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OPTIMIZED PROTOCOLS FOR THE ANALYSIS OF MITOCHONDRIAL RESPIRATORY CHAIN ENZYMES IN CELLS AND TISSUES

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1.Riassunto dell' attività svolta

I mitocondri sono organelli cellulari fondamentali con diverse funzioni critiche per la vita e la morte cellulare: la produzione di energia attraverso la catena respiratoria, la regolazione dell'apoptosi, il metabolismo del calcio, e la produzione di radicali dell'ossigeno.

La disfunzione mitocondriale è un meccanismo patogenetico ricorrente alla base di una serie di condizioni, fisiologiche e patologiche, tra cui le malattie mitocondriali, il diabete, il cancro, varie malattie neurodegenerative, e vari fenomeni di tossicità da farmaci oppure veleni. Pertanto, l'utilizzo di metodi affidabili per lo studio delle attività enzimatiche mitocondriali è un fattore critico per studiare il ruolo dei mitocondri in tali patologie. Tuttavia, recenti osservazioni hanno evidenziato la presenza di diversi problemi analitici significativi derivanti dai protocolli precedentemente pubblicati in letteratura, tali da compromettere in alcuni casi la consistenza dei risultati. Tali problemi riguardano sia la fase pre-analitica, ovvero la fase di preparazione del campione biologico, che la parte analitica vera e propria spettrofotometrica, impattando negativamente la precisione dei saggi, la specificità e la linearità delle cinetiche. I problemi analitici più significativi riportati riguardano in particolare i saggi per il complesso I, III, e CIV, che sono anche gli enzimi più comunemente deficitari nelle malattie mitocondriali.

Per sopperire a tali lacune, abbiamo sviluppato dei protocolli analitici ottimizzati per una analisi affidabile delle attività enzimatiche della catena respiratoria (complessi I-IV, I+III, II+III) attraverso un'analisi sistematica dei singoli steps analitici e pre-analitici. Per la fase di sviluppo dei protocolli abbiamo analizzato omogenati muscolari bovini con l'impiego di uno spettrofotometro a singola lunghezza d'onda.

Sia l'impiego di un'efficace protocollo di lisi del campione che la scelta specifica di ciascun reagente (buffer, substrato, adiuvanti e detergenti) in un range ristretto di concentrazioni, sono risultati fattori in grado di influenzare enormemente la sensibilità, la specificità analitica e la linearità delle cinetiche. Sulla base dei risultati ottenuti abbiamo quindi selezionato i protocolli che risultassero in profili ottimali in termini analitici, permettendo di superare gran parte dei problemi analitici riportati in particolare a livello dei complessi I, III, IV.

Abbiamo quindi effettuato, per la prima volta in letteratura, una analisi delle prestazioni analitiche di questi saggi biochimici, in termini di precisione, specificità rispetto all'aggiunta degli inibitori, e linearità delle cinetiche nel tempo attraverso la creazione di un indice di linearità, e la proporzionalità delle attività enzimatiche rispetto alla concentrazione di proteine.

In una seconda fase abbiamo validato l'impiego di questi protocolli per campioni muscolari umani, e successivamente anche per colture cellulari, altre specie animali e organismi (topo, lievito, *C. elegans*), e vari tessuti murini (fegato, cuore, cervello).

Questi protocolli permettono un' analisi affidabile delle attività enzimatiche della catena respiratoria mitocondriale con l'uso di uno spettrofotometro a singola lunghezza d'onda, e non richiedono l'impiego di mitocondri isolati da tessuti.

I protocolli implementati sono adatti per la diagnosi biochimica delle malattie mitocondriali, come per ricerche focalizzate sulla funzione mitocondriale in processi fisiologici o patologici. Questo lavoro fungerà da riferimento per futuri controlli di qualità e processi di standardizzazione nell'ambito della diagnostica specializzata in malattie mitocondriali.

2. Summary

Mitochondria are fundamental cellular organelles which decide about life and death of the cells. They are critically involved in a number of essential functions, including the production of energy through the respiratory chain enzymes, regulation of apoptosis, calcium metabolism and production of reactive oxygen species. Dysfunction of mitochondrial metabolism has been recognized a key player in a number of conditions, including mitochondrial disorders, diabetes, cancer, ageing, neurodegeneration and intoxications from drugs and poisons. Therefore, a reliable assessment of mitochondrial respiratory chain enzymatic activities is essential to investigate mitochondrial function in these disorders. However, previously published protocols were found to be inefficient due to sub-optimal tissue disruption, and results are inconsistent because of biochemical interferences leading to enzymatic inhibition, low specificity or low linearity of the kinetics, especially in the assays for complex I, III, IV.

In response to these methodological issues, we systematically develop optimized protocols for a reliable assessment of the RC enzymatic function (complex I-IV, I+III, II+III) in bovine muscle homogenates using a single-wavelength spectrophotometer. Most of the analytical pitfalls rgarding complex I, III, IV could be overcome or at least limited.

We analyzed, for the first time to our knowledge, the analytical performances of these assays, including the precision, specificity to the enzyme inhibitors, and linearity with time and protein concentrations. Next we validated these protocols to human muscle tissue and extend their applications to cultured cells, a variety of different species (mice, yeasts and *C. elegans*) and mouse tissues (i.e. liver, heart, brain). An efficient tissue disruption and the choice for each assay of specific buffers, substrates, adjuvants and detergents in a narrow concentration range, allow maximal sensitivity, specificity and linearity of the kinetics.

These protocols allow for a reliable analysis of mitochondrial respiratory chain enzymatic activities with the single-wavelength spectrophotometer, and do not require isolation of mitochondria from tissues.

These protocols are suitable for the biochemical diagnosis of mitochondrial disorders, as well as for research applications regarding mitochondrial dysfunction in health and disease. This work will be a useful reference for quality control surveys in laboratories specialized in the diagnosis of mitochondrial disorders.

3. Introduction

Mitochondria are fundamental cellular organelles involved in a variety of critical cellular reactions including the production of energy through the mitochondrial respiratory chain (RC), the regulation of cell death, calcium metabolism, and production of reactive oxygen species. The RC is comprised of four enzymatic complexes (complex I, II, III, IV) embedded in the inner mitochondrial membrane, which catalyze the transfer of reducing equivalents from high energy compounds produced by the reactions of the Krebs cycle to oxygen with the ultimate production of an electrochemical gradient through the inner mitochondrial membranes to drive the synthesis of ATP by ATP synthase.

Dysfunction of the mitochondrial RC is a key player in a variety of human disorders, including mitochondrial diseases,[1] as well as in more common conditions, such as ageing,[2] diabetes,[3] cancer,[4, 5] drug toxicity,[6,7] and several neurodegenerative diseases,[8] including Parkinson's disease,[9] and Alzheimer's disease.[10]

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.[1] Altogether, mitochondrial disorders are relatively common, with an estimated prevalence of 9.18 cases per 100,000 adults.[11] 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.

The enzymatic activities of RC complexes I-IV are assayed spectrophotometrically, and the results are commonly normalized to the total muscle protein content or to the activity of citrate synthase,[12] a mitochondrial matrix enzyme. Although a potential limitation of these assays is that the enzymatic activities are measured using *in vitro* conditions that are not physiological in terms of pH, osmolarity, substrate concentrations and cellular context and they do not allow for the evaluation of respiratory coupling, however, they still provide critical quantitative information concerning the maximal catalytic activities of the RC complexes, they are easy to reproduce and can be performed using frozen tissue and cellular samples. An ideal investigation of the mitochondrial respiratory function should also include a combination of other assays, such as polarographical measurements of oxygen consumption in intact isolated mitochondria[13] or permeabilized cells or tissues,[14] ATP synthesis,[15] oxidation studies with radiolabeled substrates,[16] and the determination of mitochondrial membrane potential,[17] which may provide a more sensitive detection of mitochondrial dysfunction.

However, these techniques have the disadvantage of requiring fresh samples with intact mitochondrial membranes, they are more complex and time-consuming, and require specific apparatuses that are not commonly found in all laboratories. Therefore, spectrophotometric assays remain a first-line technique for mitochondrial disease diagnostics and also for research applications.

Despite the importance of biochemical measurements, there is still no consensus on the optimal conditions for these assays, nor a quality assurance scheme.[18] Skeletal muscle is considered the most suitable tissue for the diagnosis of these disorders due to its availability and high metabolic rate. 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[19-23] 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. [24] 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. [25] However, the performances of some assays (CI, CIII, CI+III, CII+III, CIV) was still unsatisfactory due to unreliability, low specificity, or 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 preferable to single-wavelength spectrophotometry for mitochondrial RC assays [24] no comparative study was performed. Finally, there were no or limited informations about the utility of using isolated mitochondria or homogenates nor about the impact of freezing of the samples on RC enzymes.

The aims of our study were: *a)* to measure the impact of different conditions on RC analysis of bovine muscle homogenates, both at the homogenization phase, and at the analytical phase, in order to develop simple and reliable protocols for the analysis of RC enzyme activities *b)* to overcome the recognized analytical pitfalls in the assays for complex I, III, and IV *c)* to measure the analytical performances of the developed protocols *d)* to compare the results of the RC analysis with a single- vs double-wavelength spectrophotometry *e)* to validate these protocols on human muscles, cultured cells, and isolated mitochondria from a variety of species *g)* to compare the analytical yield of the RC analysis on muscle homogenates vs muscle isolated mitochondria *h)* to measure the impact of freezing in homogenates from different mouse organs.

4. Material and methods

4.1 Sample preparation

4.1.1 .Skeletal muscle homogenates

In order to have a sufficient quantity of the same muscle tissue and to spare limited human samples, we initially 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 homogenization buffer of the specified composition. Muscle tissue was homogenized in a glass-glass motor-driven 1ml-tapered conical tissue grinder (Wheaton Science products) held in an ice bath, with a specified number of strokes and rotational speed. The compliance between pestle and mortar was always verified very carefully before use. The homogenate was then centrifuged at 600 × g for 10 minutes at 4°C to remove nuclei and insoluble cellular debris sedimented in the pellet. The supernatant was kept on ice for the assays.

4.1.2 Muscle 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 Table 1.

Buffer type	Composition	Reference
CCA Buffer	Tris-HCl 20 mM, sucrose 250 mM, KCl 40 mM, EGTA 2 mM; pH 7.4	[24]
CCA+Heparin	Tris-HCl 20 mM, sucrose 250 mM, KCl 40 mM, EGTA 2 mM, heparin 50000 U/L; pH 7.4	[26]
CCA+BSA	Tris-HCl 20 mM, sucrose 250 mM, KCl 40 mM, EGTA 2 mM, BSA 1 mg/ml; pH 7.4	[20]
Mannitol	Tris-HCl 10 mM, mannitol 225 mM, sucrose 75 mM, EDTA 0.1 mM; PH 7.4	[25]
KCl	Tris-HCl 50 mM, KCl 150 mM	[27]
Chappel-Perry (ChP)	Tris-HCl 50 mM, KCl 100 mM, $MgSO_4$ 5 mM, EDTA 1 mM, ATP 1 mM	[28]
KP	Potassium phosphate 20 mM, pH 7.4	[29]

Table 1 Composition of the different homogenization buffers
CCA: sucrose buffer; KCl: potassium chloride buffer; KP: potassium phosphate buffer; Tris: Tris buffer; BSA: defatted bovine serum albumin; ChP: Chappel-Perry; $MgSO_4$: magnesium sulphate.

4.1.3 Preparation of total human fibroblast lysates

Two or three days before performing the experiments, 5 x 10^6 fibroblasts were plated on 150 cm^2 dishes and grown until 80%-90% confluent. The cell culture medium was removed and the cells were washed once with PBS. Cells were detached using 0,05% trypsin EDTA, and after cell detachment trypsin was blocked by adding the removed medium. The cell suspension was transfered in a 15 ml Falcon tube. Cells were centrifuged at 1000 *g* for 5 minutes at 4 °C and the surnatant was discarded. Resuspension of the cells in PBS and centrifugation at 1000 g were repeated 3 times. The fibroblast pellet was suspended in 0.4 ml hypotonic potassium phosphate buffer 20 mM, pH 7.5 and processed as specified. Typically the cells were resuspended with a 50 µl Hamilton syringe several times until dissolution, and subject to 3 cycles of freeze-thawing in liquid nitrogen.
The fibroblast lysate was keep in ice for the RC analysis.

4.1.4. Homogenates from mouse brain and liver

The tissues were immediately removed with a scalpel after sacrifice of the mice by cervical dislocation according to the national regulations. The tissues were washed with ice-cold sucrose homogenization buffer, and the gallbladder was removed from the liver. The organs were dissected in small pieces with a scalpel, diluted in 9 volumes of ice-cold sucrose homogenization buffer, and homogenized with a teflon-glass tissue grinder (Kartell Labware Division) held in an ice bath with

15 up-down strokes at 500 rpm. The homogenate was then centrifuged at 600 × *g* for 10 minutes at 4°C to remove nuclei and insoluble cellular debris sedimented in the pellet. The supernatant was kept on ice for the assays.

4.1.5. Isolation of mitochondria from mouse liver, muscle, brain, from yeast, and C. elegans

Mitochondria from mouse liver, brain, and muscle were isolated according to Frezza et al.[30] Mitochondria from human fibroblasts were isolated according Janssen et al. [31] starting from 10-15 x 10^6 human fibroblasts. Mitochondria from yeast were isolated according Grad et al,[32] while isolation of mitochondria from C. *elegans* was performed according to Rowley et al.[33] After isolation the mitochondrial pellet was resuspended in ice-cold hypotonic Tris buffer 10 mM, pH 7.6 and subject to three cycles of freeze-thawing in liquid nitrogen to disrupt the mitochondrial membranes just before use.

4.2. 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 minutes and separated on SDS-PAGE (4-12% NuPAGE bis-tris polyacrylamide denaturing gel, Invitrogen). Gels were stained with Coomassie Brilliant Blue. 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 hour 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: 10000 for 1 hour, or IgG goat anti-rabbit 1:5000, and then washed three times. The detection procedure was according to instructions from the manufacturers (GE Healthcare).

4.3 Development of optimized enzyme assays

All assays were performed using a Beckman Coulter DU-800 or a Varian Cary UV 100 spectrophotometer at 37°C in a final volume of 1 ml. Citrate synthase was assayed as previously described.[21]

Development of RC enzyme assays was performed using bovine muscle homogenates prepared as described.

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 (ε= 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 ubiquinone1 or decylubiquinone, and 5) different concentrations of the adjuvant bovine serum albumin (BSA). The reaction was started by the addition of the electron acceptor (ubiquinone1 or decylubiquinone) and the decrease in absorbance was followed for 3 minutes. 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 (ε= 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,[34] 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 minutes. 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 (ε= 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 minutes. Specific complex III activity was calculated by subtracting enzymatic rates measured in separate reactions with antimycin A (10 μg/ml). Decylubiquinol was prepared immediately before use as previously described [21] 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 2.0-3.0.

Complex IV (cytochrome c oxidase; EC 1.9.3.1) activity was measured by following the oxidation of reduced cytochrome c at 550 nm (ε= 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 minutes. Reduced cytochrome c was prepared immediately before use by adding a few grains of sodium dithionite [35] or was prepared before hand and stocked at -80°C after reduction with ascorbate and separation by Sephadex G25 chromatography.[36] 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 [25] with minor modifications. Muscle homogenate containing 30 µg muscle protein was preincubated in distilled water to induce osmotic shock[37] 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 minutes. 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 (ε= 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.[20,25,38] The reaction was started by the addition of cytochrome c and the increase in absorbance was followed for 3 minutes. 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).

4.4 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 using bovine muscle homogenates. 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 ± 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 seconds and the activity measured between 120-180 seconds (n=5 ± 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 then 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.

4.5 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 $p<0.05$.

5. Results [39]

5.1 The quality of tissue grinders may affect homogenization

In order to minimize the variability due to differences in the shape of the homogenizers, the compliance between pestle and vial of different tissue grinders (glass/glass and Teflon/glass) from different companies was carefully examined before use. Surprisingly, a large proportion of tissue grinders, either glass-glass or teflon-glass were found to have a very poor compliance between pestles and potters, especially at the bottom (**Fig. 1**), at difference with the technical specification provided by the producing companies (Wheaton Scientific and Kimble & Kontes). Defective tissue grinders were discarded from further analysis. Moreover, differently from what reported by the companies, couples of pestles and potters were not always interchangeable, due to small differences in their shape, and careful coupling was necessary before use.

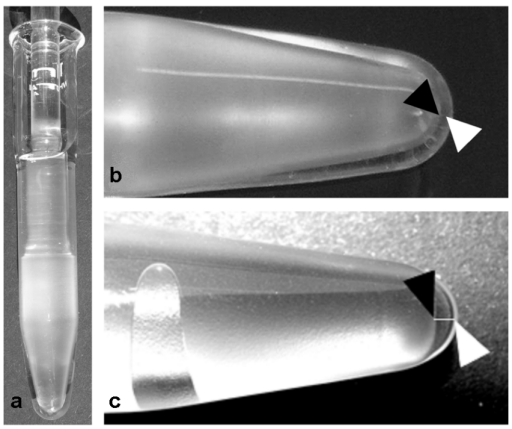

Fig. 1 Potter tissue grinder for muscle homogenization
(**A**) Glass-glass conical tissue grinder for muscle homogenization. The conical shape of the grinder improves the yield of the muscle homogenization. (**B**) Good compliance between mortar and pestle. There is no gap between the tip of the pestle and the bottom of the vial. (**C**) Poor compliance between pestle and mortar. The tip of the pestle cannot touch the bottom of the vial, which can lead to potential inconsistency in the homogenization efficacy.

5.2 Optimization of muscle homogenization protocol for RC assays

5.2.1 The composition of homogenization buffer affects the quantity and quality of supernatant's proteins

We analysed 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, see Table 1) resulted in higher total muscle protein levels compared to either sugar-based buffers and low-

osmolarity potassium phosphate buffer (**Fig. 2A**; $p < 0.0001$). This difference was explained by solubilisation of myosin in the muscle supernatants induced by ChP and KCl buffers. As seen in figure 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 were found in the pellets after centrifugation (**Fig. 2B**) indicating that a portion of RC enzymes are 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 (**Fig. 3**), 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. 2B**), whereas other buffers allowed virtually complete solubilisation of this protein.

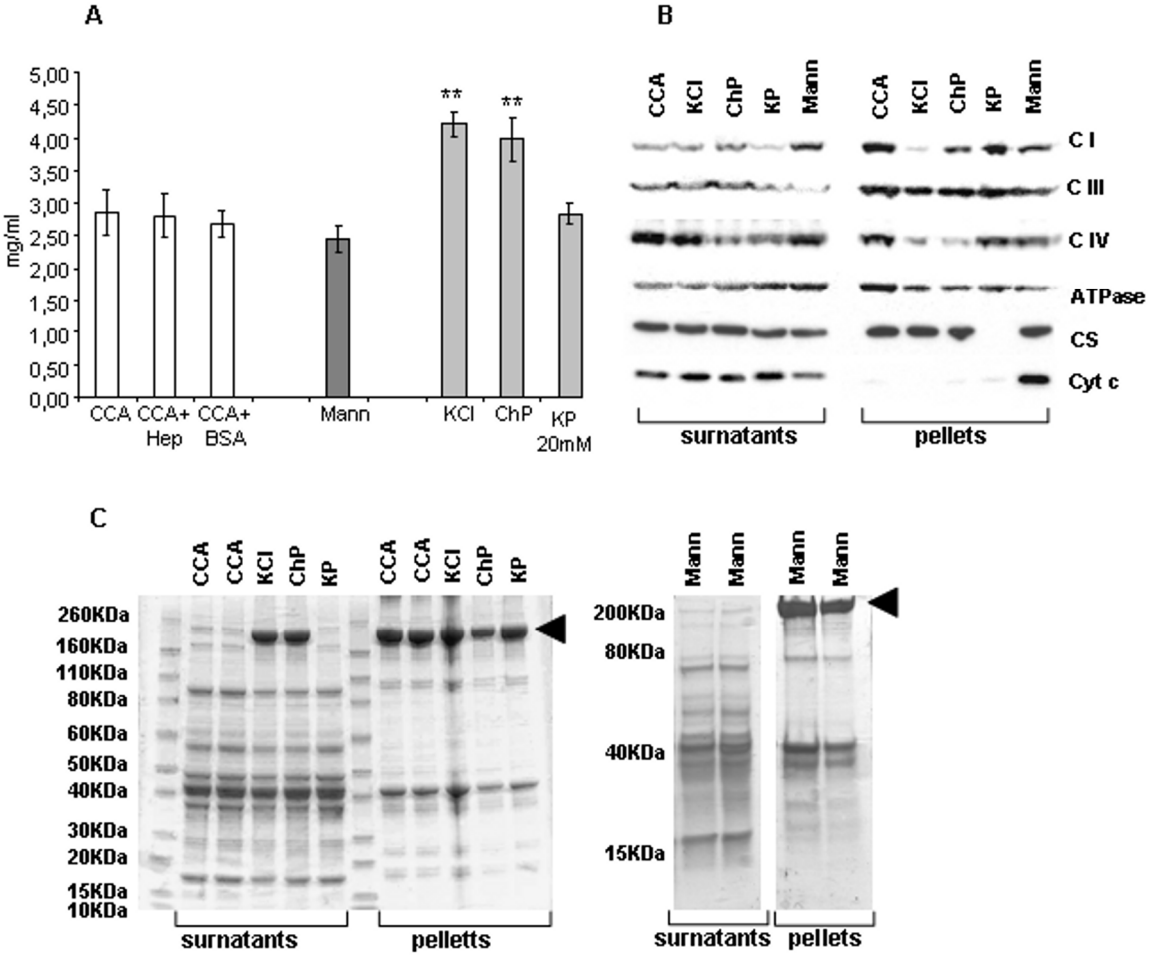

Fig. 2 Effect of different homogenization buffers on muscle homogenate protein quantity and composition. (**A**) Influence of different homogenization buffers on the total muscle protein quantity of muscle supernatants. Values are means ± SD of at least 6 measurements in at least 2 independent experiments. 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 600g. (**C**) Representative PAGE of muscle supernatants and pellets obtained after homogenization in specific buffers followed by centrifugation at 600g. Gels were stained with Coomassie blue. The arrowheads indicate the bands corresponding to myosin (molecular weight: 220 KDa).

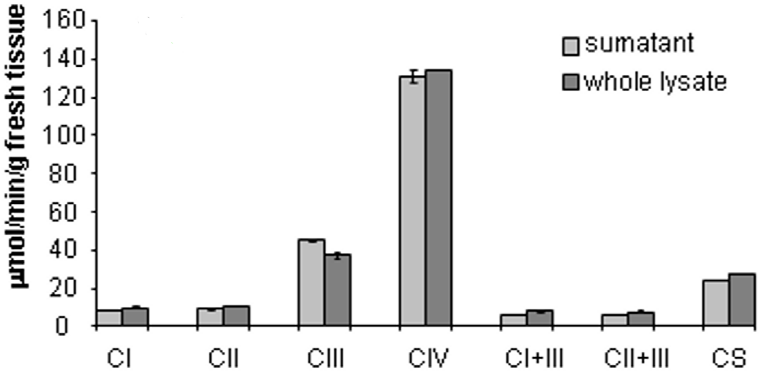

Fig. 3 Effect of the centrifugation passage (600 *g* for 10 minutes) on the RC enzymatic activities of the muscular extracts. Activities, expressed as µmol/min/g fresh tissue to overcome differences in total muscular protein content, are calculated on the same muscular extract assayed before (whole lysate), and after centrifugation (surnatant).

5.2.2 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/minutes/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 2**.

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 complex I, II, IV ($p < 0.05$ compared to all other buffers; **Fig. 4A-B, D-E**). The use of ChP buffer resulted in significantly higher CIV activities ($p< 0.01$ compared to all other buffers; **Fig. 4D**). 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. 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.

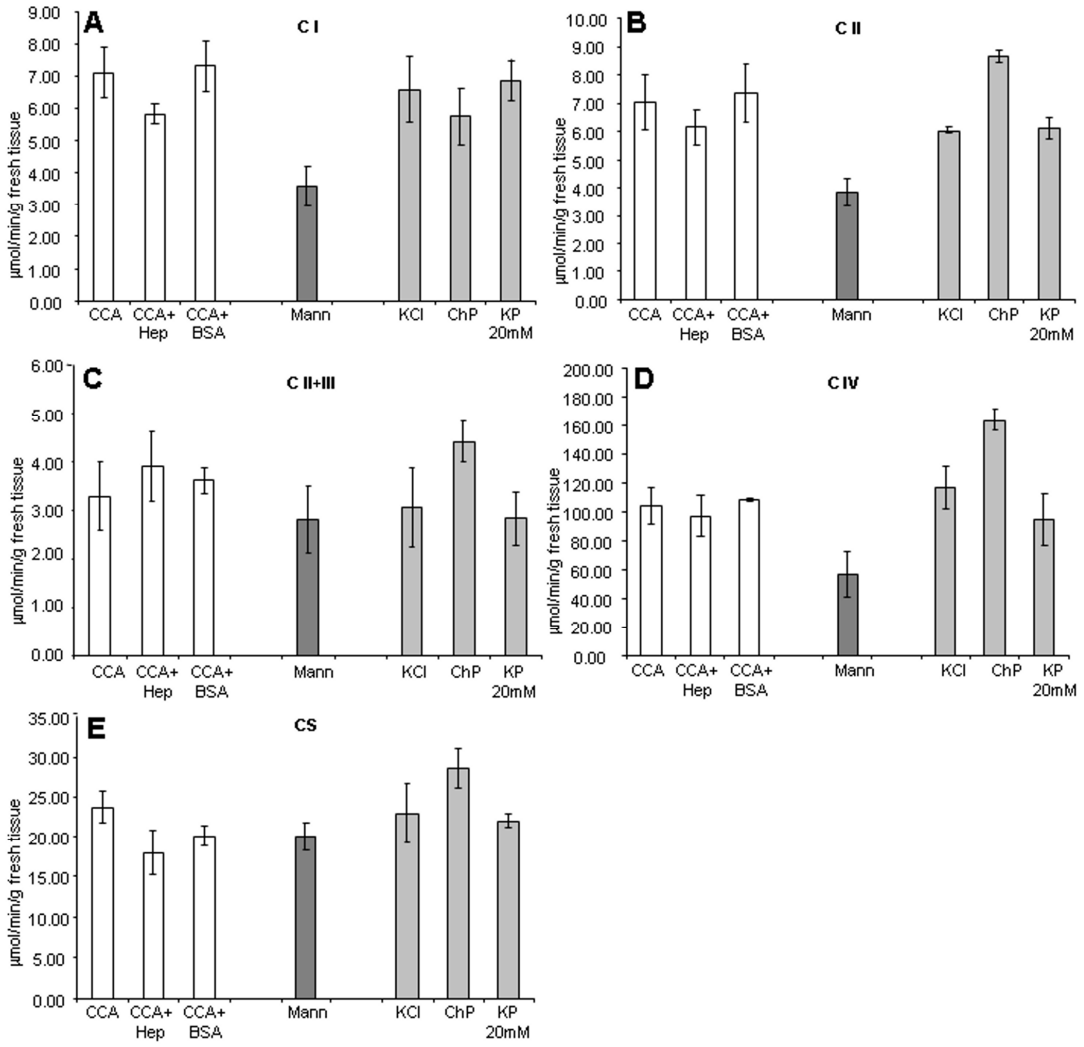

Fig. 4 Effect of different homogenization buffers on respiratory chain enzyme and citrate synthase activities, expressed as µmol/min/g fresh tissue. Values are means ± SD of at least 6 measurements in at least 2 independent experiments. (**A**) Complex I activities. (**B**) Complex II activities. (**C**) Complex II+III activities. (**D**) Complex IV activities. (**E**) Citrate synthase activities.

5.2.3 Effect of different muscle 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; **Fig. 5A-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 **Fig. 5D**). Interestingly, total protein levels in homogenates were essentially constant for all the condition tested (Rho= 0.21, p=0.42; **Fig. 5E**).

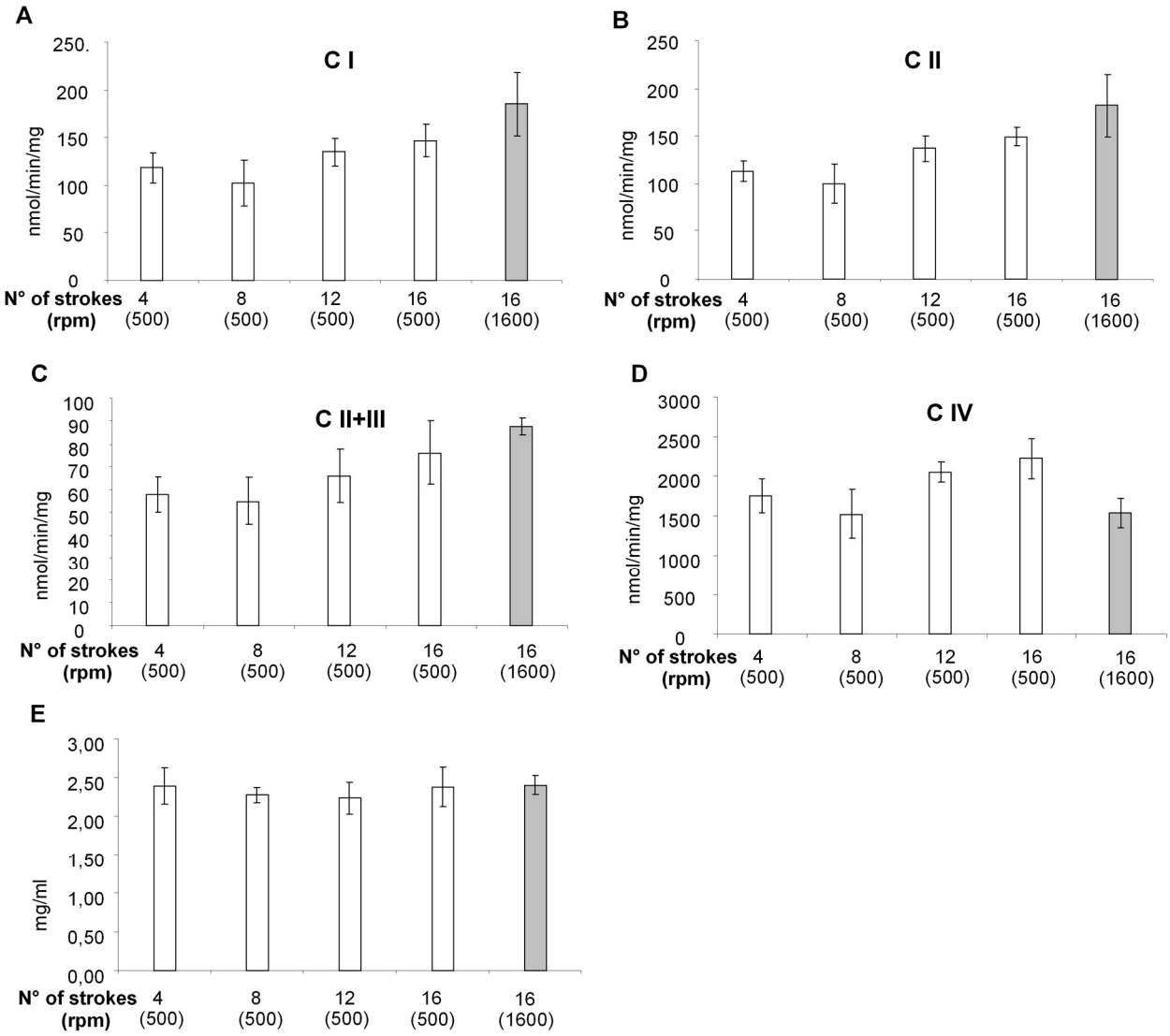

Fig. 5 Effect of different number of strokes at moderate pestle rotational speed (500 rpm) and high rotation speed (1600 rpm) during muscle homogenization on respiratory chain enzyme activities expressed as nmol/min/mg proteins and on total muscle proteins of the supernatants. (**A**) Complex I activities. (**B**) Complex II activities. (**C**) Complex II+III activities. (**D**) Complex IV activities. (**E**) Total protein quantification of the supernatants.

5.3 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 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.

Complex I (CI)

Published protocols propose the use of different pHs and concentrations of Tris-HCl or potassium phosphate (KP) in the CI assay.[20, 24, 40] We measured significantly higher enzymatic activities in potassium phosphate than in Tris-HCl at all concentrations tested (**Fig. 6A**), with optimal values between 50-250 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, ubiquinone1 or decylubiquinone. In accordance with previous data,[41] we confirmed that ubiquinone1 was preferable to decylubiquinone because decylubiquinone, despite higher initial rates, yielded invariably non-linear reactions (**Fig. 6B**) indicated by a low linearity index compared to ubiquinone1 (linearity index: decylubiquinone 0.33 ± 0.05 vs ubiquinone1 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. Ubiquinone1 concentration was saturating at 50 μM (**Fig. 6C**), while concentrations above 100 μM 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[24, 42] We found that BSA, at an optimal concentration between 3-5 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. 6D**). Repeated freeze-thaw cycles in hypotonic buffer (25 mM potassium phosphate, pH 7.4)[43] or the use of the detergent saponin[44] were reported to increase CI activity in isolated mitochondria by disrupting mitochondrial membranes. This did not apply to muscle homogenates from previously frozen muscle (data not shown), while the use of lauryl maltoside, a detergent used by some laboratories [24], dramatically inhibited complex I activity (**Fig. 6E**).

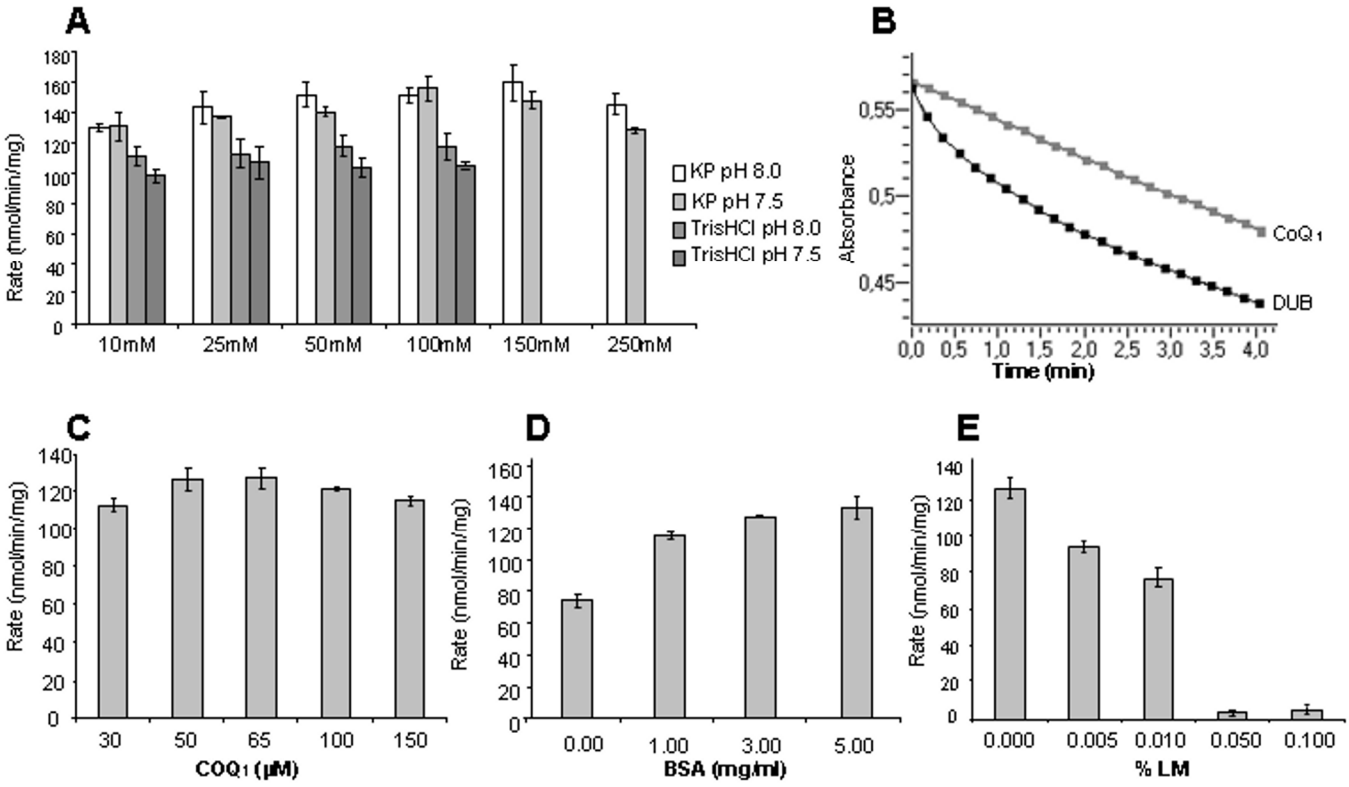

Fig. 6 Effect of different biochemical parameters on Complex I specific activity, expressed in nmol/min/mg of protein from skeletal muscle homogenate. For each assay, about 30 µg of muscle proteins was used. Specific CI activities (represented in the graph bars) were calculated after addition of rotenone 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 ubiquinone1. (**B**) Representative CI kinetics in the same muscle homogenate using the two different acceptors, ubiquinone1 (65 µM) and decylubiquinone (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 ubiquinone1. (**D**) Effect of different BSA concentrations on CI specific activity. (**E**) Effect of the detergent lauryl maltoside at different concentrations.

Complex II (CII)

In agreement with previous observations,[43] preincubation of the sample with succinate for ten minutes was mandatory to fully activate CII, and the optimal concentration of succinate was 20 mM (**Fig. 7A-B**). The use of phenazine methosulfate 1.5 mM or the ubiquinone analogue 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 (**Fig. 7C**). 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), 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 analogue, 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.

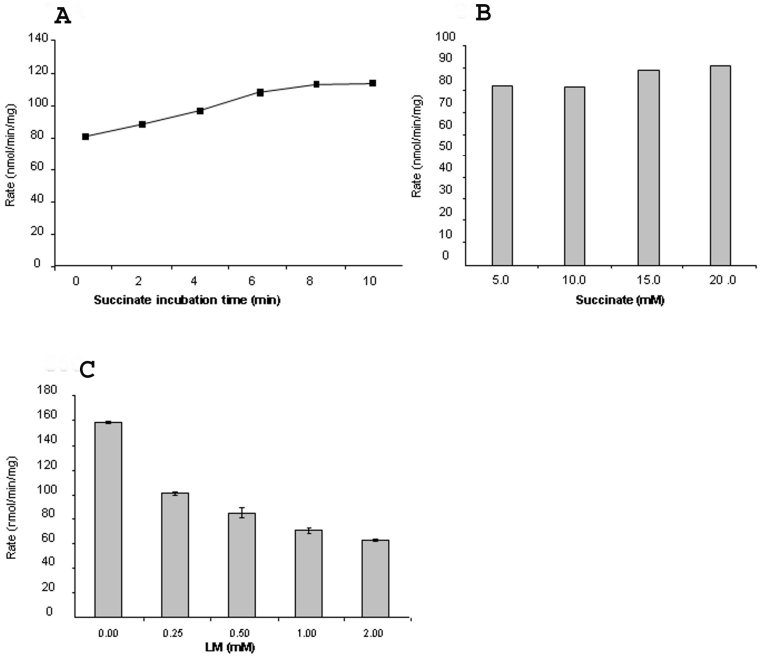

Fig. 7 Effect of succinate at different succinate incubation times (**A,** succinate 20 mM) and concentrations (**B**) on complex II activity. Values are means of 2 measurements. (**C**) Effect of the detergent lauryl maltoside on specific Complex II activity (in nmol/min/mg of skeletal muscle protein). Values are means ± SD of 3 measurements.

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; $p<0.05$, **Fig. 8A**). Addition of the complex I inhibitor rotenone, commonly used in several protocols, had no effect (**Fig. 8B**). Combined addition of 1 mg/ml BSA and 100 µM ATP[20, 25] resulted in a marginal increase in complex II+III activity (**Fig. 8C**). The addition of the adjuvant magnesium chloride, proposed by some protocols[22] 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%, $p< 0.0008$).

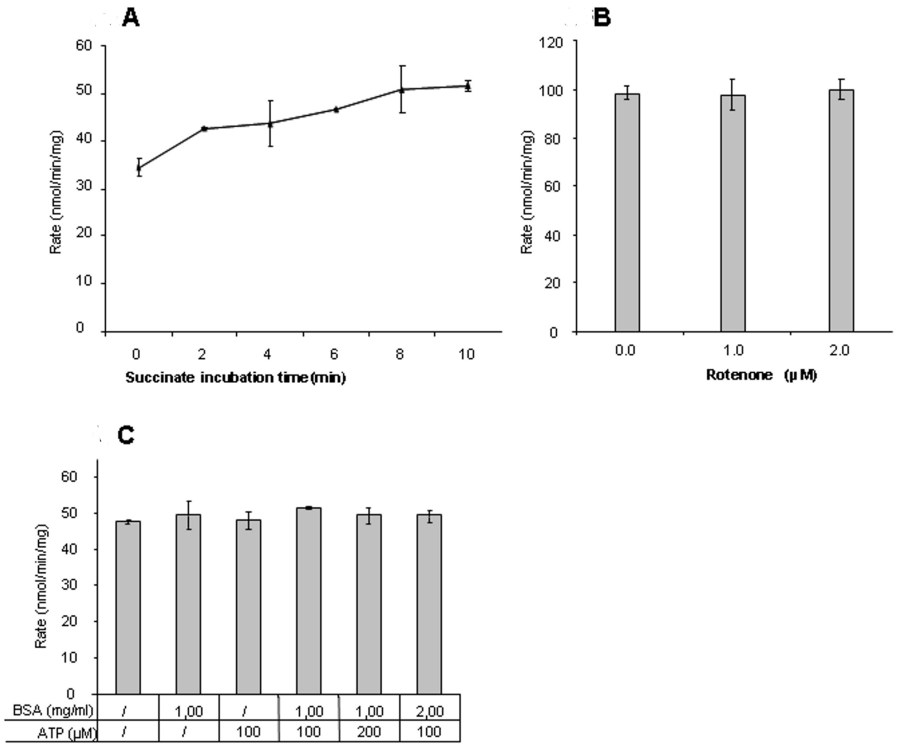

Fig. 8 Effect of different substrate concentrations, incubation times, inhibitors, and adjuvants on complex II+III activity. Values are means ± SD of 3 measurements. (**A**) Effect of different incubation times with succinate (10 mM). (**B**) Effect of the addition of the complex-I inhibitor rotenone at different concentrations. (**C**) Effect of the addition of 2 adjuvants, ATP and BSA.

Complex III (CIII)

The optimal buffer to assess CIII activity is still debated.[19, 24, 45] 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 25-50 mM (**Fig. 9A**). Both lauryl maltoside and Tween 20 have been employed in this assay,[43, 45] but a comparative analysis is not available. Only Tween 20, at an optimal concentration between 0.025-0.050% increased CIII 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. 9B**) and dramatically improved linearity (linearity index: no Tween: 0.54 ± 0.02 vs. Tween 0.025% 0.82 ± 0.03, n=3, $p<0.0005$), while lauryl maltoside had a detrimental effect on both CIII specific activity (**Fig. 9B**) 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. 9C**) and 75 µM (**Fig. 9D**), 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. 9E**). The addition of the adjuvant

BSA, commonly included in CIII protocols,[20, 24, 29] increased only background activity (**Fig. 9F**), and we therefore suggest that it should be omitted from the assay. Analytical interferences in CIII assay from contaminating cytochrome c prepared using trichloroacetic acid[46] had been previously reported. We confirmed that the use of cytochrome c produced using trichloroacetic acid resulted in higher CIII activities than using acetic acid-extracted cytochrome c (both specific and aspecific activities, **Fig. 10**). As previously reported, this effect was not explained by an aspecific reduction of cytochrome c through generation of superoxide anion during the assay, as the addition of purified superoxide dismutase 1 did not restore the enzymatic activities to the levels obtained by using acetic-acid-extracted cytochrome c.

However, this differences did not cause any problem if the protocol included the calculation of residual antimycin A-residual activities and the same batch of cytochrome c was always used for comparing different samples.

Based on these results, we compared the activities obtained from different published CIII protocols [19, 25, 29, 45] with our proposed method described in Table 2. Our protocol yielded higher specific activity (**Fig. 9G**), could be employed over a wide range of protein concentrations (**Fig. 13**), had good precision, a satisfactory percent inhibition by antimycin A, and maintained greater linearity over time (**Table 5**).

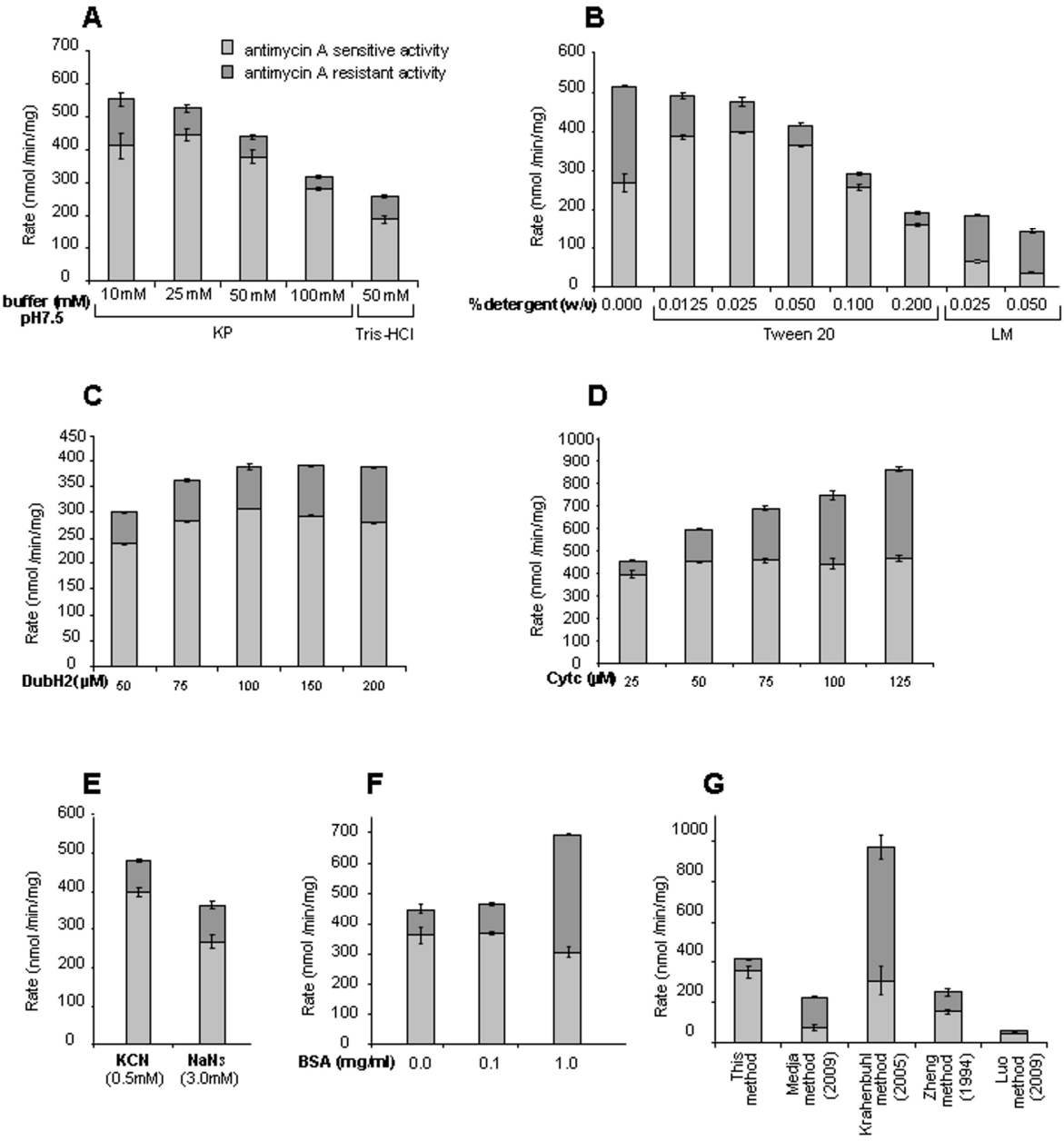

Fig. 9 Effect of different biochemical conditions on Complex III activity. Specific CIII activity (the antimicyn A sensitive activity, in light gray bars) was calculated by subtracting total enzymatic rate (dA/dt) from the residual spectrophotometric signal in antimycin A (antimycin A resistant activity, 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 (Medja et al., 2004; Krahenbull et al., 1994; Zheng et al., 1990; Luo et al., 2008).

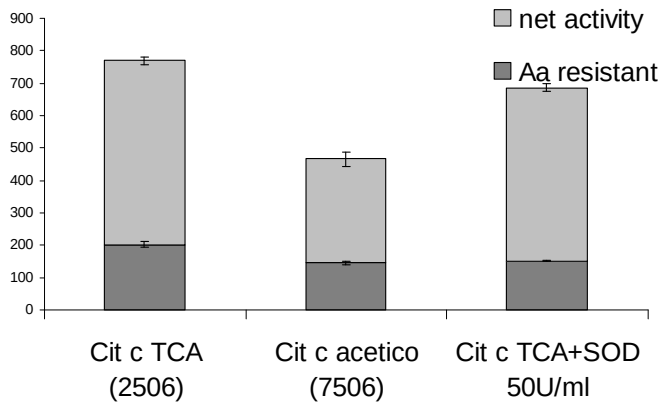

Fig. 10 Effect of different cytochrome c preparations on CIII assay. Use of cytochrome c extracted with trichloroacetic acid (Sigma 2506) results in higher specific and aspecific CIII activities than cytochrome c extracted with acetic acid (Sigma 7506). CIII activities in a separate reaction with addition of the purified enzyme superoxide dismutase (SOD, 50 U/ml) is presented on the right.

Complex IV (CIV)

Most of the protocols for CIV assay employ the buffer potassium phosphate, but its concentration and pH vary.[20, 24] 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. 11A**). 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. 11B**). Different protocols also employ variable concentrations of the substrate reduced cytochrome c. [20, 22, 24] We found that the optimal concentration was between 50 and 75 μM, while higher concentrations resulted in severe enzymatic inhibition (**Fig. 11C**). 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. 11C**). We next investigated the effects of four detergents, lauryl maltoside,[22] Tween 20,[24] Tween 80 and Triton X on CIV activity. Although lauryl maltoside, at a concentration between 0.025-0.050% increased CIV-specific activity (**Fig. 11D**), 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. 11E**). 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 [20, 24] had no significant effect on activity or kinetics (data not shown).

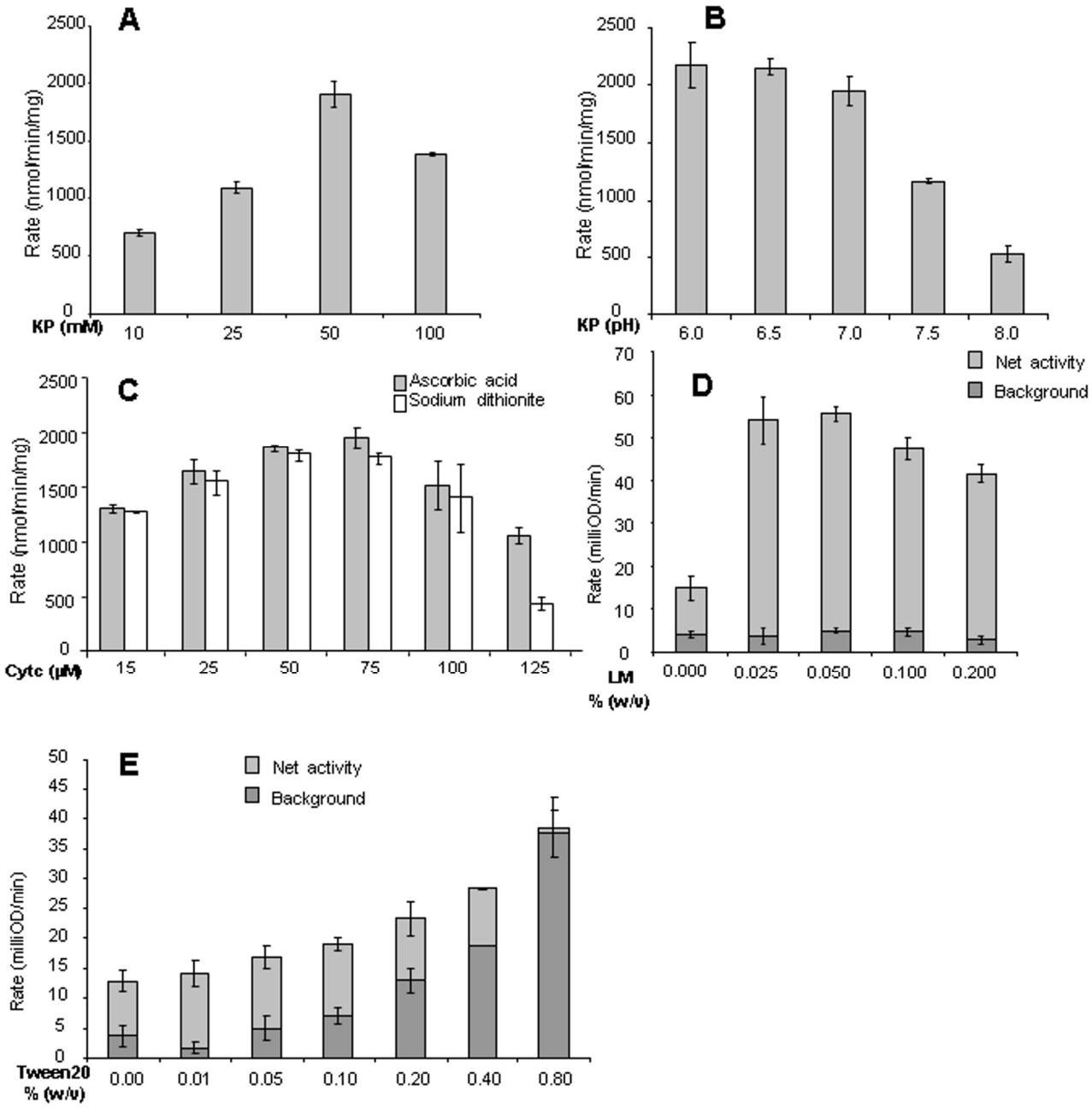

Fig. 11 Effect of different biochemical parameters on Complex IV activity, expressed as nmol/min/mg proteins or as milli optical density/min as specified. For each assay, 1.5 µg of muscle protein was used. Values are means ± SD of 3 measurements. (**A**) Effect of the concentrations of potassium phosphate 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.

5.4 Single-wavelength vs double-wavelength spectrophotometry

Based on previous results, we selected the optimal analytical conditions for the spectrophotometric assays illustrated in **Table 2**. Results of RC analysis performed with our protocols were comparable using single or double-wavelength spectrophotometry (**Table 3**), 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 Figure 12.

	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% *	-		Triton X 0.1%
Specific Inhibitor	Rotenone 10 µM	Malonate 10 mM TTFA 500 µM	Antimycin A 10 µg/ml	KCN 300 µM	Rotenone 10 µM	Malonate 10 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 240 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

Table 2 Conditions for spectrophotometric assays of respiratory chain enzymes and citrate synthase activities.

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). * Omitted when assaying human muscle tissues, in order to increase the linearity of the kinetics with time and muscle protein concentrations (range 1.5-24 µg).

	SWS		DWS	
	λ (nm)	Activities (nmol/min/mg)	λ (nm)	Activities (nmol/min/mg)
CI	340	170 ± 1.5	340-425	162.7 ± 2.7
CII	600-780	131.1 ± 2	600-780	130 ± 2.9
CIII	550	572.4 ± 19.1	550-580	619.8 ± 30.1
CIV	550	1503 ± 54	550-580	1511 ± 66
CI+III	550	80.4 ± 1	550-580	86.5 ± 1.9
CII+III	550	93.4 ± 3.6	550-580	97.2 ± 2

Table 3 Comparison of RC enzymatic results obtained with single and double-wavelength spectrophotometric analysis. SWS: single-wavelength spectrophotometry; DWS: double-wavelength spectrophotometry. λ: selected wavelength(s). Results are expressed as mean ± SD of 3 independent measurements. The same spectrophotometer was used for both analysis.

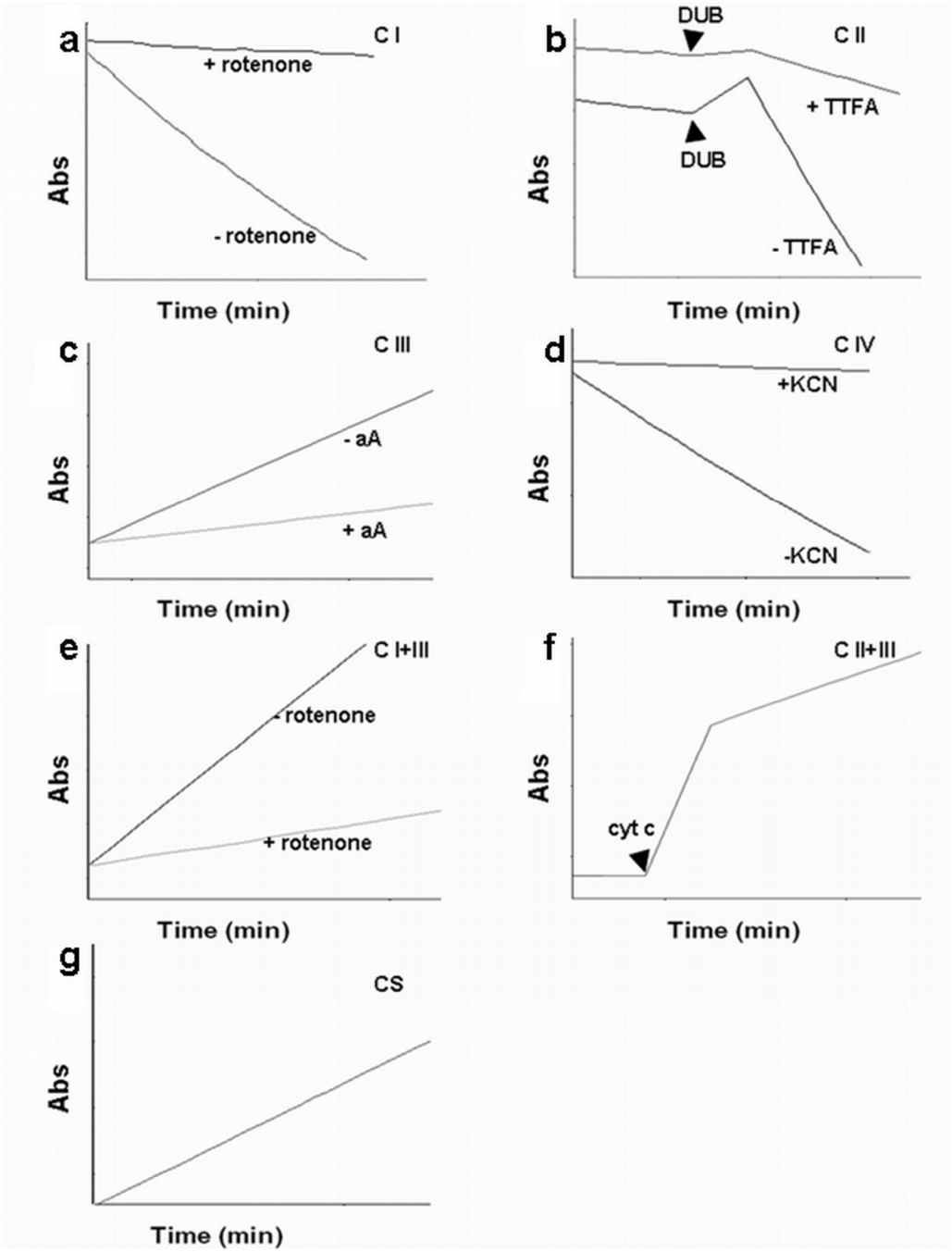

Fig. 12 Representative traces for each RC assay in muscle homogenates, obtained with the proposed protocols (Table 1) with single-wavelength spectrophotometry at 37° C
(**A**) Complex I assay. Traces of parallel reactions with and without the complex I specific inhibitor rotenone are shown. (**B**) Complex II assay. Traces of parallel reactions with and without the complex II specific inhibitor TTFA are shown. The reaction was started by the addition of decylubiquinone (DUB). (**C**) Complex III assay. Traces of parallel reactions with and without the complex III specific inhibitor antimycin A (aA) are shown. Specific CIII activity is calculated by subtraction of the two activities. (**D**) Complex IV assay. Traces of parallel reactions with and without the complex IV specific inhibitor potassium cyanide (KCN) are shown. (**E**) Complex I+III coupled assay. Traces of parallel reactions with and without the complex I specific inhibitor rotenone are shown. Specific CI+III activities are calculated by subtraction of the two activities. (**F**) Complex II+III assay. The reaction is started by the addition of cytochrome *c* (cyt c). (**G**) Citrate synthase assay.

5.5 Performance assessment of the proposed enzymatic protocols

5.5.1 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 coefficient of variations of respiratory chain enzyme activities (CI, CII, CII+III, CIV, CS) of separated homogenates obtained from the same muscle, either in the same day and in different days (**Table 4**). The tested homogenization procedure employed the use of CCA buffer, 16 up-down strokes at a rotational speed of 500 rpm. The coefficient 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 4). 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%.

	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%

Table 4 Intra-day and inter-day coefficient of variations of respiratory chain enzymes extracted by separated homogenizations.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).

5.5.2 Precision, specificity and linearity of the optimized analytical 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 2**, **Fig. 13**). The results of the performance assessment of each of these protocols, performed with a single-wavelength spectrophotometer, are summarized in **Table 5**. Intra-assay and inter-assay precisions were determined for each assay and ranged between 2.8-5.3% 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 (**Fig. 13**), 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 4**). 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.

	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

Table 5 Analytical performances of the proposed respiratory chain spectrophotometric assays on muscle homogenates: precision, specificity, and linearity.
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 parallele 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 seconds and the activity measured between 120-180 seconds. Linearity index is expressed as the mean ± SD of 3-5 independent assays.

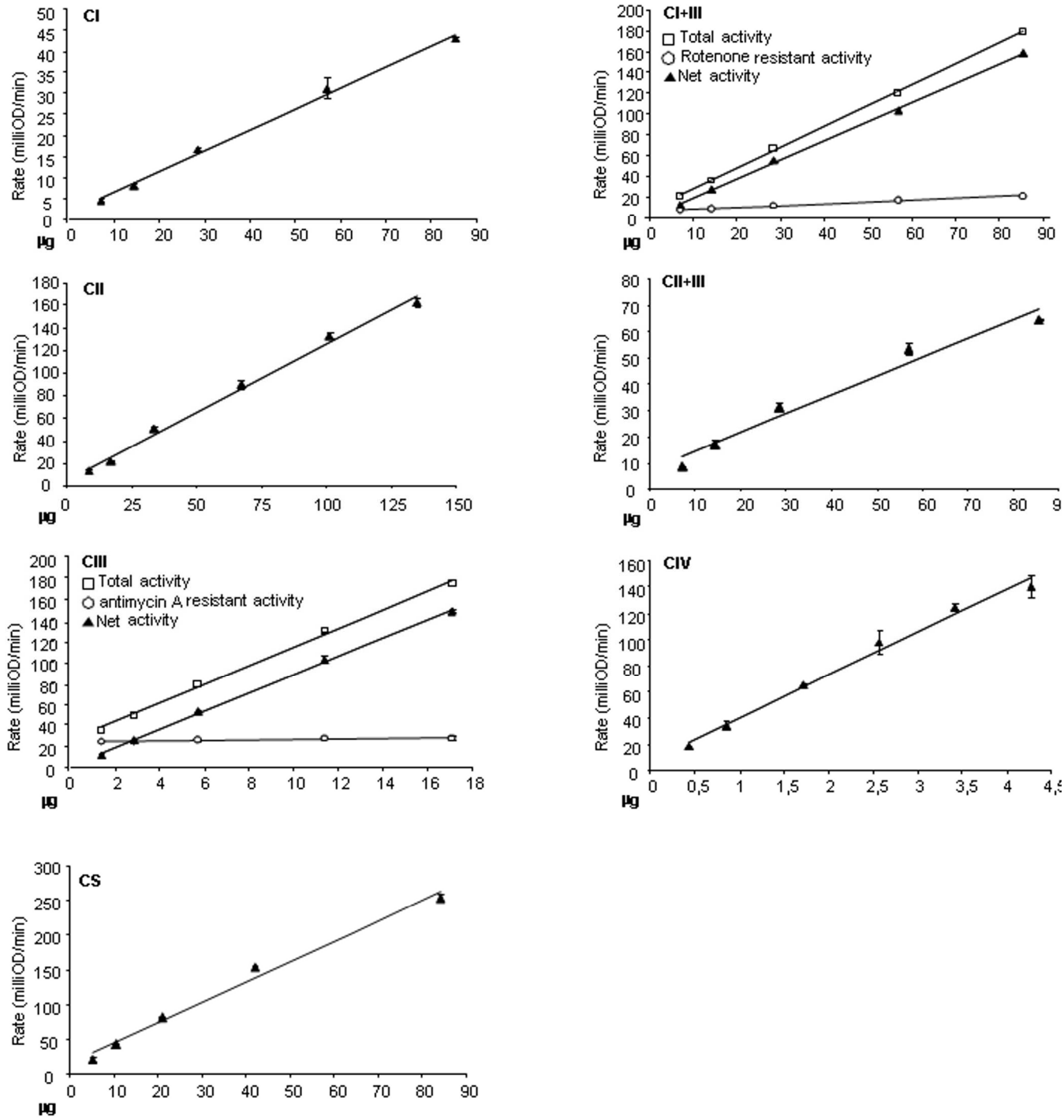

Fig. 13 Linearity between total muscle protein added to the reaction (µg/ml) and enzymatic rate (expressed as milli optical density/min) for the optimized assays for complexes I, I+III, II, II+III, III, CIV, and citrate synthase.

5.6 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 **Table 6**. The assays developed on bovine muscle were reliably applicable to human muscle

homogenates, with a unique modification concerning cytochrome c oxidase assay. In human muscle we found that lauryl maltoside did not increase the enzymatic activities as in bovine muscle, and it has a very negative effects on the linearity of the kinetics (linearity index calculated testing 6 μg of muscle proteins: no LM: 1.0 ± 0.02 vs. LM 0.05% 0.44 ± 0.04, $p<0.005$).

Moreover, a higher rotenone-insensitivity was noted for complex I assay in human muscle (around 10%) compared to bovine muscle (below 3%), but this effect did not preclude a reliable calculation of complex I activities by always running a separate cuvette containing rotenone.

	Mean ± SD (nmol/min/mg)	**Mean ± SD (% ratio to CS activity)**
Complex I	118,5 ± 42,4	32 ± 3
Complex II	184,4 ± 46,6	50,9 ± 4,7
Complex III	589,8 ± 198,7	160,5 ± 14,7
Complex IV *	413,7 ± 71,5	117 ± 22,5
Complex I+III	82,2 ± 21,5	22,6 ± 1,3
Complex II+III	111,7 ± 24,5	31 ± 3,1
Citrate synthase	368,3 ± 123,1	-

Table 6 Reference values of RC enzymatic activities in skeletal muscle homogenates from human controls (n=5). Values are expressed as mean ± standard deviations, normalized to total proteins (nmol/min/mg) or to citrate synthase activity. * Lauryl maltoside was omitted from this protocol to increase the linearity of the reaction.

5.7 Species-specific effect of lauryl maltoside on cytochrome c oxidase activity

In order to verify the hypothesis that the effect of the detergent lauryl maltoside (LM) on cytochrome c oxidase activity could be species-specific, we compared its effects in samples obtained from a number of species: bovine, human, yeast, and mouse. The behaviour of the kinetics in response to this detergent was found to be different from species to species in terms of enzymatic activity and linearity (**Fig. 14**): a) in bovine muscle homogenates LM dramatically enhanced the enzymatic activities (more than 300%), while maintaining an acceptable linearity within time (linearity index 0.77, **Fig. 14A**) b) in human muscle homogenates LM did not enhance the enzymatic activities, but it dramatically decrease the linearity of the reaction (linearity index 0.44, **Fig. 14B-C**) c) in yeast isolated mitochondria LM was inhibitory (-22% , $p<0.05$) and decreased the

linearity of the reaction (linearity index without LM: 0.95 ± 0.028 vs. LM 0.05% 0.69 ± 0.028, $p<0.05$; **Fig. 14D**) d) in mouse, LM enhanced the enzymatic activities both in muscle homogenates (+217%, **Fig. 14E**) and in muscle isolated mitochondria (+341%, **Fig. 14F**) but it also significantly decreased the linearity of the reaction (linearity index: with LM 0.51 vs 1.01 without LM). Therefore, despite high evolutionary conservation of the enzyme cytochrome c oxidase, some functional differences in the assay between species are observed. This observation can be important in the diagnostic and research practice, because a low linearity can lead to substantial imprecision in the measurements of the enzymatic activities.

Therefore, we suggest to restrict the use of LM to the assessment of CIV activities in bovine samples, and to omit it when dealing with other species, such as humans, mice or yeasts.

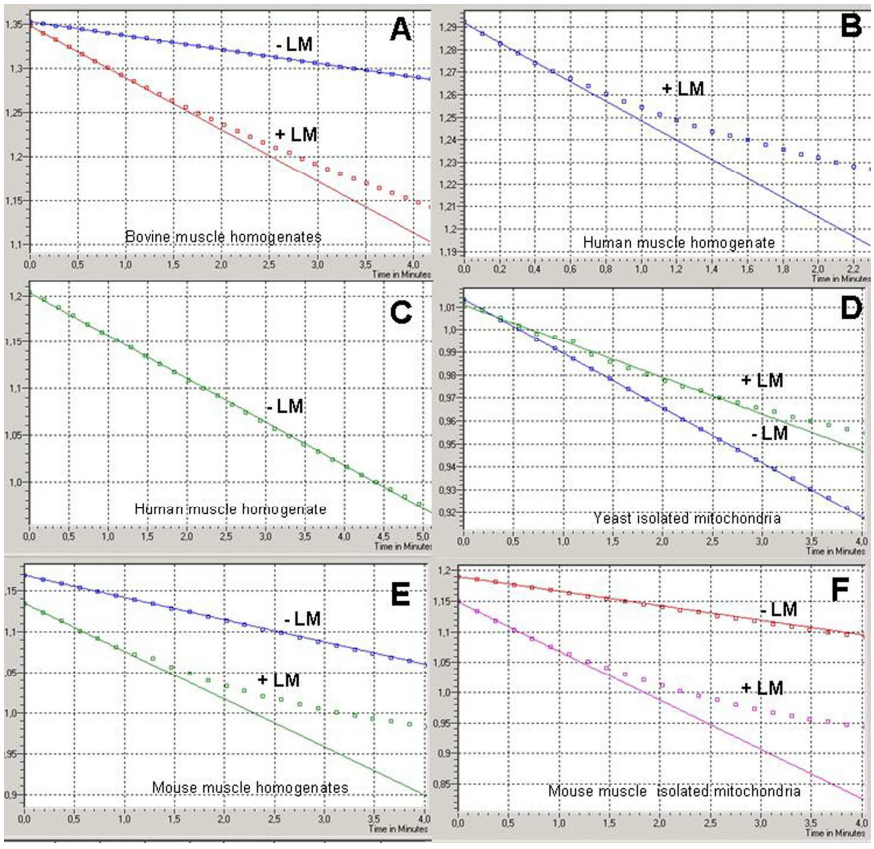

Fig. 14 Species specific effect of the detergent lauryl-maltoside on cytochrome c oxidase activity. Representative traces of reactions with (+LM) and without lauryl-maltoside (-LM) are presented (x-axis: time, y-axis: absorbance). (**A**) Bovine skeletal muscle homogenates. (**B-C**) Human muscle homogenates. (**D**) Isolated mitochondria from yeast. (**E**) Mouse muscle homogenates. (**F**) Mouse muscle isolated mitochondria.

5.8 RC protocols for cultured cells

5.8.1 Optimized lysis protocol for RC on cultured cells

In order to standardize the optimal lysis procedure for cultured cells, we compared the enzymatic activities of complex II, IV and citrate synthase in preparations of human fibroblasts obtained with different commonly employed techniques (mechanical disruption with or without cycles of freeze-thawing, sonication, and homogenization with a glass-glass tissue grinder), as well as the loss of enzymatic activities after a centrifugation passage at 600 g for 10 minutes. The results are illustrated in **Figure 15**. The highest enzymatic activities are obtained with a combination of mechanical disruption and thermal shock with one or more freeze-thawing cycles for all the tested enzymes. Importantly, sonication was detrimental for cytochrome c oxidase. Therefore we recommend to use a combination of mechanical disruption and thermal shock in a hypotonic buffer, when testing RC enzymes in cultured cells, and avoid sonication.

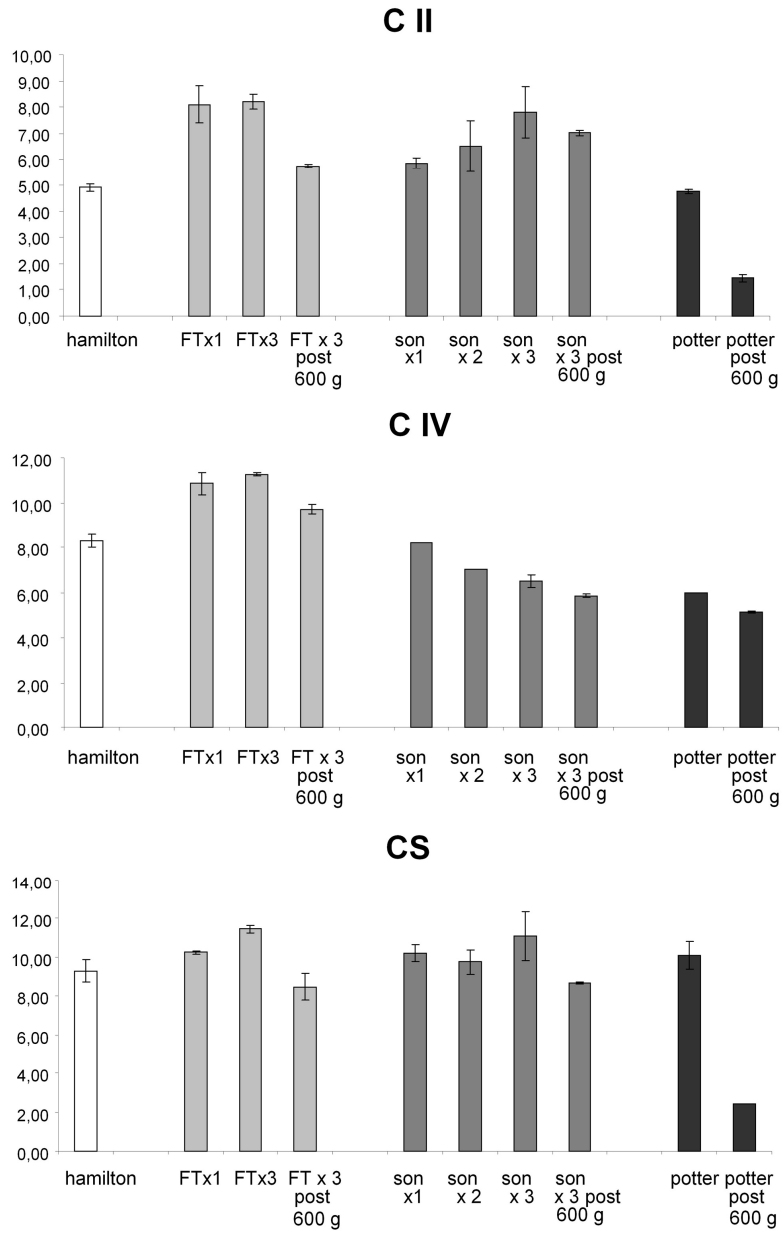

Fig. 15 Effects of different cell lysis techniques on the enzymatic activities of complex II (CII), cytochrome c oxidase (CIV), and citrate synthase (CS) of immortalized human fibroblasts. "Hamilton" bar graph represents enzymatic activities in cell extracts obtained with only mechanical lysis, through resuspension with a Hamiton syringe (white bars). "FT" bar graph refers to enzymatic activities in cell extracts obtained with a combination of mechanical disruption through resuspension with Hamilton and cycles of freeze-thawing (light grey bars; FTx1: n=1, FTx2: n=2; FTx3: n= 3). "Son" refers to enzymatic activities in cell extracts obtained through sonication cycles of 5 seconds at 40 Hz, at 1 minutes intervals (dark grey bars; son x1: n=1, son x2: n=2; son: n= 3). "Potter" refers to enzymatic activities in cell extracts obtained through homogenization of the fibroblast suspension in a glass-glass conical tissue grinder with 15 up-down strokes (black bars). "post 600 g" refers to enzymatic activities of the cell lysate after centrifugation at 600 g at 4°C for 10 minutes. Enzymatic activities are expressed mOD (Δ absorbance/min x 1000).

5.8.2 Optimized biochemical conditions for complex I assay in cultured cells

The analytical pitfalls in the assessment of complex I activities in cultured cells has been long recognized,[47] and were found to be due to extra-mitochondrial cytoplasmic dyaphorases. We confirmed that isolation of mitochondria is mandatory to reliably measure complex I activities in cultured cells, while complex I+III activities cannot be measured in even in isolated mitochondria from cells due to rotenone resistance.

In order to verify the optimal mitochondrial isolation procedure, we compared two different techniques with different chemical-physical principles, one employing a solubilization of the cell extracts with the detergent digitonin,[48] one using a combination of hypotonic and thermal shock.[31] While the enzymatic activities with these two methods were comparable, we found that the yield of mitochondria with the second method was higher, and therefore preferable.

Two different methods have been previously proposed for the measure of complex I activities: one employing ubiquinone$_1$ as electron acceptor measures the absorbance changes due to NADH oxidation at 340 nm[39,49]and a new method using a combination of DCPIP and decylubiquinone measures the absorbance changes due to DCPIP oxidation at 600 nm.[31] When comparing the two methods, CI enzymatic activities were identical (data not shown). However, we found relevant differences in terms of analytical properties of the assays: while the new DCPIP method was more sensitive (around twice sensitive compared to the classic ubiquinone$_1$ method), it was remarkably less specific in terms of rotenone-sensitivity (around 40 %, **Fig 16A** vs 62%, **Fig 16B**), and showed a more limited linearity in a range of increasing protein concentrations. We therefore suggest the use of the classic CI method.

Moreover, we investigated the effect on CI activities of repeated cycles of freeze thawing of the fibroblast isolated mitochondria in hypotonic buffer. This procedure resulted in a remarkable enhancement of complex I activities and a parallel decrease of non-specific rotenone-resistant activities (**Fig 16C** vs **16 B**), with a net increase of CI specific activities (+90%). We therefore propose to test complex I activity in isolated mitochondria obtained with the Jannsen method using our analytical protocol, after 3 cycles of freeze-thawing in hypotonic buffer.

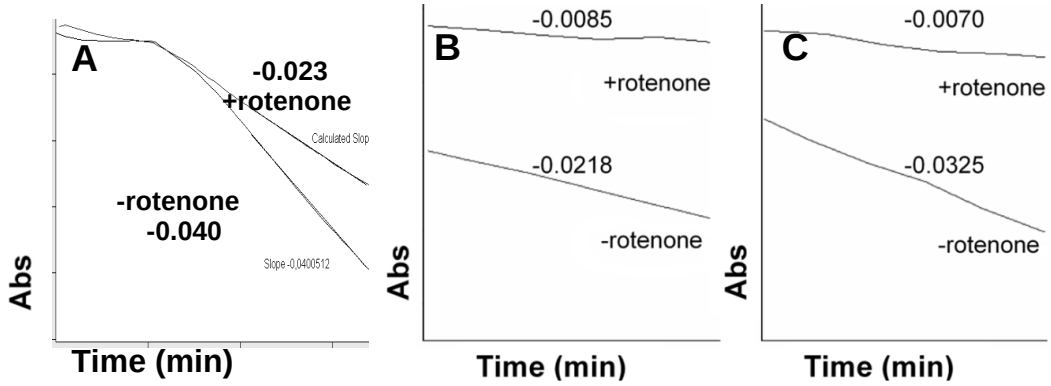

Fig. 16 Complex I assay in isolated mitochondria from human fibroblasts. (**A**) Representative traces using the Jannsen method at 600 nm (20 μg of mitochondrial proteins). (**B-C**) Effect of freeze-thaw cycles on complex I activities in fibroblast mitochondria using our method. [39] Thirty μg of mitochondria from human fibroblasts were assayed with and without rotenone in parallel cuvettes, without (**B**) or with (**C**) three freeze-thaw cycles in hypotonic buffer (KP, 20 mM, pH 7.5). Repeated freeze-thaw cycles increased the CI rotenone-sensitive activity by about 90%.

5.8.3 Adapted biochemical conditions for CII+III and CIV on cultured cells

With the exception of complex I and complex I+III, the remaining RC enzymes and citrate synthase can be measured reliably in total fibroblast lysates, prepared as described in the previous paragraph, or in isolated mitochondria (**Table 7**). Other types of cells were also reliably tested, including HeLa cells and human platelets. The biochemical conditions for this application are identical to those described for skeletal muscle with few minor modifications to increase the assay sensitivities. The potassium phosphate buffer concentration is increased up to 100 mM in complex II+III assay and decreased to 25 mM for cytochrome c oxidase, with a parallel decrease of reduced cytochrome c to 50 μM and omittance of lauryl maltoside.

5.9 Exemplificative applications of RC enzymatic assays for the diagnosis of mitochondrial disorders

We show in **Figure 17** some representative cases of biochemical defects in patients with a molecular diagnosis of mitochondrial diseases with mutations in mitochondrial DNA or in the nuclear DNA (in mitochondria-related genes), to highlight the importance of a reliable biochemical assessment of cells and tissues derived from the patients. While the biochemical defect is never pathognomonic of a defined molecular diagnosis, it can be extremely helpful in supporting a diagnosis of mitochondrial disease and in restricting the genetic analysis in the continuously grown myriad of genes responsible for mitochondrial disorders.

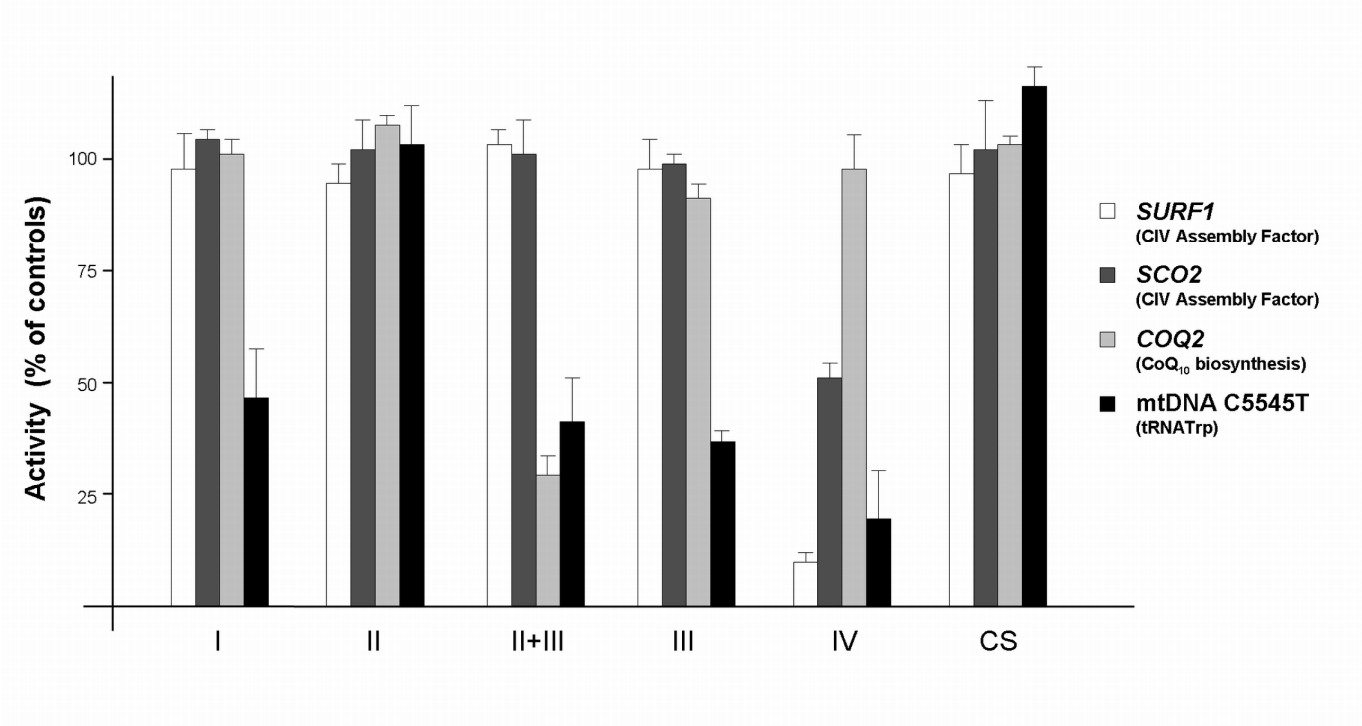

Fig. 17 Respiratory chain enzymatic activities in cultured fibroblasts from patients with different mitochondrial disorders. Patients with mutations in two different CIV assembly factors, *SURF1* (white bar) and *SCO2* (dark grey bar), have isolated CIV deficiency. The patient with mutations in *COQ2*, a gene required for CoQ_{10} biosynthesis, displays selective complex II+III deficiency (light grey bar). The patient with the *C5545T* mtDNA mutation in tRNAtrp, which impairs mitochondrial protein synthesis, has a generalized defect of complexes containing mtDNA encoded subunits (CI, II+III, III, IV; black bar).

5.10 Validation of the implemented RC enzymatic assays in mouse, yeast and *C.elegans*

Due to the widespread applications of RC enzymatic assays for research purposes in animal models and organisms, we wished to validate their applications in several species, including mouse, yeast, and *C. elegans*. We found that our optimized protocols (**Table 2**) can be reliably used for these applications without any modification.

In order to verify if isolation of mitochondria can be useful to decrease non-specific activities, we also compared the RC enzymatic in these two different preparations from mouse muscle (**table 7**). Surprisingly, we found that isolation of mitochondria does not increase the specificity of the reactions nor it does increase the precision, measured by the coefficient of variation between repeated measurements. Therefore, if an appropriate homogenization technique is used for each tissue, tissue homogenates can be reliably employed without need of isolating mitochondria, substantially sparing time, money and tissues.

	CI	CII	CIII	CIV	CI+III	CII+III	CS
Mouse skeletal muscle homogenate	447 ± 17 (32)	386 ± 30 (27)	1448 ± 195 (104)	4374 ± 249 (313)	394 ± 19 (28)	634 ± 45 (45)	1394 ± 111
Isolated mitochondria from mouse skeletal muscle	1813 ± 74 (34)	1824 ± 120 (34)	3581 ± 307 (68)	8166 ± 691 (155)	1228 ± 65 (23)	3365 ± 237 (64)	5265 ± 200
Human immortalized fibroblast total lysate	-	69 ± 3.3 (33)	110 ± 5.7 (52)	140 ± 3.3 (67)	-	52 ± 2.4 (25)	208 ± 1.5
Isolated mitochondria from human immortalized fibroblasts	118 ± 3.1 (16)	386 ± 16 (51)	482 ± 5.6 (64)	606 ± 3.7 (81)	-	235 ± 1 (31)	751 ± 10

Table 7 Respiratory chain enzyme activities in illustrative examples of control preparations in muscle and cultured fibroblasts. Enzymatic rates are expressed as nmol/min/mg protein ± standard deviations and as % ratio to CS activity in brackets. (-) Enzymatic activities cannot be reliably measured in these preparations due to very high non-specific rotenone-insensitive signal.

5.11 Effect of freezing on RC activities in mouse muscle, liver, brain

We found that freezing the muscle, brain liver before homogenization results in increased or unchanged enzymatic activities in most cases, with few exceptions for CII+III in muscle and CIII in brain (**Fig. 18**). The increased enzymatic activities observed with freezing are probably due to a greater accessibility of the substrates to the enzymes, due to mitochondrial membranes disruption. Loss of CII+III activities in muscle might be due to coenzyme Q10 loss due to muscle membranes destabilization.

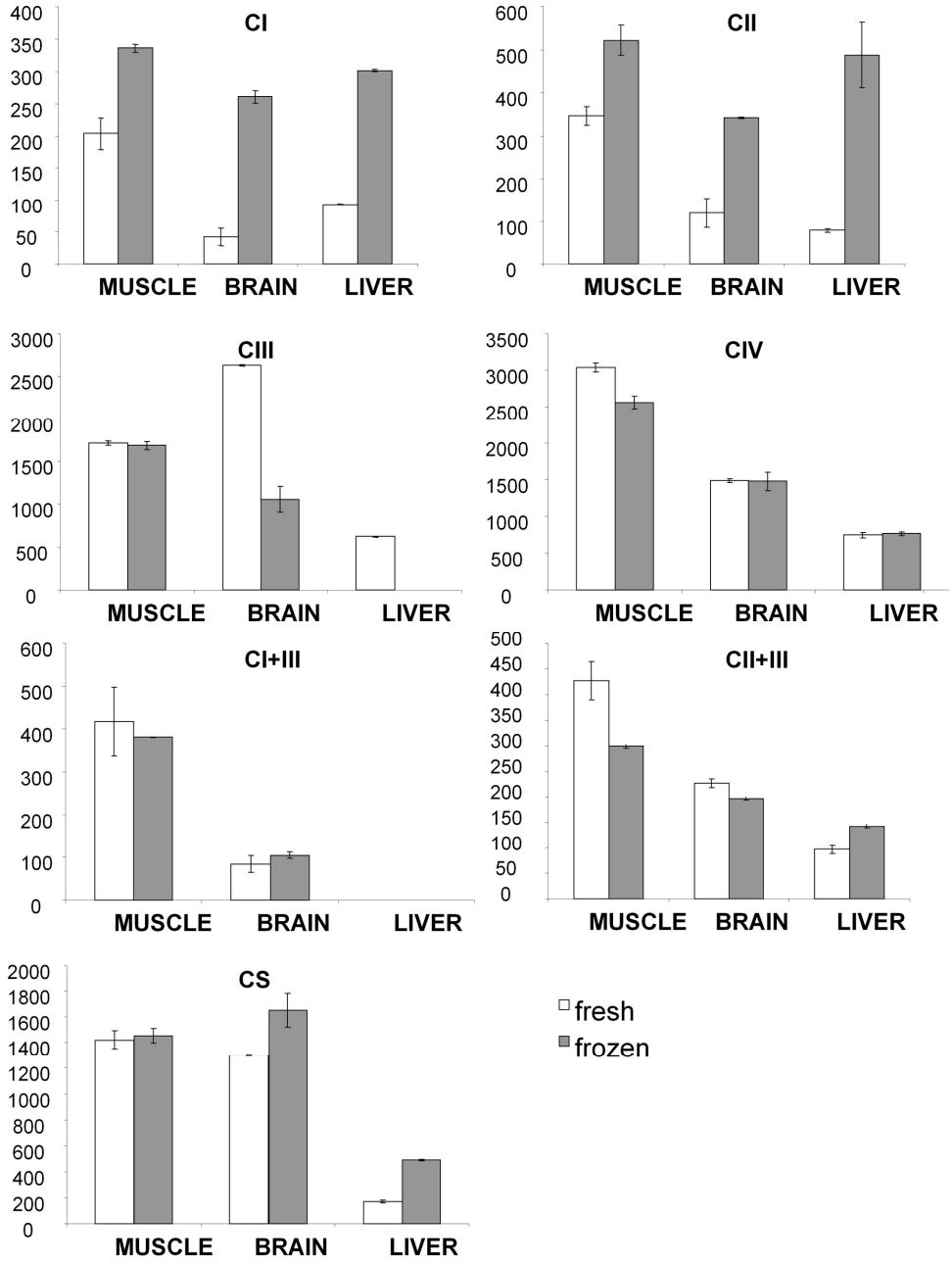

Fig. 18 Effect of freezing on RC enzymatic activities of mouse muscle, liver and brain homogenates. White bars represent enzymatic activities from homogenates obtained from fresh tissues. Gray bars represents enzymatic activities from tissues from the same mouse frozen in liquid nitrogen and then thawed before the homogenization. Enzymatic activities are expressed as nmol/min/mg proteins. CIII activities of frozen liver homogenates are not available. CI+III activities in liver cannot be measured due to overwhelming specific rotenone-resistant activities.

6. Discussion

At difference with other inborn errors of metabolism, residual enzymatic activities can be considerable in mitochondrial diseases.[18, 50] 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.

6.1 Standardization of the sample lysis 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 sample, release of the mitochondria, breakage of the mitochondrial membranes and solubilisation 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 lysis protocols have been proposed throughout the years for muscle and cells but no systematic assessment of their performance has been conducted.

Surprisingly, we showed that a remarkable proportion of tissue grinders from different companies had very poor compliance between mortar and pestles, and were therefore not suitable for a reliable homogenization. This is a worrisome concern, which suggests extreme care in the selection of this equipment before use. A different technology and quality control is probably needed by the manifacturers to ensure a good quality of these products, employed for diagnostic purposes.

Moreover, we showed that several factors influence this phase of the RC analysis in muscle, either by affecting directly the enzymatic activities of individual complexes or by altering total muscle protein content which is commonly employed for the normalisation of the results. In fact, only ionic buffers with relatively high osmolarity were able to solubilise myosin in the muscle surnatants, 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.[25]

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, might 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[26] or BSA[51] had not any remarkable effect, and therefore they can be safely omitted when performing RC assays on muscle homogenates.

While it is a general assumption that mitochondria are retained in the supernatants after the first centrifugation at 600 g,[51] 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.[19, 24, 52] We focused on the glass-on-glass pestle homogenization technique, which was previously shown to produce higher enzyme activities in skeletal muscle.[52] While many protocols homogenize with a limited number of strokes, often between 6 and 7,[20, 24, 25] 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 in muscle and negatively affected by mechanical stress induced by sonication,[19] while the activities of CI and CII are maximized and the same phenomenum was observed in cultured cells in the present study. 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 (12-16), 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 solubilised; 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.[12] Each of these normalization strategy 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.[53, 54]

The optimization of the lysis protocol was found to be crucial for RC analysis in cultured cells as well. A combination of mechanical disruption, hypotonic and thermal shock resulted in the highest enzymatic activities. We suggest that sonication, commonly employed in some specialized diagnostic laboratories,[55] should not be employed for the analysis of cytochrome c oxidase activity to avoid false positive results.

6.2 Optimization of analytical protocols

We have examined the impact of different biochemical conditions that affect the analytical performances of each RC assay on bovine muscle homogenates. 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.[56] 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.[56] 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 type of substrate was also critical, especially for CI assay, where we recommend the use of ubiquinone1 rather than decylubiquinone to improve linearity of the reaction with time.

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.[57]

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.[25, 57] Some centers do not routinely analyse this complex, but rather rely only on the combined assays of CI+III and CII+III, which rely on the endogenous CoQ10 pools. We now propose a new optimized protocol, based on a previously published method,[45] 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 and 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.

6.3 Analytical assessment of the proposed protocols

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.

Protocols where 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 depend upon several factors, such as the fractional saturation of the enzyme with substrate, the buffering capacity of the medium, and product inhibition.[58]

6.4 Single wavelength or double wavelength spectrophotometry?

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 fluctuations of the light intensity of the old-generations spectrophotometers. 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.[20, 22]

6.5 Validation of the optimized protocols in human, mouse, yeast and C. elegans

The protocols so defined were reliably applied to a number of species without any modification except omittance of the detergent lauryl maltoside. While this detergent in bovine muscle leads to a dramatic enhancement of the enzymatic activities of cytochrome c oxidase through oligomerization of the enzyme[59] without a remarkable loss of linearity of the reaction, it caused a pronounced decrease in the linearity in the other species, and a significant enzymatic inhibition in yeast. The effect of this detergent on bovine muscle was specific for CIV, as it profoundly inhibited the other RC enzymes.

We therefore suggest to omit this reagent in the diagnostic practice and in all the applications focused on non-bovine tissues.

6.6 Tissue homogenates or isolated mitochondria?

Although isolated mitochondria have the advantage of allowing measurements of oxygen consumption and ATP production in addition to RC enzymatic analysis, isolation requires large amounts of fresh tissue, [21] and is not practical for routine diagnosis, especially for small children. Instead, muscle homogenates can be obtained from frozen biopsies shipped from peripheral hospitals. To verify if isolation of mitochondria would lead to more accurate and specific results we compared the RC analysis of mouse muscle homogenates and mouse muscle isolated mitochondria. Isolation of mitochondria did not increase the precision nor the specificity of the results in muscle tissue. Therefore, we suggest that isolation of mitochondria is not necessary if an appropriate homogenization protocol is performed.

6.7 Validation of the optimized protocols in cultured cells

Our optimized protocols were also validated on cultured cells, with some minor modifications.
Most of the assays can be reliably performed on cell lysates prepared as described, with the exception of complex I and I+III. For the assessment of complex I, preparation of isolated mitochondria is essential to decrease the overwhelming non-specific cytosolic oxido-reductases.
While the results obtained with the new DCPIP-complex I method described by Jannsen are identical to those obtained with our protocols, the former assay is less rotenone-sensitive and displays lower proportionality of the results with increasing protein concentrations.

6.8 Fresh tissue or frozen tissue?

While in most diagnostic laboratories the RC analysis is performed on frozen tissues, very limited informations exist on the effects of freezing of the samples on the activities of the mitochondrial respiratory chain. We showed that freezing has complex effects on different enzymes in different tissues. Overall, we suggest that freezing of the tissues should not only be a concern in the diagnostic analysis of muscle, and it may be a necessary step to improve the sensitivity of the assays with few exceptions. We therefore suggest to consider freezing of the tissue depending on the specific applications.

7. Conclusions

Different homogenization protocols and analytical conditions have a dramatic impact on the results of respiratory chain spectrophotometric assays 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 with a commercial single-wavelength spectrophotometer in cells and tissues form a variety of species.

These protocols allow to overcome most of the reported analytical pitfalls described for complex I, III, and CIV. Use of the detergent Tween 20 is critical for a reliable CIII assay. 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.

Isolation of mitochondria is not necessary for measuring RC enzymes in tissues with these protocols, but it is required to measure complex I activities in cultured cells.

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.

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