

Revisiting Pitfalls, Problems and Tentative Solutions for Assaying Mitochondrial Respiratory Chain Complex III in Human Samples

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Abstract: The assessment of mitochondrial respiratory chain enzyme activity in human samples is a difficult task due to both the small amount of tissue generally available and the frequent need to perform enzyme activity measurement in crude mitochondrial fraction. This is particularly true for the measurement of complex III activity which partial deficiency can be easily overlooked. In this review, we first consider the several interfering reactions occurring when measuring this activity. We subsequently describe the use of an alkyl glycoside detergent, lauryl maltoside, to keep these interfering reactions to a very low level. Next, we quantify the effect of the detergent on the actual measurement of complex III in various human tissue samples and cells. Finally, we also demonstrate that the use of the detergent allows (i) a better detection of an inherited partial defect affecting cytochrome *b*, a catalytic subunit of the mitochondrial complex III, (ii) to possibly discriminate decreased complex III activity resulting from an abnormal complex III assembly (BCS1 gene mutation) from an hampered catalytic activity originating from a cytochrome *b* mutation. This detailed review of the problems associated with complex III assessment and of their tentative solution highlights the difficulties still encountered in the measurements of mitochondrial respiratory chain in humans.

Key Words: Respiratory chain; Mitochondrial disease; Complex III; Lauryl maltoside; BCS1; Cytochrome *b*; quinone.

INTRODUCTION

Mitochondrial complex III (CIII; cytochrome *bc*₁ complex; ubiquinol cytochrome *c* reductase; EC 1.10.2.2) transfers electrons from the ubiquinone pool to cytochrome *c* in the respiratory chain [1]. Along with electron transfer, a cyclic reaction mechanism, known as the Q cycle, allows protons to be pumped from the mitochondrial matrix to the inter-membrane space (Fig. 1) [2]. The crystal structure of the bovine heart mitochondria CIII has been resolved [3] showing that the closely interacting monomers are arranged as symmetric dimers forming several cavities accommodating quinone substrates and inhibitors (antimycin, myxothiazol). Cytochrome *c*₁, and possibly subunit 8 which mediates the interaction with cytochrome *c*, protrudes in the inter-membrane space [3]. Under the current conditions of quinol cytochrome *c* reductase activity assay, CIII is likely to be in a dimeric state, although cooperation between monomers in this dimeric structure remains to be shown [4]. An active monomeric form of the complex is obtained when using a high concentration of various non-denaturing detergents [5]. In keeping with this, it has been shown that an alkyl glycoside detergent (*n*-dodecyl- β -*D*-maltoside; lauryl maltoside; LM) markedly increased CIII activity in human skeletal muscle mitochondria when used in excess of the critical miscellar concentration (0.16 mM) [6].

Steady-state kinetics studies on the bovine heart native CIII have provided evidence for (i) a ping-pong mechanism with one quinol molecule and two ferricytochrome *c* molecules as substrates [7,8] and (ii) a diffusion-limited step for cytochrome *c* (but not ubiquinone) in the electron transfer in CIII [9]. Finally, ferrocycytochrome *c* non-competitively inhibits the enzyme reaction against either quinol or ferricytochrome *c* [8].

Deficiency of CIII is a comparatively rare condition in humans [10]. Mutations in the mitochondrial (mt) gene encoding cytochrome *b* have been identified in a few patients with CIII deficiency [11], but no mutation in nuclear genes encoding 12 of the 13 subunits of CIII has been reported so far [12], except for the HUMQPC-encoding gene [13]. Finally, mutations in the BCS1 gene involved in the CIII assembly have been recently shown to cause neonatal hepatic failure with renal involvement and encephalomyopathy [14].

Surprisingly enough, mt tRNA mutations generally cause major impairment of CI and/or CIV activities without significant alteration of CIII activity [15]. A differential use of codons for the synthesis of the various RC complex subunits may be advocated to account for this apparent discrepancy. However, a similar observation has been reported for patients with large deletion of the mtDNA encompassing several mt tRNAs [16, 17]. Even more puzzling is the recent observation that CIII activity is much less decreased as compared to the activity of the other respiratory chain complexes in heart tissue of mice with depletion of mtDNA caused by a disrupted mitochondrial transcription factor A gene [18]. In keeping with this, isolated cytochrome *c* oxidase deficiency has been reported in patients with mtDNA depletion, with only partial

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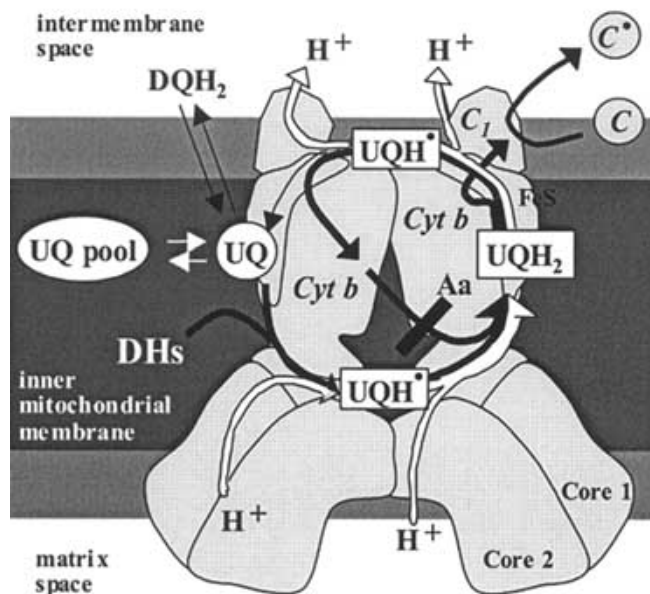


Fig. (1). Schematic representation of fluxes through mitochondrial complex III. Dark arrows indicate electron flow through the complex, proton extrusion from the mitochondrial matrix space to inter-membrane space being represented with white arrows.

decreased activities of other respiratory chain complexes in some of the patients [19].

We first review the factors that may explain these striking observations. We then discuss the conditions of CIII activity assays used in screening procedures for respiratory chain deficiency in patients at risk and illustrate the importance of using a detergent when performing this assay.

REDOX INTERACTIONS BETWEEN COMPONENTS OF COMPLEX III ASSAY MEDIUM

Short chain homologues of ubiquinone, such as reduced decylubiquinol (DQH₂), are widely used to provide electrons for the measurement of complex III activity

through the reduction of cytochrome *c* by the antimycin-sensitive quinol cytochrome *c* reductase activity. However most of these reduced quinones are known to readily auto-oxidize producing both superoxide and hydrogen peroxide [20] prone to reduce cytochrome *c*, exchanging electrons directly with cytochrome *c* in solution [21]. For obvious reasons, these reactions have to be kept as low as possible when assaying complex III activity, although the use of antimycin often allow to recognize complex III-catalyzed cytochrome *c* reduction from such interfering reactions. The auto-oxidation of decylubiquinol largely depends on its protonation status, as shown by the pH dependence of the reaction (Fig. 2A) [21]. The auto-catalytic reaction yields H₂O₂ as a terminal product (Fig. 2A, inset; one H₂O₂ per DQH₂ oxidized) and, being triggered by metal traces, can be

Table 1. Effect of Superoxide Dismutase, Bovine Serum Albumin, Cytochrome *c* and Lauryl Maltoside on Decylubiquinol Auto-Oxidation

Polarographic Measurements were Performed As Described in [17].

Condition	Decylubiquinol (50 μM) auto-oxidation Oxygen uptake (nmol/min)
KH ₂ PO ₄ 10 mM; pH 7.8	50 ± 3
+ Superoxide dismutase (0.5 U/ml)	1 ± 1
+ Bovine serum albumin (1 mg/ml)	113 ± 6
+ Bovine serum albumin (1 mg/ml) + Superoxide dismutase (0.5 U/ml)	3 ± 1
+ Bovine serum albumin (1 mg/ml) + cytochrome <i>c</i> (40 μM)	3 ± 1
+ Bovine serum albumin (1 mg/ml) + Lauryl maltoside (750 μM)	2 ± 1

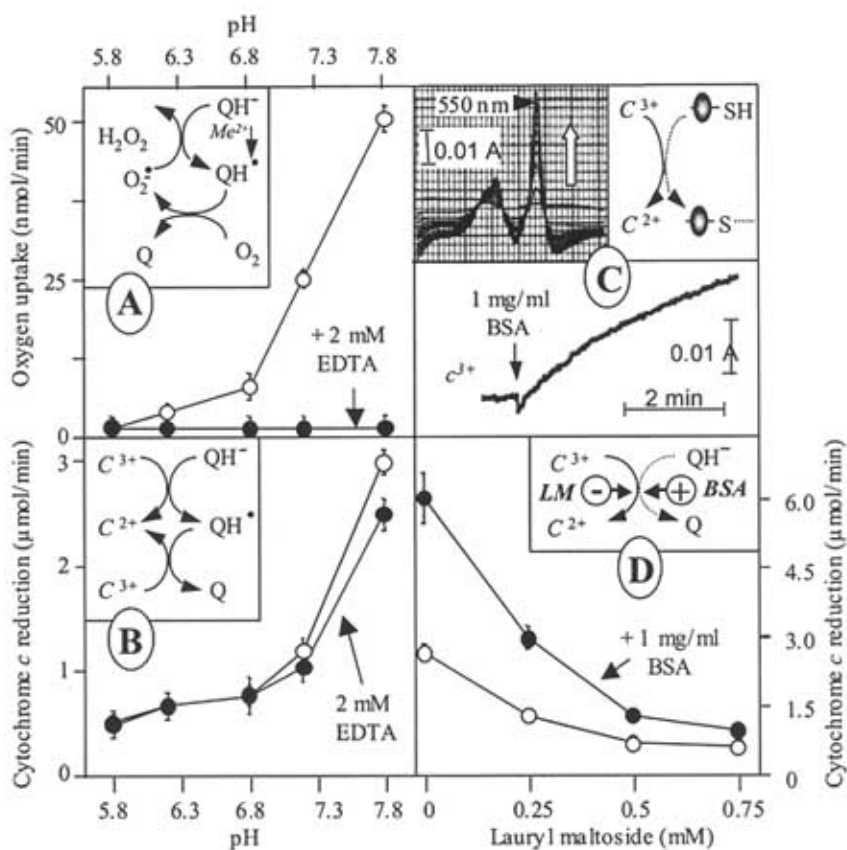


Fig. (2). Chemical redox reactions involving components used in the mitochondrial complex III assay. **A:** pH dependency of decylubiquinol autooxidation in the presence or absence of EDTA (2 mM); *inset:* scheme showing the one-electron reduction of oxygen by reduced quinone. **B:** Effect of pH and EDTA on the reduction by decylubiquinol of cytochrome *c* in solution; *inset:* Schematic representation of cytochrome *c* reduction by quinol and semi-quinol. **C:** Spectrophotometric recording of the reduction of cytochrome *c* brought about by bovine serum albumin at 550 nm; *insets:* spectral study showing the reduction of cytochrome *c* by BSA. **D:** Effect of lauryl maltoside on reduction by decylubiquinol of cytochrome *c* in solution in the presence or absence of BSA. *inset:* schematic representation of the interactions between quinol, cytochrome *c*, BSA and lauryl maltoside. Dotted lines indicate potential multi-step reactions. Decylubiquinol: 50 μM; Cytochrome *c*: 40 μM; BSA: 5 mg/ml. Spectral studies were performed on a double-wavelength spectrophotometer (DW2000; Aminco/SLM, USA) in a 1 ml-cell thermostated at 37°C.

fully inhibited by the addition of a chelator even at high pH value (2 mM EDTA; Fig. 2A). This autocatalytic process requires the intermediate production of superoxide radicals that can be quenched by the addition of either superoxide dismutase or cytochrome *c* (Table 1). The reduction of cytochrome *c* in solution by DQH₂ is also strongly dependent on the protonation status of the quinol and thus shows strong pH dependency (Fig. 2B) [21]. However, in contrast with DQH₂ autooxidation, it does not involve metal and a chelator such as EDTA is inefficient in decreasing this reaction (Fig. 2B). Bovine serum albumin (BSA) has often to be added when measuring the activity of mitochondrial membranous enzymes, yet BSA also triggers DQH₂ autooxidation (Table 1). Not only, BSA is reactive toward DQH₂, but also readily reduces cytochrome *c* in solution (Fig. 2C). It has been suggested [22] that the numerous cystein residues of the BSA (5% of the total amino-acids of the protein) mediate this latter reduction (Fig. 2C, inset), as they do in the rapid reduction of Ellman's reagent, 5,5'-

dithiobis 2-nitrobenzoic acid (DTNB) or through auto-oxidation and superoxide production. Cytochrome *c* reduction in solution by both DQH₂ plus BSA can be quite significant at the pH values generally used for measurement of complex III activity (pH>7.2; Fig. 2D) and can be problematic when studying human samples to be screened for respiratory chain defect. An alkyl glycoside detergent, lauryl maltoside (LM), previously shown to be compatible with the measurement of the antimycin-sensitive DQH₂ cytochrome *c* reductase activity in human skeletal muscle samples [6], proved to decrease these interfering reactions. A concentration of 0.75 mM LM has been used since it has been shown to result in maximal CIII activity [6]. The initial rationale was that interacting/sequestering reactions might stabilize quinol and consequently reduced interfering reactions. It was actually first observed that LM strongly reduced quinol autooxidation (Table 1). Unexpectedly, the electron exchange between cytochrome *c* in solution and BSA were reduced as well (Fig. 2D). This suggested that

complex molecular interactions occur between all these compounds that were investigated further.

MOLECULAR INTERACTIONS BETWEEN COMPONENTS OF COMPLEX III ASSAY MEDIUM

In order to investigate the possible interactions between decylubiquinone, BSA, LM and cytochrome *c* in solution, it can be taken advantage of the UV absorption spectra of both oxidized quinone (decylubiquinone; DQ) and cytochrome *c*. LM did not show any specific absorbance peak in the UV-region studied (not shown). The effect of LM on the DQ spectrum between 330 and 250 nm is shown in Fig. 3. Upon LM addition to a DQ-containing cuvette, a 5 nm shift towards far UV was observed (Fig. 3A; spectra b and c) reflecting the direct interaction between the detergent and the quinone. Interestingly enough, the spectral image of DQ was also affected by the presence of BSA (Fig. 3A, comparison between spectrum b and 3B spectrum b). Adding LM, in the

presence of BSA again shifted the quinol absorption peak to 278 nm, as observed in the absence of BSA (Fig. 3A, spectrum c and 3B spectrum c). This suggests that LM was reducing the interaction between BSA and DQ. Finally, DQ addition to a cuvette containing both BSA and cytochrome *c* in solution (Fig. 3C spectrum b) results in an absorbance increase at 283 nm which was only one third of that predictable from experiment with DQ and BSA alone (Fig. 3B, spectrum b). Again, this shows a direct interaction between cytochrome *c* and the oxidized quinone. Finally, the subsequent addition of LM results in an absorption peak at 278 nm corresponding to the exact additive absorbance of cytochrome *c* (Fig. 3C, spectrum a) and DQ, observed in the presence of BSA (Fig. 3B, spectrum c). This set of UV spectral studies suggests that i) DQ physically interacts with both BSA and cytochrome *c* in solution and that ii) LM is able to displace these interactions in all studied situations. This provided a basis to account for the observed inhibition by LM of quinone autoxidation, and of electron exchange between DQH₂, BSA and cytochrome *c* in solution (Fig. 2).

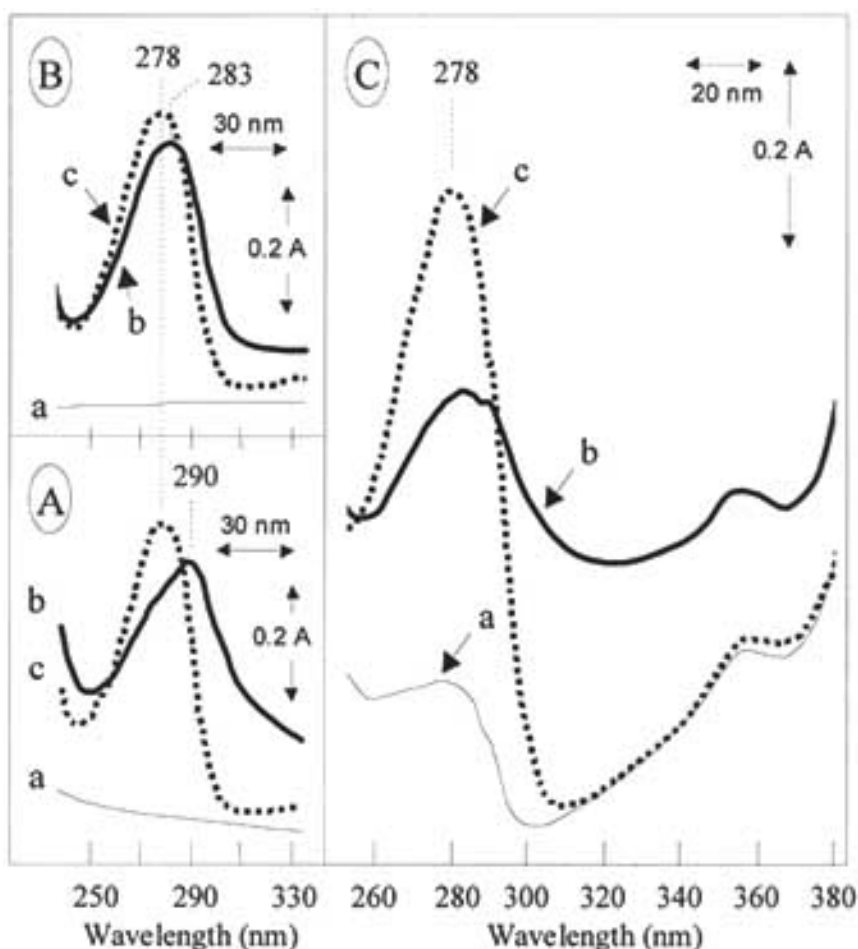


Fig. (3). UV spectral studies of molecular interaction between cytochrome *c*, bovine serum albumin, lauryl maltoside and decylubiquinone.

A: Spectrum of decylubiquinone (30 μ M) in the absence (trace **b**) or in the presence (trace **c**) of lauryl maltoside (750 μ M) in 10 mM KH₂PO₄ (pH 7.8). Base line (trace **a**). Reference cuvette: 10 mM KH₂PO₄; **B:** Similar to **A**, but in the presence of 1 mg/ml BSA; Reference cuvette: 10 mM KH₂PO₄ + 1 mg/ml BSA; **C:** UV spectrum of purified horse heart cytochrome *c* (12.5 μ M; trace **a**) in the presence of 1 mg/ml BSA; Spectrum of decylubiquinone *plus* cytochrome *c* in the absence (trace **b**) or in the presence (trace **c**) of laurylmaltoside. Reference cuvette: 10 mM KH₂PO₄ + 1 mg/ml BSA. Notice the different absorbance scale used for this last experiment.

KINETIC PARAMETERS OF COMPLEX III IN HUMAN SAMPLES

Because of the spectacular effect of LM on the reactions potentially interfering when measuring complex III activity, it is of importance to recognize the effect of LM on the kinetic properties of CIII on human samples under the conditions routinely used in standard screening procedures for respiratory chain dysfunction [23]. This is reported for both crude homogenates of different human tissues, or lymphocytes and cultured skin fibroblasts made permeable by two freeze-thaw cycles in Table 2. Obviously, none of these crude preparations allowed us to determine actual kinetic parameters, but only apparent values for these. In all tissues investigated, as previously reported for isolated skeletal muscle mitochondria [6], we observed a significant increase of CIII activity under V_{max} conditions in the presence of 750 μM LM (Table 2). The rate of the antimycin A-insensitive DQH_2 cytochrome *c* reduction was in parallel reduced by LM. Noticeably, since antimycin-A is highly hydrophobic and tends to bind to a number of untreated plastic surfaces, a saturating concentration of this inhibitor was used all throughout this study. Beside, we did not observe significant interactions between antimycin-A and the components of the assay, except with the biological membrane themselves.

Finally, the apparent K_m for both DQH_2 and cytochrome *c* were studied and the V_{max}/K_m ratio calculated in heart and fibroblast preparations (Table 3). Quinol isotherms in the presence of LM did not show real saturation prohibiting the calculation of confident apparent K_m values. Nevertheless, the analysis of the V_{max}/K_m ratios, known to better detect potential changes in substrate-protein affinity [24], allowed us to show that LM did not change the affinity of complex

III for its substrates, except for cytochrome *c* in the case of the heart homogenate. In this case, the K_m itself was decrease by more than an order of magnitude.

COMPLEX III ACTIVITY IN PATIENT'S TISSUE SAMPLES

We next investigated the effect of LM on the residual complex III activity of tissue samples from patients harboring a mutation in either the mitochondrial gene encoding cytochrome *b* [12] or the nuclear gene encoding BCS1, a protein required for correct assembly of complex III [14]. In the presence of LM, the activity of CIII in skeletal muscle homogenate from patient 1 (BCS1 mutation) responded similarly to controls, *i.e.* increased CIII activity. As result, the presence of LM did not significantly change the residual activity measured in BCS1-mutant fibroblasts as compared to control (roughly 8%). Conversely, the residual CIII activity measured in the heart homogenate of patient 2 (cytochrome *b* mutation) tends to be further reduced when measured in the presence of LM (Table 4). As a result, the residual activity that represented about 30% of control activity in the absence of LM was reduced to 13% of control activity in the presence of the detergent.

CONCLUSION

As illustrated above, numerous redox and molecular interactions possibly interfere with the assay of the mitochondrial respiratory chain CIII. Most were strongly decreased in the presence of lauryl maltoside, in excess of its critical miscellar concentration. Beside its already known effect on CIII [6], it is recalled that the detergent binds both

Table 2. Antimycin-Sensitive and -Insensitive Quinol Cytochrome *c* Reductase Activity Measured Under V_{max} Condition in the Absence or Presence of Lauryl Maltoside (750 μM) in Various Human Tissues or Cells

Biopsies were immediately frozen and stored in liquid nitrogen. Noticeably, a poor handling of tissue samples, particularly following freezing, resulted in the rapid loss of CIII activity. Homogenates from frozen samples were prepared in an extraction medium (1-10/50 w/v) consisting of 20 mM Tris-HCl (pH 7.2), 0.25 M sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml BSA [17]. Circulating lymphocytes were isolated from 10 ml of blood on a Ficoll cushion as described [17]. Cultured skin fibroblasts were grown in RPMI 1640 supplemented with 10% undialyzed calf serum, 2 mM glutamine, 2.5 mM sodium pyruvate, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin and 200 μM uridine under standard conditions [18]. Medium for CIII assay consisted in 10 mM phosphate buffer (KH_2PO_4 ; pH 7.8), 2 mM EDTA, 1 mg/ml bovine serum albumin (BSA), 0.3 mM KCN and 40 μM cytochrome *c* [17]. The reaction was initiated by the addition of 50 μM decylubiquinol and inhibited by 1 μM antimycin.

Sample	Antimycin-sensitive quinol cytochrome <i>c</i> reductase activity		Antimycin-insensitive quinol cytochrome <i>c</i> reductase activity	
	- lauryl maltoside	+ lauryl maltoside	- lauryl maltoside	+ lauryl maltoside
<i>Homogenates</i>				
Skeletal muscle (n=7)	153 \pm 39	284 \pm 97	113 \pm 84	66 \pm 46
Liver (n=5)	189 \pm 67	303 \pm 94	73 \pm 23	46 \pm 7
Heart (n=5)	538 \pm 256	913 \pm 516	189 \pm 67	117 \pm 66
<i>Cells</i>				
Cultured skin fibroblasts (n=4)	96 \pm 10	140 \pm 22	85 \pm 9	33 \pm 7
Circulating lymphocytes (n=4)	71 \pm 5	122 \pm 10	37 \pm 6	31 \pm 6

Table 3. Complex III Apparent Kinetic Parameters in Human Heart Homogenate and Freeze-Thaw Permeabilized Cultured Skin Fibroblasts in the Presence and Absence of Lauryl Maltoside (750 μM)

Complex III activity (decylubiquinol cytochrome *c* reductase) was measured as described under Materials and Methods. Kinetic parameters were determined by fitting the rates obtained at various substrate concentrations to the Michaelis-Menten equation modified to provide estimates of V_{max} and K_{m} as well as of the Hill coefficient [19]. The activity of complex III was measured as described under Materials and Methods.

Condition	V_{max}	K_{m} cyt <i>c</i>	$V_{\text{max}}/K_{\text{m}}$	K_{m} DQH2	$V_{\text{max}}/K_{\text{m}}$
		(μM)		(μM)	
<i>Heart homogenate</i>					
- lauryl maltoside	576 \pm 23	9.6 \pm 4.2	60	n.m. ^a	n.m.
+ lauryl maltoside (750 μM)	990 \pm 42	0.2 \pm 0.2	4950	n.m.	n.m.
<i>Skin fibroblasts</i>					
- lauryl maltoside	98 \pm 12	4.7 \pm 3.6	21	19.3 \pm 1.7	5.2
+ lauryl maltoside (750 μM)	141 \pm 11	5.2 \pm 3.0	27	3.1 \pm 0.8	45.5

^a not confidently measurable

quinone and cytochrome *c*, hence decreasing their potential redox interaction. However, decreasing molecular interactions and redox exchange between quinol and cytochrome *c* did not affect their reactivity toward the respiratory chain complex III. Instead, the presence of lauryl maltoside induces a clear V_{max} effect, roughly doubling antimycin-sensitive complex III activity in most human tissues or cells studied. We also illustrated the non-saturating kinetics for quinone (in the concentration range studied) when measuring CIII activity in heart homogenates,

but not in fibroblasts. The sensitivity of this non-saturating reaction to antimycin established that this actually took place through CIII. This abnormal kinetic behavior might result from a complex equilibration of the exogenously added quinol with the endogenous ubiquinone pool or from the direct interaction with additional CIII components at high quinol concentration.

Diffusion of electron donors and acceptors, in particular cytochrome *c* is known to potentially limit CIII activity [9].

Table 4. The Differential Effect of Lauryl Maltoside (750 μM) on Antimycin-Sensitive Quinol Cytochrome *c* Reductase Measured in Patients' tissue Homogenates Harboring A Mutation in Either the Complex III Assembly Gene, BCS1, or in the Cytochrome *b* Gene

Biopsy specimens referred is controls were obtained from patients who underwent diagnostic biopsy but no evidence of respiratory chain dysfunction. Informed consent was obtained *prior* to biopsy. A diagnostic endomyocardial biopsy was performed in patient 1 presenting with cardiac hypertrophy who was subsequently shown to harbor an heteroplasmic mutation at nt 15243G>A in the mitochondrial cytochrome *b* gene causing a severe complex III defect in heart (90% mutant mitochondrial DNA in heart) [11]. A diagnostic skeletal muscle biopsy was performed in patient 2 who presented with hepatic involvement at birth and experienced an episode of acute myoglobinuria at one month of age. A severe defect of complex III was identified in the skeletal muscle of this patient harboring two heterozygous mutations at nt 464G>C and nt 1057G>A of the complex III assembly gene, BCS1 [12]. Details on clinical presentation have been previously reported for both patients [11,12].

Sample	Antimycin-sensitive quinol cytochrome <i>c</i> reductase activity	
	- lauryl maltoside	+ lauryl maltoside
<i>Patient homogenates</i>		
Patient 1 Skeletal muscle (BCS1 mutation)	15	22 (+47%) ^a
Patient 2 Heart (Cytochrome <i>b</i> mutation)	162	120 (-26%) ^a
<i>Control homogenates</i>		
Skeletal muscle (n=7)	153 \pm 39	284 \pm 97
Heart (n=5)	538 \pm 256	913 \pm 516

^aPercent change of the rate measured in the absence of the detergent

This may hamper the detection of partial CIII defect in human samples, the activity of catalytic subunits being not necessarily limiting for electron flow under standard assay conditions. This was illustrated by the comparative study of the effect of LM on either cytochrome *b*-mutant or BCS1-mutant tissue samples. Both predictably result in CIII activity decrease. However, the former mutation specifically hits the cytochrome *b*-component of complex III, while the second mutation presumably results in a decreased number of normally assembled complex III. CIII assay in the absence or presence of LM reveals that the mutation in cytochrome *b* appears more deleterious to the activity when measured in the presence of the detergent possibly due to decreased control exerted on CIII activity by limiting diffusion steps [9]. By analogy, it is tempting to assume that the decreased cytochrome *b* content predictably resulting from either large deletion, or depletion of mitochondrial DNA, or mutation in the tRNA mt genes, is often poorly detected due to assay conditions where substrate diffusion controls the CIII activity rates actually measured.

The presence of LM was shown to strongly reduce most interfering redox reactions with cytochrome *c* in solution, thus increasing antimycin sensitivity of the quinol cytochrome *c* reductase activity measured in biological samples. When assaying CIII activity in minute amount of human biopsies, this can be quite important since non-enzymatic reduction may be sufficiently important (relative to the enzymatic reduction) as to hamper a confident measurement of CIII activity. On the other hand, LM does not affect the ability to detect defects resulting from poor CIII assembly, as shown in the skeletal muscle of the patient harboring mutations in the BCS1 gene.

It can therefore be suggested to routinely including LM (750 μ M) detergent in the assay medium for the measurement of CIII activity together with a metal cation chelator, such as EDTA, which concurs to reduce superoxide production through quinol autoxidation.

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