at a concentration of 1 mg/ml (+ 8.2%). The addition of ATP had a slightly inhibitory effect (data not shown), while lauryl maltoside was also detrimental (Supplementary Fig. 5C). The rate of DCPIP reduction in the absence of decylubiquinone represented a small fraction of that obtained after addition of decylubiquinone (about 6% when testing 24 μg of total muscle proteins; Fig. 6B), and the degree of enzymatic inhibition after addition of the complex II inhibitors TTFA, a chemical interfering with ubiquinone binding, as well as malonate, a succinate analog, was high (85% and 97%, respectively), suggesting that the overall succinate–ubiquinone reductase activity of complex II was effectively measured, rather than only the succinate dehydrogenation reaction.

3.2.3. Complex II + III (CII + III)

As with the complex II reaction, preincubation of the sample with succinate for ten minutes was necessary to fully activate complex II + III (34.5 ± 1.8 nmol/min/mg protein without succinate preincubation vs. 51.8 ± 1.2 nmol/min/mg protein with succinate preincubation; $p<0.05$, Supplementary Fig. 6A). Addition of the complex I inhibitor rotenone, commonly used in several protocols, had no effect (Supplementary Fig. 6B). Combined addition of 1 mg/ml BSA and 100 μM ATP (Medja et al., 2009; Rustin et al., 1994) resulted in a marginal increase in complex II + III activity (Supplementary Fig. 6C). The addition of the adjuvant magnesium chloride, proposed by some protocols (Kirby et al., 2007) resulted in a significant inhibition of CII + III activities, when used at a concentration of either 5 mM (−18%, $p<0.0003$) or 10 mM (−15.4%, $p<0.0008$).

3.2.4. Complex III (CIII)

The optimal buffer to assess CIII activity is still debated (Gellerich et al., 2004; Luo et al., 2008; Zheng et al., 1990). Use of potassium phosphate (50 mM, pH 7.5) resulted in higher specific activity than Tris–HCl at the same pH and concentration (189.9 ± 12.5 nmol/min/mg protein in Tris–HCl vs. 379.7 ± 19.3 nmol/min/mg protein in potassium phosphate; $p<0.005$). The optimal concentration of potassium phosphate was between 25 and 50 mM (Fig. 4A). Both lauryl maltoside and Tween 20 have been employed in this assay (Birch-Machin et al., 1994; Luo et al., 2008), but a comparative analysis is not available. Only Tween 20, at an optimal concentration between 0.025 and 0.050% increased CIII

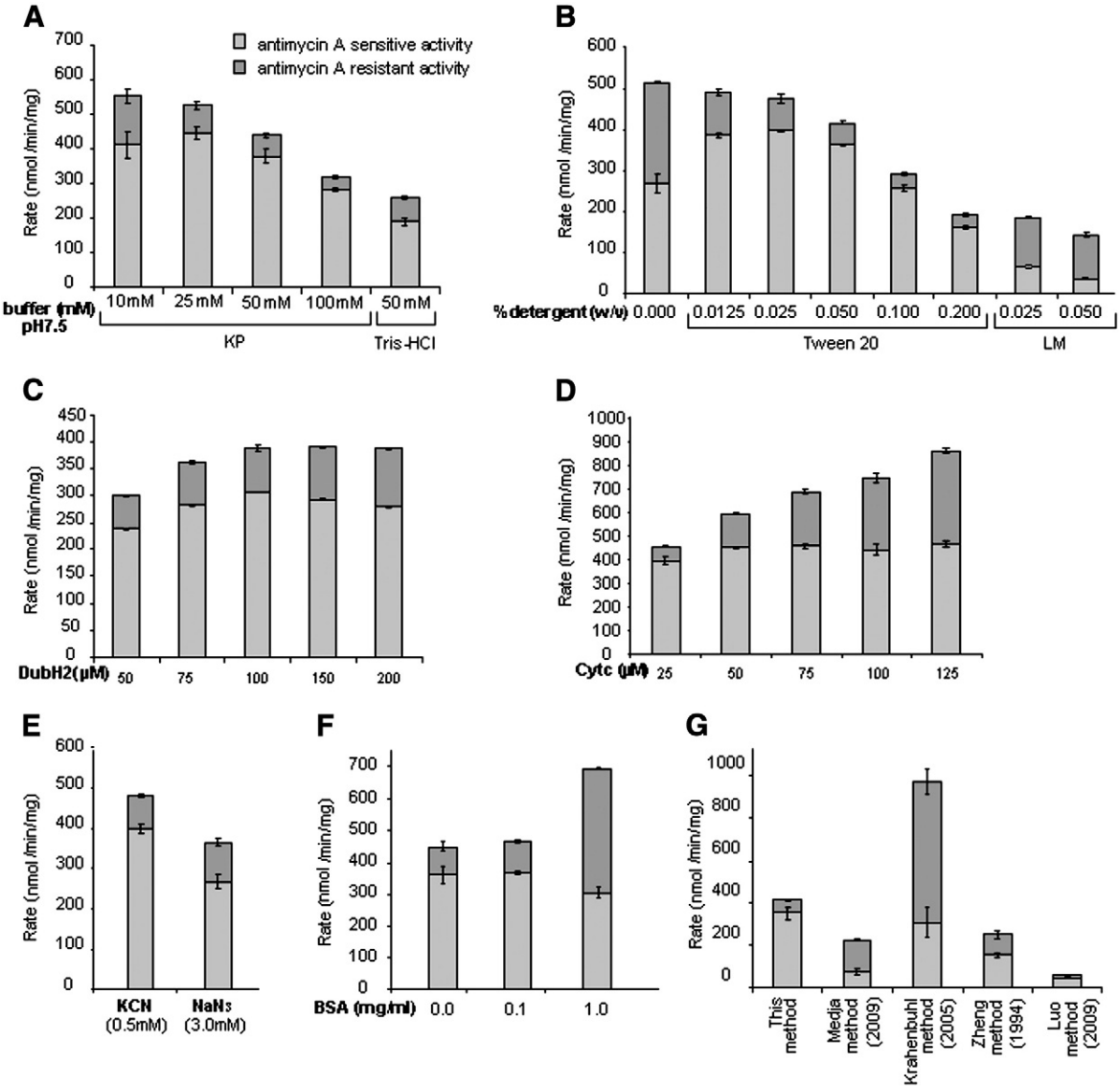

Fig. 4. Effect of different biochemical conditions on complex III activity. Specific CIII activity (the antimicyn A sensitive activity, in light gray bars) was calculated by subtracting total enzymatic rate (dA/dt) from the residual spectrophotometric signal in antimycin A (antimycin A resistant activity, dark gray bars). For each assay, 6 μg of muscle protein was used. Values are means ± SD of 3 measurements. (A) Influence of the buffer type (potassium phosphate, KP or Tris–HCl both at pH 7.5) and concentration on CIII enzymatic activity. Reaction conditions were 75 μM cytochrome *c*, 500 μM potassium cyanide, 0.1 mg/ml BSA, 0.025% Tween 20, and 100 μM decylubiquinol. Buffer type and concentration are specified on the abscissas. (B) Effect of the detergents Tween 20 and lauryl maltoside. Reaction conditions were the same as in (A) except for the buffer, 25 mM KP at pH 7.5. Tween 20 and lauryl maltoside concentrations are specified on the abscissas. (C) Saturation curve for the substrate decylubiquinol. Reaction conditions were the same as previously indicated except for decylubiquinol concentrations (specified on the abscissas). (D) Saturation curve for cytochrome *c*. Reaction conditions were the same as previously indicated except for cytochrome *c* concentration (specified on the abscissas). (E) Effect of two different CIV inhibitors, potassium cyanide (500 μM) and sodium azide (3 mM), on CIII activity. (F) Effect of different concentrations of BSA. (G) Comparison between the proposed CIII assay (described in Table 1) and previously published methods (Krahenbuhl et al., 1994; Luo et al., 2008; Medja et al., 2009; Zheng et al., 1990).

specific activity (no Tween: 269.3 ± 19.2 nmol/min/mg protein vs. 397.7 ± 10.1 nmol/min/mg protein in 0.025% Tween, $p<0.05$; Fig. 4B) and dramatically improved linearity (*linearity index:* no Tween: 0.054 ± 0.02 vs. Tween 0.025% 0.082 ± 0.03, $n=3$, $p<0.0005$), while lauryl maltoside had a detrimental effect on both CIII specific activity (Fig. 4B) and linearity. Higher concentrations of Tween 20 resulted in a further decrease in antimycin A insensitive activity, but also reduced CIII-specific activity. Decylubiquinol and cytochrome *c* concentrations were saturating at 100 μM (Fig. 4C) and 50 μM (Fig. 4D), respectively, while higher concentrations resulted only in increased nonspecific activity. The choice of potassium cyanide rather than sodium azide as the complex IV inhibitor resulted in higher specific CIII activity (Fig. 4E). The addition of the adjuvant BSA, commonly included in CIII protocols (Gellerich et al., 2004; Krahenbuhl et al., 1994; Rustin et al., 1994), increased only background activity (Fig. 4F), and we therefore suggest that it should be omitted from the assay.

Based on these results, we compared the activities obtained from different published CIII protocols (Krahenbuhl et al., 1994; Luo et al., 2008; Medja et al., 2009; Zheng et al., 1990) with our proposed method described in Table 1. Our protocol yielded higher specific activity (Fig. 4G), could be employed over a wide range of protein concentrations (Supplementary Fig. 7), had good precision, a satisfactory percent inhibition by antimycin A, and maintained greater linearity over time (Table 3).

3.2.5. Complex IV (CIV)

Most of the protocols for CIV assay employ the buffer potassium phosphate, but its concentration and pH vary (Gellerich et al., 2004;

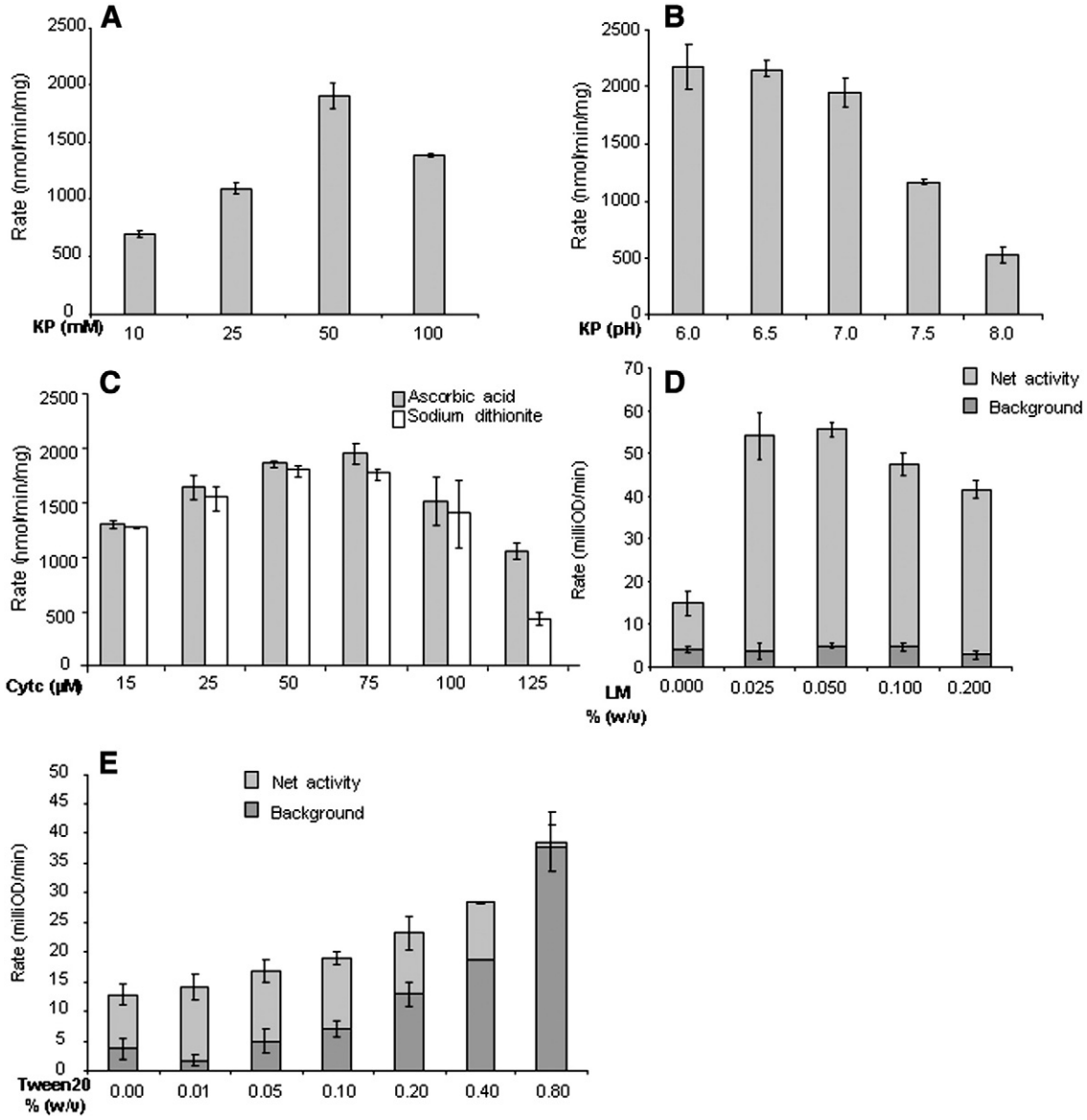

Fig. 5. Effect of different biochemical parameters on complex IV activity, expressed as nmol/min/mg proteins or as milli optical density/min as specified. For each assay, 1.5 μg of muscle protein was used. Values are means ± SD of 3 measurements. (A) Effect of the concentrations of potassium phosphate buffer (KP), pH 7.0. (B) Impact of pH variations in KP 50 mM. (C) Saturation curve for reduced cytochrome *c*. Reduced cytochrome *c* concentrations, prepared either immediately before use with sodium dithionite or with ascorbate followed by column separation, are indicated in the abscissas. (C–D) Effect of the detergents lauryl maltoside (D) and Tween 20 (E) on CIV activity. Background activity (represented by dark gray bars) represents reduced cytochrome *c* oxidation before addition of the muscle homogenate, while net activity (represented by light gray bars) represents enzymatic rates calculated by subtraction of the activity obtained after addition of the homogenate minus background activity.

Rustin et al., 1994). The concentration of potassium phosphate buffer that resulted in the maximal enzymatic activities was 50 mM, while higher or lower concentrations resulted in significant enzymatic inhibition (Fig. 5A). Moreover, we found that the optimal pH value was between 6.0 and 7.0, while higher values result in a dramatic inhibition of enzymatic activity (1963 ± 168 nmol/min/mg protein at pH 7.0 vs. 1164.3 ± 11 nmol/min/mg protein at pH 7.5, $p < 0.05$; Fig. 5B). Different protocols also employ variable concentrations of the substrate reduced cytochrome *c* (Gellerich et al., 2004; Kirby et al., 2007; Rustin et al., 1994). We found that the optimal concentration was between 50 and 75 μM, while higher concentrations resulted in severe enzymatic inhibition (Fig. 5C). The technique for cytochrome *c* reduction, either by addition of a few crystals of sodium dithionite or by the more laborious method using ascorbate followed by separation with column chromatography, did not have a significant impact on enzymatic rate at the optimal cytochrome *c* concentration (Fig. 5C). We next investigated the effects of four detergents, lauryl maltoside (Kirby et al., 2007), Tween 20 (Gellerich et al., 2004), Tween 80 and Triton X on CIV activity. Although lauryl maltoside, at a concentration between 0.025 and 0.050% remarkably increased CIV-specific activity (Fig. 5D), linearity was significantly diminished by this detergent, especially in human muscle. Conversely, the apparent enhancement caused by Tween 20 was due only to increased background activity that also occurred in the absence of muscle protein (Fig. 5E). Use of Tween 80 at a concentration of 0.1% and 0.5% had a similar unwanted effect to that observed with Tween 20, and the addition of 0.1% Triton X resulted in a dramatic CIV inhibition (data not shown). Bovine serum albumin at a concentration of 1 mg/ml (Gellerich et al., 2004; Rustin et al., 1994) had no significant effect on activity or kinetics (data not shown).

3.3. Single-wavelength vs double-wavelength spectrophotometry

Based on previous results, we selected the optimal analytical conditions for the spectrophotometric assays illustrated in Table 1.

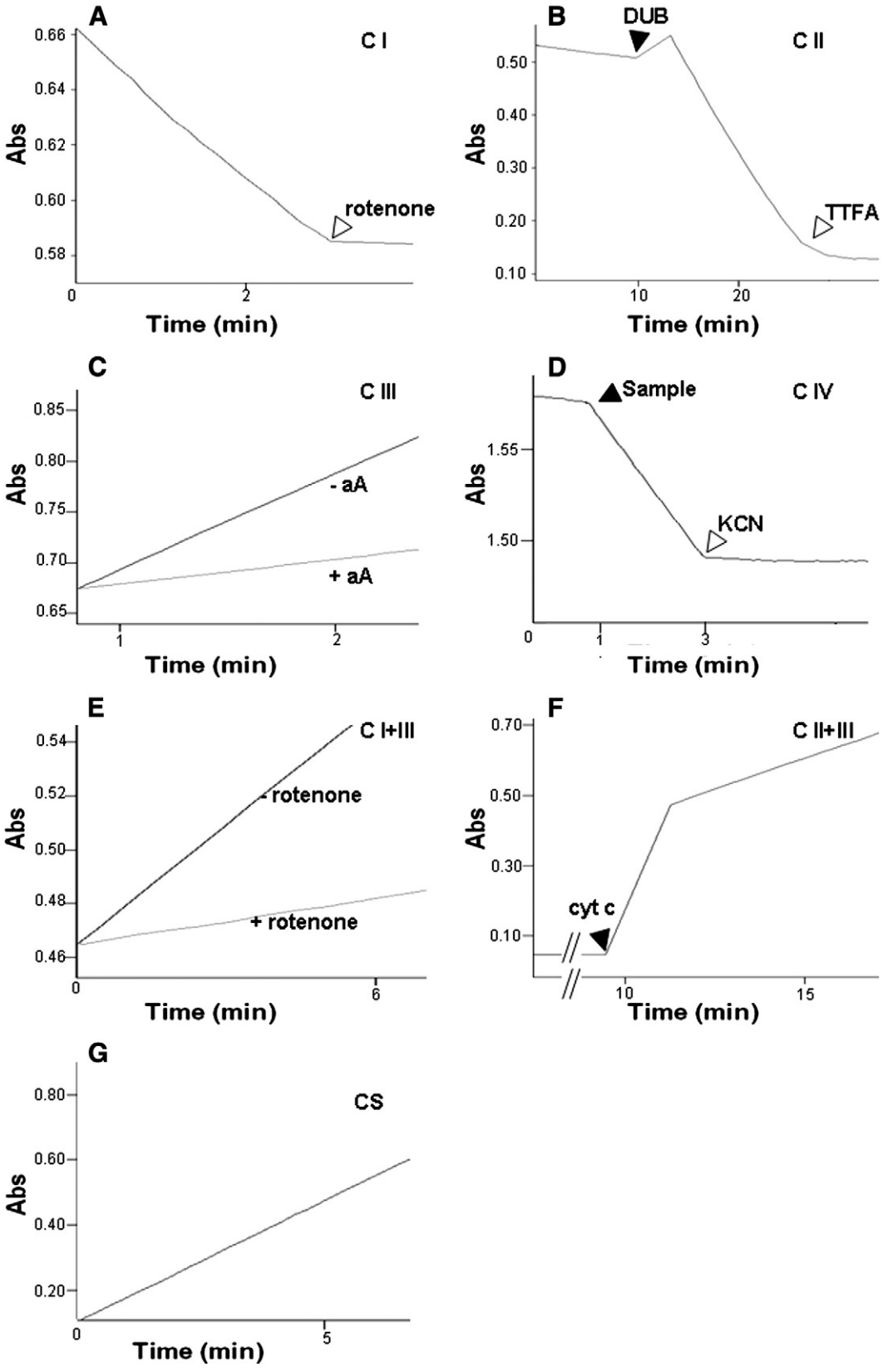

Fig. 6. Representative traces for each RC assay, obtained with the proposed protocols (Table 1) with single-wavelength spectrophotometry at 37 ° C. Where indicated, the specific complex inhibitor was added to demonstrate the specificity of the reaction. (A) Complex I assay; addition of the specific CI inhibitor, rotenone, is indicated by the white arrow. (B) Complex II assay; the reaction is started by addition of decylubiquinone (black arrow), and inhibited by addition of 2-thenoyltrifluoroacetone (500 μM). (C) Complex III assay; the two traces represent two separate reactions (cuvettes), one in the absence and one in the presence of the specific CIII inhibitor, antimycin A. Specific CIII activity is calculated by subtraction of the two activities. (D) Complex IV assay; the reaction is started by addition of the muscle supernatant (1.5 μg) and inhibited by addition of potassium cyanide (100 μM). (E) Complex I + III assay; the two traces represent two separate reactions (cuvettes), one in the absence and one in the presence of rotenone. Specific CI + III activities are calculated by subtraction of the two activities. (F) Complex II + III assay; the reaction is started by addition of the substrate cytochrome *c*. (G) Citrate synthase assay.

Table 3
Analytical performances of the proposed respiratory chain spectrophotometric assays on muscle homogenates: precision, specificity, and linearity.

	CI	CII	CIII	CIV	CI + III	CII + III	CS
Muscle proteins (μg)	30	24	6	1.5	30	30	15
CV intra-assay (n = 10)	3%	5.2%	5.2%	3%	1.7%	2.8%	5.3%
CV inter-assay (n = 10)	4.1%	4%	7.4%	3.7%	6.4%	6.7%	5.4%
% inhibition (n = 3)	98%	97% (mal) 85% (TTFA)	67%	100%	79%	99% (mal) 92% (Aa)	–
Linearity index (n = 5)	0.89 ± 0.02	0.95 ± 0.05	0.82 ± 0.03	0.77 ± 0.08	0.85 ± 0.012	1,01 ± 0,09	1,01 ± 0,07

Muscle proteins: amount of muscle protein from homogenates used for the performance assessment of the assays; CV intra-assay: coefficient of variation intra-assay, calculated as the ratio of standard deviation/mean of ten measurements of the same sample performed on the same day; CV inter-assay: coefficient of variation, calculated as the ratio of standard deviation/mean of ten measurements on the same sample, separated on aliquots and frozen at performed in different days; % inhibition: percentage of inhibition, calculated by the ratio of the activity measured in two parallel cuvettes, with and without the addition of the specific complex inhibitor (mean of 3 independent measurements); mal: malonate 5 mM; TTFA: 2-thenoyltrifluoroacetone 500 μM; Aa: antimycin A 10 μg/ml; linearity index: ratio of the enzyme activity observed within the first 60 s and the activity measured between 120 and 180 s. Linearity index is expressed as the mean ± SD of 3–5 independent assays.

Results of RC analysis performed with our protocols were comparable using single or double-wavelength spectrophotometry (Supplementary Table 2), and double-wavelength spectrophotometry did not increase the analytical precision of the assays, although considerably more time consuming, as only one cuvette could be assayed at once. Representative traces for each RC assay are illustrated in Fig. 6.

3.4. Performance assessment of the proposed enzymatic protocols

The range of muscle proteins that can be assessed in each assay while still maintaining proportionality between enzymatic rate and the amount of sample tested was determined (Table 1, Supplementary Fig. 8). The results of the performance assessment of each of these protocols, performed with a single-wavelength spectrophotometer, are summarized in Table 3. Intra-assay and inter-assay precisions were determined for each assay and ranged between 1.7–5.3% and 3.7–7.4%.

The specificity of the enzymatic reactions, measured as the degree of inhibition using specific complex inhibitors, varied from 100% to 67%.

The CIII reaction displayed the highest nonspecific activity, although this was substantially reduced by the addition of Tween 20. Residual antimicyn A-resistant activity was independent of the amount of muscle protein added (Supplementary Fig. 7), suggesting that this non-specific activity was not simply due to incomplete inhibition. Furthermore, nonspecific activity did not affect the intra-assay CV (5.2%) or the inter-assay CV (7.4%).

Linearity also differed between assays (Table 3). The CII + III and citrate synthase reactions displayed very linear kinetics with negligible decay over the first three minutes (linearity indices of 1.01 ± 0.09 and 1.01 ± 0.07). CIV assay showed the shortest window of linearity when lauryl maltoside was employed (linearity index with lauryl maltoside 0.77 ± 0.08 vs 1.03 ± 0.02 without lauryl maltoside). This lauryl-maltoside-induced non-linearity was especially pronounced in human muscle tissue (data not shown).

3.5. Validation of RC protocols in human muscle homogenates

To validate the proposed RC protocols in human muscle tissue, we performed the RC analysis on frozen muscle biopsies from five individual free of known muscle diseases. The results are shown in Supplementary Table 3.

4. Discussion

At difference with other inborn errors of metabolism, residual enzymatic activities can be considerable in mitochondrial diseases (Trijbels et al., 1993; Thorburn et al., 2004). Therefore, to be clinically useful, mitochondrial RC enzymatic assays must be sufficiently sensitive to detect partial loss of function and sufficiently specific and precise to limit false positive results. Moreover, these methods should be sensitive enough to be performed in small amounts of tissue, especially in case of pediatric patients. Yet the methods must be relatively simple and not prohibitively expensive in order to be employed for the routine diagnosis of RC disorders. We utilized muscle homogenates because although isolated mitochondria from fresh muscle have the advantage of allowing complementary measurements of oxygen consumption and ATP production in addition to RC enzymatic analysis, isolation requires large amounts of fresh tissue (Trounce et al., 1996), and is not practical for routine diagnosis, especially for small children. Instead, muscle biopsies can be performed in peripheral hospitals, and the frozen samples can be easily shipped to the diagnostic laboratory.

4.1. Standardization of the muscle homogenization protocol is necessary for reliable RC analysis

Sample preparation is a critical step of the biochemical analysis. An optimal protocol must allow sufficient disruption of a hard tissue such as skeletal muscle, release of the mitochondria, breakage of the mitochondrial membranes and solubilization of the RC complexes, in order to make them accessible to the specific substrates, but at the same time it must protect the RC enzymes from either chemical or physical inactivation. A good yield in terms of enzymatic activity is essential to increase sensitivity of the analysis and to reduce the amount of sample employed. To this purpose several homogenization protocols have been proposed throughout the years but no systematic assessment of their performance has been conducted.

We showed that several factors influence this phase of the RC analysis, either by affecting directly the enzymatic activities of individual complexes or by altering total muscle protein content which is commonly employed for the normalization of the results. In fact, only ionic buffers with relatively high osmolarity were able to solubilize myosin in the muscle supernatants, resulting in higher total protein concentrations.

When we expressed data as μmol/min/g of fresh tissue, absolute enzymatic activities were roughly comparable with most buffers except with mannitol. Mannitol buffer performed poorly for most RC assays especially for CI, CII, and CIV. It should be noted that this widely used buffer was found to be optimal for RC assays when used for the dilution of previously isolated heart mitochondria (Medja et al., 2009). Notably, after centrifugation, cytochrome *c*, a soluble protein of the mitochondrial intermembrane space, was found in the pellets obtained after centrifugation only in the samples processed in mannitol buffer. This finding suggests that this buffer, at difference with the others, maintain more effectively mitochondrial integrity precluding breakup of mitochondrial membranes, which is essential for the measurements of RC enzyme activities. Therefore the choice of the most appropriate homogenization buffer must be made depending on its use (i.e. for RC assays in muscle homogenates or for other biochemical studies requiring mitochondrial integrity, i.e. measurements of respiratory control index).

Addition of several commonly used adjuvants such as heparin (Max et al., 1972) or BSA (Sherratt et al., 1988) had not any remarkable effect, and therefore they can be safely omitted when performing RC assays on muscle homogenates.

While it is a common assumption that mitochondria are retained in the supernatants after the first centrifugation at 600×*g* (Sherratt et al., 1988), we showed that a substantial amount of subunits of all RC complexes are lost in the pellets after this passage with all methods. However, loss of RC enzymatic activities after centrifugation did not exceed 10–20%, and we therefore suggest to maintain this passage in order to obtain purer muscular extracts.

The protocol of mechanical tissue disruption also has an impact on the yield of respiratory chain enzyme activities. Several techniques are available including the use of tissue grinders with pestles made of glass or teflon, mechanical blenders, detergents, sonicators (Gellerich et al., 2004; Zheng et al., 1990). We focused on the glass-on-glass pestle homogenization technique, which was previously shown to produce higher enzyme activities in skeletal muscle (Grace et al., 1996). While many protocols homogenize with a limited number of strokes, often between 6 and 7 (Gellerich et al., 2004; Medja et al., 2009; Rustin et al., 1994), we noted that an increased number of strokes results in an increment of RC enzymatic activities, probably because it improves breakage of the tissue and release of the enzymes, and therefore an insufficient number of strokes may lead to underestimation of the RC enzymatic activities. A harsh homogenization obtained with high rotational speeds is beneficial for most RC enzymes, except for CIV which is subject to a considerable loss of activity. These data are in accordance with a previous study, showing that CIV is more labile and negatively affected by mechanical stress induced by sonication (Zheng et al., 1990), while the activities of CI and CII are maximized. Use of excessive mechanical force might affect the stability of delicate enzymes, such as CIV, due to shearing forces and generation of excessive heat. We therefore suggest that a reasonable compromise might employ a sufficient number of strokes (around 16), at low rotational speed (500 rpm). Interestingly, however, total protein levels in supernatants did not vary significantly with different stoke numbers, indicating that myofibrillar proteins (which constitute the majority of muscle proteins) are promptly solubilized; therefore total protein levels cannot be used to evaluate the efficacy of the homogenization process with respect to mitochondrial proteins.

Overall, the pre-analytical variability significantly exceeded the one associated with the analytical phase. However, a strict standardization of the homogenization phase led to an acceptable degree of replicability, as shown by consistent coefficients of variation.

Normalization of the RC enzymatic activities for total muscle proteins or citrate synthase did not significantly change this variability. In the diagnostic practice, we suggest to express the results of RC analysis using both normalization for total proteins and for citrate synthase, which is a mitochondrial matrix enzyme commonly used as an index of mitochondrial abundance (Reisch and Elpeleg, 2007). Each of these normalization strategies might be useful in different pathological contexts, especially in conditions associated with a change of mitochondrial quantity. Normalization for citrate synthase activity will facilitate the detection of partial enzymatic defects in diseases with compensatory mitochondrial proliferation, while normalization for total proteins could unmask a RC enzymatic defects in some cases of mitochondrial DNA depletion syndromes, associated with reduced citrate synthase activities (Sarzi et al., 2007; Tesarova et al., 2004).

4.2. Optimization of analytical protocols

We have examined the impact of different biochemical conditions that affect the analytical performances of each RC assay. Our goal was to identify optimized protocols displaying maximized specific activities while minimizing inhibitor-insensitive reactions, good linearity with time, and a consistent relationship between measured activity and sample protein concentrations.

In general, our protocols were conceived to be as simple as possible, and we selected only reagents with significant and reproducible effects on the reaction.

The choice of buffer (composition, concentration, and pH), substrates, detergents, adjuvants, and inhibitors had a dramatic impact on each RC assay. Therefore, there is no single optimal reaction medium for all assays. Buffers are essential to maintain a stable pH during an enzyme-catalyzed reaction. However, concentrations of buffers usually far exceed that of any other component in the reaction mixture, and unwanted interactions can occur between buffers and the enzymes tested or substrates, leading to enzymatic inhibition (Blanchard, 1984). In general, we observed that potassium phosphate resulted in higher activities than Tris–HCl. Furthermore, Tris–HCl has the additional disadvantage of high variations in pH depending on temperature (Blanchard, 1984). Moreover, the pH and ionic strength can significantly influence the electrostatic interactions between substrate and enzyme.

To achieve maximal enzymatic rates, we determined the maximum saturating substrate concentration that did not cause enzymatic inhibition. This substrate optimization was particularly important for the CIV assay, which was subject to a dramatic substrate inhibition at excessive concentrations of reduced cytochrome *c*. The choice of the type of substrate was also critical, especially for CI assay, where we recommend the use of ubiquinone$_1$ rather than decylubiquinone to improve linearity of the reaction with time.

Specific detergents allowed to increase the enzymatic rates in CIII and CIV assays, but they have deleterious effects for other complexes. For example, lauryl maltoside enhanced CIV activity, while it profoundly inhibited the other RC enzymes. This detergent has a specific effect on cytochrome *c* oxidase oligomerization (Sinjorgo et al., 1987), although it leads to a substantial decrease in the linearity of the reactions, especially pronounced in human muscle, and we therefore suggest to omit this reagent in the diagnostic practice. Similarly, while the addition of the adjuvant BSA, commonly used to enhance enzyme stability, was mandatory to maximize CI activity and rotenone sensitivity, it had undesirable effects on the CIII assay by enhancing nonspecific activity due to spontaneous reduction of cytochrome *c* by the numerous cystein residues of BSA in presence of reduced quinones (Chretien et al., 2004).

Activity of complex III has been always considered the most difficult to measure due to the preparation of reduced quinones, the high non-specific activity, and insufficient reliability and linearity (Chretien et al., 2004; Medja et al., 2009). Some centers do not routinely analyze this complex, but rather rely only on the combined assays of CI + III and CII + III although a reliable assay for isolated CIII had been found necessary for the identification of partial CIII defects, which can fail to be recognized when using the coupled assays (Taylor et al., 1993). We now propose a new optimized protocol, based on a previously published method (Luo et al., 2008), but with optimal concentrations of potassium phosphate buffer, saturating concentrations of decylubiquinol, low concentrations of the detergent Tween 20, potassium cyanide instead of sodium azide, and exclusion of BSA. This protocol minimized nonspecific activity, yielded greater sensitivity compared to the original protocol and it had a substantially greater linearity with time compared to other published methods. It was precise and linear over time and with increasing sample quantity.

Finally, we provided an assessment of the analytical performances of the proposed protocols based on precision, specificity of the enzyme inhibitors, linearity, and proportionality of enzymatic activities over a range of protein sample concentrations. To the best of our knowledge, this information has not been systematically provided along with previously published protocols.

In general, the proposed protocols displayed high sensitivity, and required only microgram quantities of muscle proteins per reaction. The full set of RC enzymes can be easily measured using minute quantities of muscle biopsy tissue (around 20–30 mg) in several replicates. Double-beam spectrophotometers, allowing monitoring absorbance changes with time at two different wavelengths, were considered necessary to investigate reliably the function of respiratory chain enzymes to overcome artifacts caused by light scattering due to turbidity of mitochondrial preparations and substrates and

fluctuations of the light intensity of the old-generations spectrophotometers (Chance, 1957; Trounce et al., 1996). We showed that the degree of analytical precision was very high for all the assays with the use of a modern single-wavelength spectrophotometer, indicating that measurements of respiratory chain enzymes can be reliably performed without need of expensive, time consuming and commercially unavailable double-wavelength spectrophotometers (Gellerich et al., 2004; Rustin et al., 1994).

Protocols were designed to achieve high sensitivity with high linearity of the reactions. Obtaining a reliable initial velocity in an enzymatic assay is of prime importance for obtaining a faithful measure of the enzymatic rate, especially when multiple samples are assayed at the same time. This parameter depends upon several factors, such as the fractional saturation of the enzyme with substrate, the buffering capacity of the medium, and product inhibition (Allison and Purich, 1979).

5. Conclusions

Different homogenization protocols and analytical conditions have a dramatic impact on the results of respiratory chain spectrophotometric assays used for the diagnosis of mitochondrial diseases and significantly affect their sensitivity and reliability. We presented protocols with optimized analytical performances that allow for the assay of electron transport complexes in mitochondria from muscle homogenates with a commercial single-wavelength spectrophotometer. Use of the detergent Tween 20 is critical to measure maximal specific activities for CIII. Surprisingly, many reagents, including adjuvants, detergents and inhibitors proposed by previous protocols were found to have negligible or even deleterious effects on the RC assays. The variability associated with the homogenization step of a hard tissue such as muscle, significantly exceeds that due to the spectrophotometric analysis when performed with our protocols, although it can be limited to acceptable levels with a strict standardization.

We believe that the criteria established here could provide both objective measures for quality assurance within diagnostic centers and a rational foundation for the standardization of the protocols between different centers.

Supplementary materials related to this article can be found online at doi:10.1016/j.mito.2011.07.006.

Acknowledgments

This work has been supported by a donation from the Stevanato Group SPA, in memory of its founder Giovanni Stevanato, from Telethon Italy grant GGP09207 and a grant from Fondazione Cariparo. We thank the Neuromuscular bank of Padova—Telethon Genetic Biobank Network (project no. GTB07001) supported by the Italian Telethon grants and Eurobiobank for providing us with human muscle specimens. This research is part of a project of the Telethon founded Italian Collaborative Network on Mitochondrial Disorders (GUP09004). There was no role of the funding source in the conduction of the study. We are grateful to Dr. D. Chretien, Dr. A. Lombès, Dr. M. Zeviani, Dr. AJM Janssen, Dr. E. Pegoraro, Dr. S. Di Mauro, and Dr. P. Bernardi for helpful discussion and to Mrs. L. Santinello for her assistance as librarian.

References

Allison, R.D., Purich, D.L., 1979. Practical considerations in the design of initial velocity enzyme rate assays. Methods Enzymol. 63, 3–22.
Barrientos, A., Fontanesi, F., Diaz, F., 2009. Evaluation of the mitochondrial respiratory chain and oxidative phosphorylation system using polarography and spectrophotometric enzyme assays. Curr. Protoc. Hum. Genet. Chapter 19, Unit 19.3, pp. 1–13.
Birch-Machin, M.A., Briggs, H.L., Saborido, A.A., Bindoff, L.A., Turnbull, D.M., 1994. An evaluation of the measurement of the activities of complexes I–IV in the respiratory chain of human skeletal muscle mitochondria. Biochem. Med. Metab Biol. 51, 35–42.
Blanchard, J.S., 1984. Buffers for enzymes. Methods Enzymol. 104, 404–414.
Bugiani, M., Invernizzi, F., Alberio, S., Briem, E., Lamantea, E., Carrara, F., Moroni, I., Farina, L., Spada, M., Donati, M.A., Uziel, G., Zeviani, M., 2004. Clinical and molecular findings in children with complex I deficiency. Biochim. Biophys. Acta 1659, 136–147.
Chance, B., 1957. Techniques for the assay of the respiratory enzymes. Methods Enzymol. 4, 273–329.
Chretien, D., Bourgeron, T., Rotig, A., Munnich, A., Rustin, P., 1990. The measurement of the rotenone-sensitive NADH cytochrome *c* reductase activity in mitochondria isolated from minute amount of human skeletal muscle. Biochem. Biophys. Res. Commun. 173, 26–33.
Chretien, D., Slama, A., Briere, J.J., Munnich, A., Rotig, A., Rustin, P., 2004. Revisiting pitfalls, problems and tentative solutions for assaying mitochondrial respiratory chain complex III in human samples. Curr. Med. Chem. 11, 233–239.
DiMauro, S., Schon, E.A., 2003. Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348, 2656–2668.
DiMauro, S., Servidei, S., Zeviani, M., DiRocco, M., DeVivo, D.C., DiDonato, S., Uziel, G., Berry, K., Hoganson, G., Johnsen, S.D., Johnson, P.C., 1987. Cytochrome *c* oxidase deficiency in Leigh syndrome. Ann. Neurol. 22, 498–506.
Estornell, E., Fato, R., Pallotti, F., Lenaz, G., 1993. Assay conditions for the mitochondrial NADH:coenzyme Q oxidoreductase. FEBS Lett. 332, 127–131.
Gellerich, F.N., Mayr, J.A., Reuter, S., Sperl, W., Zierz, S., 2004. The problem of interlab variation in methods for mitochondrial disease diagnosis: enzymatic measurement of respiratory chain complexes. Mitochondrion 4, 427–439.
Grace, M., Fletcher, L., Powers, S.K., Hughes, M., Coombes, J., 1996. A comparison of maximal bioenergetic enzyme activities obtained with commonly used homogenization techniques. J. Sports Med. Phys. Fitness 36, 281–286.
Hatefi, Y., Stiggall, D.L., 1978. Preparation and properties of succinate: ubiquinone oxidoreductase (complex II). Methods Enzymol. 53, 21–27.
Kirby, D.M., Thorburn, D.R., Turnbull, D.M., Taylor, R.W., 2007. Biochemical assays of respiratory chain complex activity. Methods Cell Biol. 80, 93–119.
Krahenbuhl, S., Talos, C., Wiesmann, U., Hoppel, C.L., 1994. Development and evaluation of a spectrophotometric assay for complex III in isolated mitochondria, tissues and fibroblasts from rats and humans. Clin. Chim. Acta 230, 177–187.
Long, J., Ma, J., Luo, C., Mo, X., Sun, L., Zang, W., Liu, J., 2009. Comparison of two methods for assaying complex I activity in mitochondria isolated from rat liver, brain and heart. Life Sci. 85, 276–280.
Luo, C., Long, J., Liu, J., 2008. An improved spectrophotometric method for a more specific and accurate assay of mitochondrial complex III activity. Clin. Chim. Acta 395, 38–41.
Max, S.R., Garbus, J., Wehman, H.J., 1972. Simple procedure for rapid isolation of functionally intact mitochondria from human and rat skeletal muscle. Anal. Biochem. 46, 576–584.
Medja, F., Allouche, S., Frachon, P., Jardel, C., Malgat, M., Mousson de, C.B., Slama, A., Lunardi, J., Mazat, J.P., Lombes, A., 2009. Development and implementation of standardized respiratory chain spectrophotometric assays for clinical diagnosis. Mitochondrion 9, 331–339.
Reisch, A.S., Elpeleg, O., 2007. Biochemical assays for mitochondrial activity: assays of TCA cycle enzymes and PDHc. Methods Cell Biol. 80, 199–222.
Rustin, P., Chretien, D., Bourgeron, T., Gerard, B., Rotig, A., Saudubray, J.M., Munnich, A., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35–51.
Salviati, G., Betto, R., Danieli, Betto D., 1982. Polymorphism of myofibrillar proteins of rabbit skeletal-muscle fibres. An electrophoretic study of single fibres. Biochem. J. 207, 261–272.
Salviati, L., Hernandez-Rosa, E., Walker, W.F., Sacconi, S., DiMauro, S., Schon, E.A., Davidson, M.M., 2002. Copper supplementation restores cytochrome *c* oxidase activity in cultured cells from patients with SCO2 mutations. Biochem. J. 363, 321–327.
Sarzi, E., Bourdon, A., Chretien, D., Zarhrate, M., Corcos, J., Slama, A., Cormier-Daire, V., de, L., P., Munnich, A., Rotig, A., 2007. Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. J. Pediatr. 150 (531–4), 534.
Schaefer, A.M., McFarland, R., Blakely, E.L., He, L., Whittaker, R.G., Taylor, R.W., Chinnery, P.F., Turnbull, D.M., 2008. Prevalence of mitochondrial DNA disease in adults. Ann. Neurol. 63, 35–39.
Sherratt, H.S., Watmough, N.J., Johnson, M.A., Turnbull, D.M., 1988. Methods for study of normal and abnormal skeletal muscle mitochondria. Methods Biochem. Anal. 33, 243–335.
Sinjorgo, K.M.C., Durak, I., Dekker, H.L., Edel, C.M., Bieleman, A.H.L., Back, N.B.T., Hakvoort, T.B.M., Muijsers, A.O., 1987. The effect of detergents on bovine cytochrome c oxidase: a kinetic approach. Biochim. Biophys. Acta 893, 241–250.
Taylor, R.W., Birch-Machin, M.A., Bartlett, K., Turnbull, D.M., 1993. Succinate-cytochrome c reductase: assessment of its value in the investigation of defects of the respiratory chain. Biochim. Biophys Acta. 1181, 261–265.
Tesarova, M., Mayr, J.A., Wenchich, L., Hansikova, H., Elleder, M., Blahova, K., Sperl, W., Zeman, J., 2004. Mitochondrial DNA depletion in Alpers syndrome. Neuropediatrics 35, 217–223.
Thorburn, D.R., Chow, C.W., Kirby, D.M., 2004. Respiratory chain enzyme analysis in muscle and liver. Mitochondrion 4, 363–375.
Trijbels, J.M., Scholte, H.R., Ruitenbeek, W., Sengers, R.C., Janssen, A.J., Busch, H.F., 1993. Problems with the biochemical diagnosis in mitochondrial encephalo-myopathies. Eur. J. Pediatr. 152, 178–184.
Trounce, I.A., Kim, Y.L., Jun, A.S., Wallace, D.C., 1996. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. Methods Enzymol. 264, 484–509.
Wibom, R., Hagenfeldt, L., von D., U., 2002. Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples. Anal. Biochem. 311, 139–151.
Zheng, X.X., Shoffner, J.M., Voljavec, A.S., Wallace, D.C., 1990. Evaluation of procedures for assaying oxidative phosphorylation enzyme activities in mitochondrial myopathy muscle biopsies. Biochim. Biophys. Acta 1019, 1–10.