Biochemical Assessment of Coenzyme Q10 Deficiency

Juan Carlos Rodríguez-Aguilera,¹,² Ana Belén Cortés,¹,² Daniel J. M. Fernández-Ayala,²,³ and Plácido Navas²,³,*

Iain P. Hargreaves, Academic Editor

¹Laboratorio de Fisiopatología Celular y Bioenergética, 41013 Sevilla, Spain; jcrrodagu@upo.es (J.C.R.-A.); abcorrod@upo.es (A.B.C.)
²Centro de Investigación Biomédica en Red de Enfermedades Raras, Instituto de Salud Carlos III, Universidad Pablo de Olavide-CISC, 41013 Sevilla, Spain; dmofer@upo.es
³Centro Andaluz de Biología del Desarrollo, 41013 Sevilla, Spain
*Correspondence: pnavas@upo.es; Tel.: +34-954-349-385

Received 2017 Jan 18; Accepted 2017 Feb 28.

Copyright © 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Abstract

Coenzyme Q10 (CoQ10) deficiency syndrome includes clinically heterogeneous mitochondrial diseases that show a variety of severe and debilitating symptoms. A multiprotein complex encoded by nuclear genes carries out CoQ10 biosynthesis. Mutations in any of these genes are responsible for the primary CoQ10 deficiency, but there are also different conditions that induce secondary CoQ10 deficiency including mitochondrial DNA (mtDNA) depletion and mutations in genes involved in the fatty acid β-oxidation pathway. The diagnosis of CoQ10 deficiencies is determined by the decrease of its content in skeletal muscle and/or dermal skin fibroblasts. Dietary CoQ10 supplementation is the only available treatment for these deficiencies that require a rapid and distinct diagnosis. Here we review methods for determining CoQ10 content by HPLC separation and identification using alternative approaches including electrochemical detection and mass spectrometry. Also, we review procedures to determine the CoQ10 biosynthesis rate using labeled precursors.

Keywords: coenzyme Q10, CoQ10 deficiency syndrome, CoQ10 biosynthesis, mitochondria diseases

1. Introduction

The mitochondrial respiratory chain (MRC) generates most of the cellular ATP and is comprised of five multi-subunit enzyme complexes. Both the mitochondrial DNA (mtDNA) and the nuclear DNA (nDNA) encode for polypeptides of these complexes and also proteins involved in mitochondrial function. Besides MRC enzyme complexes, two electron carriers, coenzyme Q (CoQ) and cytochrome c, are vital for mitochondrial synthesis of ATP. Mutations in genes of either genome may cause mitochondrial diseases, which are common among inherited metabolic and neurological disorders [1].

CoQ is a lipid-soluble component of virtually all cell membranes. It is composed of a benzoquinone ring with a polyprenyl side chain, the number of isoprene units being a characteristic of given specie, e.g., 10 in humans (CoQ10). CoQ10 transports electrons from MRC Complexes I and II to Complex III. These
electrons come from either NADH or succinate [2] although CoQ10 can be alternatively reduced with electrons provided by different redox reactions in mitochondria [3]. Consequently, CoQ10 is essential for ATP production inside mitochondria, although it is also an indispensible antioxidant in extramitochondrial membranes and a key factor for pyrimidine nucleotide synthesis [4].

CoQ biosynthesis depends on a pathway that involves at least 11 genes (COQ genes), showing a high degree of conservation among species, and is carried out by a putative multi-subunit enzyme complex [5]. Most of the information about the CoQ biosynthesis pathway comes from yeast, and maintains a high homology with mammal gene components (Table 1) [6]. The CoQ10 biosynthesis pathway is highly regulated by transcription factors PPARα and NfκB [7,8,9]. HuR and hnRNP C1/C2 binding proteins stabilize COQ7 mRNA as another CoQ10 biosynthesis regulatory mechanism [10].

Coq7p is post-translationally regulated in yeast that involves mitochondrial phosphatase Ptc7 [11,12]. Ptc7 human orthologue (PPTC7) is related to cellular bioenergetics and stress resistance [13]. Coq7p activity is a key regulator of the CoQ biosynthesis complex [6,14], which may depend on the interaction with Coq9p contributing to the stabilization of the biosynthesis complex [15,16,17,18]. The level of CoQ is highly regulated inside cells and tissues but its concentration is different in each tissue and organ, and depends on dietary conditions and age [19,20]. CoQ also varies greatly in human diseases such as Alzheimer’s disease, cardiomyopathy, Niemann-Pick and diabetes.

2. CoQ10 Deficiency Syndrome

CoQ10 deficiency syndrome includes diverse inherited pathological diseases defined by the decrease of CoQ10 content in muscle and/or cultured skin fibroblasts. CoQ10 deficiency impairs oxidative phosphorylation and causes clinically heterogeneous mitochondrial diseases [21,22]. When the decrease in CoQ10 content is due to mutations in genes encoding proteins of the CoQ biosynthesis pathway or its regulation (COQ genes), it causes primary CoQ10 deficiency [23,24]. Secondary CoQ10 deficiencies may be due to defects in genes unrelated to the CoQ10 biosynthetic pathway. Secondary CoQ10 deficiency is a common finding in oxidative phosphorylation (OXPHOS) and non-OXPHOS disorders [25]. A low mitochondrial CoQ10 content is described in mtDNA depletion [26], mutations in the DNA repairing aprataxin [27], mutations of the enzyme ETFDH of the β-oxidation of fatty acids [28], recurrent food intolerance and allergies [29], methylmalonic aciduria [30], myalgic encephalomyelitis chronic fatigue syndrome [31], and propionic acidemia [32]. We propose that cases of secondary CoQ10 deficiency associated with OXPHOS defects could be adaptive mechanisms to maintain a balanced OXPHOS which is required to keep cells alive, although the mechanisms explaining these deficiencies and the pathophysiological role in the disease are unknown.

The clinical phenotypes of primary CoQ10-deficient patients are broader than initially reported in 1989 [33], including (i) a multisystem disorder with steroid-resistant nephrotic syndrome as the main clinical manifestation (COQ1-PDSS2) [34], (COQ2) [35], (COQ6) [36] and (ADCK4) [37]; (ii) a multisystem disorder without nephrotic syndrome (COQ1-PDSS1) [38], (COQ9) [39] and (COQ7) [40]; (iii) cerebellar ataxia (COQ8-ADCK3) [41,42,43,44,45,46,47]; and (iv) myopathy and encephalopathy (COQ4) [48,49,50].

3. Primary CoQ10 Deficiency Therapy

Primary CoQ10 deficiency is unique among mitochondrial diseases because an effective therapy is available for patients, which is the supplementation of CoQ10. Ubiquinol, the reduced form of CoQ10, was recently approved as an orphan drug for primary CoQ10 deficiency [51].

While this approach is quite successful in some patients, with a clear improvement of the pathological
phenotype \([52]\), some cases do not show any clinical relief as would be expected \([53]\), probably because they are suffering secondary CoQ\(_{10}\) deficiency. High-dose oral CoQ\(_{10}\) supplementation can stop the progression of the encephalopathy and allows the recovery of renal damage \([52]\). High-dose CoQ\(_{10}\) supplementation was also able to prevent the onset of renal symptoms in PDSS2-deficient mice \([54]\). Furthermore, CoQ\(_{10}\) but not other quinones can restore mitochondrial function in deficient human fibroblasts \([55]\). Due to the therapeutic possibility of CoQ\(_{10}\) supplementation for these patients, a rapid and unequivocal diagnosis of the deficiency is essential.

### 4. CoQ\(_{10}\) Determination in Cells and Tissues

Content of CoQ\(_{10}\) has been determined in plasma, white blood cells, skin fibroblasts and skeletal muscle biopsies to assess a deficiency diagnosis \([56,57,58]\), and recently useful determination in the urine of pediatric patients was demonstrated \([59]\). Although CoQ can be measured in plasma and white blood cells, you cannot use it for the diagnosis of mitochondrial diseases since CoQ content in plasma and white blood cells is often not decreased in these conditions.

CoQ\(_{10}\) content is mainly analyzed by the injection of lipid extracts in HPLC and detected by either electrochemical and/or UV-vis detectors, or mass spectrometry. Electrochemical detection has significant advantages compared to UV-vis detection; these include higher sensitivity and also the ability to measure oxidized and reduced forms of CoQ, either separately or combined, according to differential positioning of the conditioning cell (before or after the injector valve, respectively).

CoQ\(_{10}\) extraction from biological samples (0.5 mg protein) requires the disruption of hydrophobic elements (lipid bilayers and lipoproteins) by adding SDS (1% final concentration). Lipids are dispersed with an alcohol cocktail (2-propanol 5% in ethanol) mixed with the disrupted biological sample (ratio 1:2 \(v/v\)), and they undergo subsequent triplicated hexane extraction (dispersed sample:hexane ratio 3:5 \(v/v\)). Hexane fractions are mixed and dried under vacuum, and then reconstituted in ethanol prior to HPLC analysis. To estimate CoQ\(_{10}\) recovery, 100 pmol CoQ\(_{9}\) was included in the alcohol cocktail (2-propanol 5% in ethanol). Trace amounts of CoQ\(_{9}\) may have eventually been found in human tissues (probably from dietary uptake), but this does not interfere with the significant amount of internal standard added.

For convenience in high-throughput analysis, volumes are scaled down for extraction and vortex in 1.5 mL polypropylene tubes or 2 mL cryo vials.

Separation in C18 RP-HPLC columns (5 \(\mu\)m, 150 \(\times\) 4.6 mm) requires 20 mM AcNH\(_4\) pH 4.4 in methanol (solvent A) and 20 mM AcNH\(_4\) pH 4.4 in propanol (solvent B). A gradient method with a 85:15 solvent mixture (A:B ratio), and a flow rate of 1.2 mL/min, is regularly used as the starting conditions. The mobile phase turns to a 50:50 A:B ratio starting in minute 6 and completed in minute 8, as the flow rate decreases to 1.1 mL/min. After 20 min (run time) at 40 °C, the columns are re-equilibrated to the initial conditions for three additional minutes.

The detection of total CoQ\(_{10}\) can be achieved either by UV-vis (set to 275 nm) or electrochemical (ECD) detectors (channel 1 set to −700 mV and channel 2 set to +500 mV, conditioning guard cell after injection valve). For complex samples including many peaks, the CoQ\(_{10}\) peak is confirmed by spectral information (UV-vis) or by the redox area ratio (ECD detector, −700/+500 area ratio), compared to pure CoQ\(_{10}\).

**Figure 1** illustrates two chromatograms that correspond to normal age-matched human dermal fibroblasts (black plot) compared to patient dermal fibroblasts with CoQ\(_{10}\) deficiency (red plot).

### 5. Analysis of CoQ\(_{10}\) Biosynthesis
Another important approach to assess CoQ\textsubscript{10} deficiency in cells is to determine the rate of biosynthesis by the level of incorporation of labeled of CoQ\textsubscript{10} precursors such as \textit{para}-hydroxybenzoate (\textit{p}-HB) labeled with either \textsuperscript{13}C\textit{p}-HB or \textsuperscript{14}C\textit{p}-HB, which is the precursor of the benzoquinone ring, or \textsuperscript{2}H-mevalonate, which is the precursor of the isoprenyl side chain [10,60].

Polyprenyl-\textit{p}HB transferase activity was assayed by measuring the incorporation of \textsuperscript{14}C\textit{p}-HB into nonaprenyl-4-hydroxybenzoate [35]. Isolated mitochondria (0.1–1 mg protein) were mixed with assay buffer (50 mM phosphate buffer, pH 7.5, 10 mM MgCl\textsubscript{2}, 5 mM EGTA containing 1 mM PMSF, 20 µg/mL each of the protease inhibitors chymostatin, leupeptin, antipain, and pepstatin A, 5 µM solanesyl pyrophosphate solubilized in detergent solution (1% in water), and 10\textsuperscript{5} DPM of \textsuperscript{14}C\textit{p}-HB). A sufficient volume of a 10% detergent stock solution was also added to the reaction medium to achieve a final detergent concentration of 1%. The following detergents were tested: Triton X-100, Chaps, sodium cholate, sodium deoxycholate, lysophosphatidyl choline, and octylglucoside. After incubation for 30 min at 37 °C with gentle stirring, the reaction was stopped by chilling samples to 4 °C. Prenylated \textsuperscript{14}C\textit{p}-HB was separated by organic extraction with hexane and then measured using a liquid scintillation counter. Specific activity was expressed as disintegrations per minute (DPM) min\textsuperscript{−1} mg\textsuperscript{−1} protein.

Biosynthesis of \textsuperscript{14}C-CoQ\textsubscript{10} has been quantified in any type of cell culture, such as cancer cells, human skin fibroblasts, and murine embryonic fibroblast and stem cells [10,61]. Previously, cultures were incubated with 4.5 nM \textsuperscript{14}C\textit{p}-HB for one to three days, depending on the cell-specific rate of growth. The \textsuperscript{14}C\textit{p}-HB was chemically synthesized in our laboratory from \textsuperscript{14}C-thyrosine [61]. Labeled-CoQ\textsubscript{10} content is analyzed by lipid extract injection in HPLC and detected by the radio-flow detector LB 509 with a solid cell YG 150 Al-U4D (Berthold Technologies, Bad Wildbad, Germany) in parallel with either electrochemical or UV-vis detectors. Lipid extraction is done as we described above for CoQ\textsubscript{10} determination in cells and tissues, but isocratic HPLC analysis lipid separation is performed with methanol:propanol (65:35) plus 20 mM AcNH\textsubscript{4} pH 4.4 at a constant flow rate of 1 mL/min (Figure 2).

Alternatively, a non-radioactive protocol to analyze CoQ\textsubscript{10} biosynthesis was developed using either \textsuperscript{2}H-mevalonate or \textsuperscript{13}C-\textit{p}hydroxybenzoate as CoQ\textsubscript{10} precursors as described by Buján et al. (2014) [60]. Human fibroblasts at 60%–70% were incubated with these precursors for 24–72 h at different concentrations. After incubation, cells were trypsinized and washed twice with isotonic buffer. Pelleted cells were resuspended with 300 µL of a buffer solution containing 0.25 mmol/L sucrose, 2 mmol/L EDTA, 10 mmol/L Tris and 100 UI/mL heparin, pH 7.4, and sonicated twice for 5 s. These homogenates were used to determine CoQ\textsubscript{10} biosynthesis measuring by HPLC-MS/MS, as described in Arias et al. (2012) [62]. Briefly, HPLC separation was as indicated above and extracted peaks were analyzed by MS/MS in a Micromass Quattro micro\textsuperscript{TM} (Waters/Micromass, Manchester, UK). The MS/MS was operated in the electrospray positive ion mode with a cone voltage (CV), and collision energy (CE) of 15 V and 20 eV, respectively. The following multiple-reaction monitoring transitions were selected: \textit{m/z} 900 > 203 and 897 > 197 for \textsuperscript{13}C-CoQ\textsubscript{10} or \textsuperscript{2}H-CoQ\textsubscript{10}, respectively, 894 > 197 for the physiological CoQ\textsubscript{10} and 826 > 197 for CoQ\textsubscript{9} (internal standard). The dwell time for each transition was 200 ms and the run-time was 16 min. Nitrogen (at a flow rate of 50 L/h) and argon (adjusted to obtain a vacuum of 3–10\textsuperscript{−3} bar) were used as the nebulizing and collision gas, respectively.

### 6. Concluding Remarks

Coenzyme \textsubscript{Q}10 deficiency syndrome includes a group of mitochondrial diseases showing diverse inherited pathological phenotypes. The common aspect of them is the lower content of CoQ\textsubscript{10} in tissues and organs. Primary deficiency is caused by defects in proteins encoded by \textit{COQ} genes, which are components of the biosynthesis pathway or its regulation. CoQ\textsubscript{10} supplementation is the current treatment of primary CoQ\textsubscript{10}
deficiency, which highly improves symptoms. A rapid and distinct characterization of the deficiency is important, and it is mainly determined in skeletal muscle and/or skin dermal fibroblasts. The main approach is to analyze the total content of CoQ₁₀ in lipid extracts by HPLC and UV and/or electrochemical detection. Alternatively, the CoQ₁₀ biosynthesis rate in cultured cells can be determined by incubation with radiolabeled precursors.

**Acknowledgments**

This work has been funded by the Instituto de Salud Carlos III FIS PI14-01962 grant. Authors were also funded by the Junta de Andalucía BIO177 research group.

**Author Contributions**

J.C.R.-A., A.B.C., D.J.M.F.-A., and P.N. have contributed to writing and editing the review and figures.

**Conflicts of Interest**

The authors declare no conflict of interest.

**References**


Figures and Tables
HPLC elution profile of lipid extracts from human skeletal muscular tissue. Patient pathological profile (red plot) shows that CoQ\textsubscript{10} is clearly diminished compared to healthy control volunteers (black plot). CoQ\textsubscript{9} is used as internal standard for normalization.
HPLC elution profile of lipid extracts from human fibroblasts cultured with the radiolabeled precursor $^{14}\text{C}-\text{p-HB}$. Patient pathological profile (red plot) shows that CoQ$_{10}$ is clearly diminished compared to control cells from healthy humans (blue plot). Left Y-axis shows the radio-flow detector scale (volts). Right Y-axis shows the UV-detector scale (absorbance units) for a standard pool of CoQ$_{10}$ and CoQ$_{9}$ (black plot). Notice that the only peak detected in this analysis corresponded with CoQ$_{10}$. 
Table 1

Yeast COQ genes and their characterized human homologues.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Human</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>COQ1</td>
<td>PDSS1 */PDSS2 *</td>
<td>Synthesis of polyprenyl-diphosphate</td>
</tr>
<tr>
<td>COQ2</td>
<td>COQ2 *</td>
<td>pHB-prenyl-transferase</td>
</tr>
<tr>
<td>COQ3</td>
<td>COQ3 *</td>
<td>Methyltransferase</td>
</tr>
<tr>
<td>COQ4</td>
<td>COQ4 *</td>
<td>Organization of the multi-enzyme complex</td>
</tr>
<tr>
<td>COQ5</td>
<td>COQ5</td>
<td>Methyltransferase</td>
</tr>
<tr>
<td>COQ6</td>
<td>COQ6 *</td>
<td>Mono-oxygenase</td>
</tr>
<tr>
<td>COQ7</td>
<td>COQ7 *</td>
<td>Hydroxylase</td>
</tr>
<tr>
<td>COQ8</td>
<td>ADCK3 */ADCK4 *</td>
<td>Unorthodox kinase (regulatory)</td>
</tr>
<tr>
<td>COQ9</td>
<td>COQ9 *</td>
<td>Lipid binding protein</td>
</tr>
<tr>
<td>COQ10</td>
<td>COQ10a/COQ10b</td>
<td>CoQ chaperone</td>
</tr>
<tr>
<td>PTC7</td>
<td>PPTC7</td>
<td>Phosphatase (regulatory)</td>
</tr>
</tbody>
</table>

* These genes were mutated in human causing primary CoQ10 deficiency.

Articles from Journal of Clinical Medicine are provided here courtesy of Multidisciplinary Digital Publishing Institute (MDPI)