A sensitive mass spectrometric assay for mitochondrial CoQ pool redox state in vivo

Nils Burger, Angela Logan, Tracy A. Prime, Amin Mottahedin, Stuart T. Caldwell, Thomas Krieg, Richard C. Hartley, Andrew M. James, Michael P. Murphy

PII: S0891-5849(19)31565-5
DOI: https://doi.org/10.1016/j.freeradbiomed.2019.11.028
Reference: FRB 14499

To appear in: Free Radical Biology and Medicine

Received Date: 18 September 2019
Revised Date: 17 November 2019
Accepted Date: 21 November 2019


This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 Published by Elsevier Inc.
CoQ extraction

Hexane, acidified Methanol

CoQ redox state determination via LC-MS/MS

Correlation with metabolomic data sets
Acquiring data about the bioenergetic state of the cell
Characterise the CoQ redox state and its modulation in vivo
A sensitive mass spectrometric assay for mitochondrial CoQ pool redox state \textit{in vivo}

Nils Burger\textsuperscript{a}, Angela Logan\textsuperscript{a}, Tracy A. Prime\textsuperscript{a}, Amin Mottahedin\textsuperscript{a,b,c}, Stuart T. Caldwell\textsuperscript{d}, Thomas Krieg\textsuperscript{b}, Richard C. Hartley\textsuperscript{d}, Andrew M. James\textsuperscript{a}, Michael P. Murphy\textsuperscript{a,b,*}

\textsuperscript{a}MRC Mitochondrial Biology Unit, University of Cambridge Hills Road, Cambridge CB2 0XY, UK
\textsuperscript{b}Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 0QQ, UK
\textsuperscript{c}Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
\textsuperscript{d}School of Chemistry, University of Glasgow, Glasgow G12 8QQ, UK

*Corresponding author
Prof Michael P. Murphy: mpm@mrc-mbu.cam.ac.uk
Phone: +44 1223 252900

Abstract
Coenzyme Q (CoQ) is an essential cofactor, primarily found in the mitochondrial inner membrane where it functions as an electron carrier in the respiratory chain, and a lipophilic antioxidant. The redox state of the CoQ pool is the ratio of its oxidised (ubiquinone) and reduced (ubiquinol) forms, and is a key indicator of mitochondrial bioenergetic and antioxidant status. However, the role of CoQ redox state in vivo is poorly understood, because determining its value is technically challenging due to redox changes during isolation, extraction and analysis. To address these problems, we have developed a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay that enables us to extract and analyse both the CoQ redox state and the magnitude of the CoQ pool with negligible changes to redox state from small amounts of tissue. This will enable the physiological and pathophysiological roles of the CoQ redox state to be investigated in vivo.

**Highlights**

- Sensitive LC-MS/MS assay to quantify CoQ redox state
- Changes in CoQ redox state can be measured in mitochondria, cells and in vivo
- CoQ redox state is stable during extraction and analysis

**Keywords**

CoQ<sub>10</sub>, CoQ<sub>9</sub>, mass spectrometry, redox state, oxidative stress, mitochondria, Coenzyme Q.
1. INTRODUCTION

Coenzyme Q (CoQ) is a hydrophobic respiratory chain component within the mitochondrial inner membrane that is also found to a far lesser extent in other membranes [1] (Figure 1a). The final steps of CoQ biosynthesis take place in mitochondria, where the redox active benzoquinone headgroup is linked to an isoprenyl chain in which the number of isoprenoid units varies with species: in yeast it is CoQ$_6$, in rodents it is mainly CoQ$_9$ with some CoQ$_{10}$, while in humans CoQ$_{10}$ predominates over CoQ$_9$ [1–5]. Within the mitochondrial inner membrane CoQ collects electrons from dehydrogenases, notably complexes I and II (Figure 1a), during which the ubiquinone form, CoQ, undergoes two-electron reduction to ubiquinol (CoQH$_2$) (Figure 1b). The CoQH$_2$ is then reoxidised to CoQ at complex III to enable the respiratory chain to reduce O$_2$ to water while pumping protons across the inner membrane to establish the protonmotive force (Δp) that drives ATP synthesis by oxidative phosphorylation [1,6] (Figure 1a). In addition to complexes I and II, a number of other dehydrogenases also feed electrons into the CoQ pool from diverse metabolic pathways [1,7] (Figure 1c). As well as its role in the respiratory chain, CoQH$_2$ is also an important antioxidant within the mitochondrial inner membrane where it acts as a chain breaking antioxidant to prevent lipid peroxidation [1-5].

Thus, the CoQ pool plays a central role in mitochondrial function and is the organelle’s principal point of contact with many other metabolic pathways. Consequently CoQ deficiency contributes to mitochondrial dysfunction, disease and ageing [8–10]. The redox state of the CoQ pool is the ratio of its oxidised (CoQ) and reduced (CoQH$_2$) forms, and is a key indicator of mitochondrial bioenergetic and antioxidant status. The CoQ redox state alters dynamically in response to its relative rates of reduction by dehydrogenases and oxidation by complex III or by reactive oxygen species (ROS). Changes in the CoQ redox state are central to mitochondrial redox signalling in oxygen sensing [11] and inflammation [12,13] and also to the tissue damage associated with ischaemia reperfusion injury [14–16]. Therefore, the CoQ redox state is central to health and disease and measuring it in vivo is vital.

While assessing the size of the CoQ pool in tissues in vivo is straightforward, measuring its redox state in vivo is technically challenging. This is mainly due to the difficulty of stabilising the redox state of the CoQ pool during isolation, extraction and analysis. In addition, for many analytical methods large amounts of material are required, limiting applicability. Methods based on liquid chromatography coupled to electrochemical
detection were used extensively in the past to investigate CoQ levels and the CoQ redox state in biological samples [17–20]. More recently several LC-MS/MS approaches for analysis of the CoQ redox state have been described [17–23]. In these protocols single-phase CoQ extraction with relatively polar alcohols such as methanol or propanol are used [17,20–23]. However, as the samples change redox state during isolation and storage, samples have to be analysed rapidly and in small batches. Recently, an improved protocol was developed for the determination of the CoQ redox state in tissues by extraction into non-polar hexane followed by analysis in acidified ethanol [18]. While this limits oxidation, separate standard curves were still required for the two redox states, with the CoQH$_2$ standard curve being a potential limitation due to oxidation of CoQH$_2$ standards distorting the CoQ redox state. Here we describe a simplified two-phase extraction that utilises a single internal standard (IS) that generates a stable extract in which both the redox state of the CoQ pool and its amount can be determined. This will facilitate the analysis of both *in vitro* and *in vivo* models and expand our understanding of the role of the CoQ pool in health and disease.

## 2. RESULTS

### 2.1 CoQ LC-MS/MS assay

To establish CoQ redox state detection by LC-MS/MS, CoQ$_{9/10}$, CoQ$_{9/10}$H$_2$ and $d_6$-CoQ$_{10}$ internal standard (IS) were prepared (Figure 2a). Precursor scans of direct infusion of methanol/ammonium formate solutions of these compounds detected the H$^+$ and NH$_4^+$ adducts that gave product ions at 197 m/z for both ubiquinone and ubiquinol, and at 203 m/z for the IS (Figure 2b). We chose to use NH$_4^+$ adducts for LC-MS/MS analysis as they were more abundant, presumably due to higher concentrations of NH$_4^+$ compared to H$^+$ within the buffer and because for both redox forms the H$^+$ adducts showed the same mass for CoQ$_9$ and CoQ$_{10}$, making CoQ redox state analysis impossible. The proposed fragmentations mechanism is shown in Figure S1. LC-MS/MS analysis of pure samples of each compound showed that the CoQ and CoQH$_2$ were detected in the appropriate channel (Figure 2c). While there was some bleed through of ubiquinone samples into the ubiquinol m/z channel, ubiquinone and ubiquinol were readily separated by LC (Figure 2c). Hence, this LC-MS/MS analysis can easily separate the two redox forms, despite a mass difference of only 2.

### 2.2 CoQ redox state determination
To determine the redox state of the CoQ pool it would be most convenient to measure the ratio of the mass spectrometer response to the two redox forms, to avoid having to construct standard curves for both redox forms of CoQ\textsubscript{9} and CoQ\textsubscript{10}. For this to work the relative mass spectrometer response to CoQ and CoQH\textsubscript{2} must be stable over a range of concentrations and ratios of both forms. To see if this was the case, the peak areas following analysis of equal amounts of CoQ and CoQH\textsubscript{2} at a range of increasing total concentrations were compared (Figure 3a). This analysis indicated that both redox forms of CoQ\textsubscript{9} and CoQ\textsubscript{10} had similar mass spectrometric responses. Furthermore, when samples containing a range of CoQH\textsubscript{2}/CoQ ratios were assessed the LC-MS/MS analysis also detected these ratios accurately (Figure 3b). As the CoQH\textsubscript{2}/CoQ ratio also determines the % reduction of the CoQ pool, we replotted these data as % reduction (Figure 3c) and also the reduction potential for the CoQ/CoQH\textsubscript{2} redox couple [24] (Figure 3d). Of note, for CoQH\textsubscript{2}/CoQ ratios of 100:1, in contrast to ratios ≤ 50:1, the measured value did not match that of the input sample, indicating that at very low CoQ reduction potentials some CoQH\textsubscript{2} oxidation will inevitably occur. However, these highly reduced ratios are beyond the biologically relevant range. These analyses showed that measurement of the uncorrected ratio of the mass spectrometric response to both redox forms enables simultaneous and accurate determination of the redox states of both the CoQ\textsubscript{9} and CoQ\textsubscript{10} pools. We also wanted to investigate if CoQH\textsubscript{2} has the potential to transfer electrons to CoQ following extraction, and thereby distort the measured ratios. To do this we mixed CoQ\textsubscript{9}H\textsubscript{2} and CoQ\textsubscript{10}, or CoQ\textsubscript{10}H\textsubscript{2} and CoQ\textsubscript{9}, in methanol containing 2 mM ammonium formate and incubated for 2 hours at 37˚C (Figure S2a,b). This showed some slow oxidation of CoQH\textsubscript{2}, but this was not associated with direct reduction of the ubiquinone of the other species (Figure S2a,b). This analysis showed that the oxidation of the ubiquinols was slow under our conditions, even at 37˚C, and that this oxidation was not associated with ubiquinone reduction. Therefore, during cold storage of the extracts oxidation of CoQH\textsubscript{2} will have minimal effect on the CoQH\textsubscript{2}/CoQ ratio.

2.3 CoQ\textsubscript{9} and CoQ\textsubscript{10} quantification

The above analysis showed that the CoQH\textsubscript{2}/CoQ redox ratio could be measured without the requirement for standard curves, simplifying the analysis and removing potential confounding factors of ubiquinol oxidation during standard curve preparation. However, it is also important to determine the sizes of the CoQ\textsubscript{9} and CoQ\textsubscript{10} pools. To do this we used \textit{d}_{6}-CoQ\textsubscript{10} as an IS which was synthesised by base-catalysed exchange of the methoxy groups of CoQ\textsubscript{10} dissolved in hexane-CD\textsubscript{3}OD. This generated pure compound that could be used as an
internal standard. The two-stage preparation described previously [19] proved unnecessary. Using \( d_6\)-CoQ\(_{10}\) as IS we constructed standard curves for CoQ\(_9\) and CoQ\(_{10}\) (Figure 4a,b). This enables the total amounts of CoQ\(_9\) and CoQ\(_{10}\) in a sample to be determined, in parallel with measurement of their redox ratios.

2.4 Measurement of the CoQ redox state in mitochondria and cells

Next, we extended the CoQ extraction method to the analysis of isolated mitochondria and cells. For bovine heart mitochondrial membranes and isolated rat heart mitochondria we used a two-phase extraction with ice-cold hexane and acidified methanol (Figure 5a). This precipitated proteins and retained many potential redox active polar metabolites in the aqueous-methanol phase, while acidification helped prevent oxidation of ubiquinol. Finally, extracting the CoQ pool into hexane generated a stable solution which was then processed for mass spectrometry.

To assess the efficacy of this procedure we measured the CoQ redox state in bovine mitochondrial membranes, which contain predominantly CoQ\(_{10}\), respiring on NADH or succinate in the presence of various inhibitors (Figure 5b). Membranes respiring on NADH or succinate had a CoQ pool of about 56 – 60% reduced. With NADH the complex I inhibitor rotenone decreased this to ~ 4% reduced (Figure 5c), while with succinate the complex II inhibitor malonate also maintained an oxidised CoQ pool of ~12% reduced (Figure 5d). Addition of the complex IV inhibitor KCN led the CoQ pool to be ~ 80% reduced (Figure 5c,d). Incubating membranes at 37˚C in the absence of substrates led to complete oxidation of the CoQ pool, which can subsequently be reduced to ~ 60% by NADH (Figure 5e).

We next examined isolated rat heart mitochondria, which contain mainly CoQ\(_9\). Mitochondria respiring on glutamate/malate to generate NADH led to reduction of the CoQ pool (Figure 5f). The CoQ pool was oxidised by blocking complex I with rotenone and increased to ~77% reduction upon addition of KCN. Using succinate as a substrate also led to CoQ reduction that was decreased by malonate and increased by KCN. Addition of the uncoupler FCCP to abolish \( \Delta p \) and thereby stimulate respiration oxidised the CoQ pool (Figure 5g).

To assess CoQ in a monolayer of C2C12 cells in culture we first rapidly washed the cell layer and then scraped the cells into cold PBS before extraction. As C2C12 cells are derived from mice, CoQ\(_9\) is the predominant form of CoQ. Cells showed a similar steady state CoQ redox state to isolated mitochondria, and this was oxidised by blocking complexes
I and II with rotenone and TTFA, respectively (Figure 5h). Uncoupling mitochondria with
FCCP oxidises the CoQ pool due to increased respiration, and blocking complex III with
antimycin led to reduction of the CoQ pool (Figure 5h).

In all three systems, there was a dominant CoQ species, either CoQ$_9$ or CoQ$_{10}$, along
with a far smaller amount of the other form. The redox state of the major form is reported
above, but the method detected both forms accurately. In all cases the redox state of the
major and minor CoQ species were the same, suggesting that both pools come to the same
redox state (Figure S3a-f).

2.5 Measurement of the CoQ redox state in vivo
We next extended the two-phase extraction to assess CoQ redox state in vivo. To do this we
focused on the mouse heart and liver as these are important tissues in which the CoQ redox
state is important in health and disease. In assessing tissues, it is vital to freeze the tissue as
rapidly as possible so that the measured CoQ redox state accurately reflects that in vivo. To
illustrate this, heart or liver tissue was excised either from terminally anaesthetised mice after
thoracotomy, or after cervical dislocation and then rapidly frozen in a Wollenberger clamp at
liquid nitrogen temperature. The frozen tissue was then homogenised in ice-cold acidified
methanol and hexane and analysed by LC-MS/MS (Figure 6a).

We first determined how well this procedure extracted CoQ from the tissue (Figure
6b, S4a,b). This was done by re-extracting the heart and liver tissue and seeing how much
residual CoQ was extracted (Figure 6b, S4a,b). This showed that the first extraction removed
~75-80% of the CoQ pool. While subsequent extractions could remove a bit more, it was
decided not to include a second extraction routinely.

We next quantified the CoQ pool in heart and liver and found ~1.2 nmol CoQ$_9$ and
~0.12 nmol CoQ$_{10}$ per mg protein in heart (Figure 6c). In the liver the CoQ pools are much
smaller with ~250 pmol CoQ$_9$ and ~6 pmol CoQ$_{10}$ per mg protein (Figure 6c). These values
are consistent with the previously reported values [25,26]. Interestingly the CoQ$_9$/CoQ$_{10}$ ratio
varied significantly between organs, being ~10 in heart and ~42 in liver (Figure 6d).

An important constraint in analysing the CoQ redox state is the oxidation of extracts
by air before analysis. This often requires the rapid analysis of sample which are processed in
small batches. To see if this was a concern, we reanalysed the CoQ redox state of samples
stored in the LC-MS/MS autosampler at 8˚C for up to 24 hours in argon-flushed tubes. The
CoQ redox state was stable for at least 24 hours in the autosampler, confirming that large
sample sets can be analysed in one run without artefactual oxidation (Figure 6e, S4c). The
CoQ redox state was stable without argon flushing (Figure S4d). Nevertheless, we continued to flush samples with argon as a precaution. We next investigated the stability of the CoQ redox state of extracts during long-term storage at -20˚C in argon-flushed tubes. This showed that the CoQ redox state was stable upon storage for up to at least 65 days (Figure 6f). This allows to store and combine separate CoQ extractions in a single analysis run.

We noticed that CoQ extracts from mouse hearts were far more reduced than previously reported values (Figure 6e) [18]. Consequently, we wanted to assess whether there was any artifactual reduction of CoQ during extraction. To do this we set up a detection protocol for $d_6$-CoQ$_{10}$ and $d_6$CoQ$_{10}$H$_2$ as described in section 2.1 (Figure S4e,f). We then measured the reduction of $d_6$-CoQ$_{10}$ when it was spiked into the tissue extraction solution and detected ~6% of the added $d_6$-CoQ$_{10}$ as $d_6$-CoQ$_{10}$H$_2$ in our extracts (Figure S4g). In a mock extraction without tissue ~0.8% of the added $d_6$-CoQ$_{10}$ was converted to $d_6$-CoQ$_{10}$H$_2$. No signal for $d_6$-CoQ$_{10}$ or $d_6$-CoQ$_{10}$H$_2$ was detected in unspiked tissue extracts (Figure S4g). Therefore, we conclude that a small amount of CoQ reduction can occur during extraction. However, completely oxidised $d_6$-CoQ$_{10}$ is thermodynamically much more prone to reduction than the already largely reduced CoQ pools found in vivo and even if the oxidized CoQ pool was underestimated by 5% this would have a minor effect on the overall redox state.

Next, we wanted to determine the CoQ redox state in clamp frozen heart. In mice killed by cervical dislocation the CoQ pool was highly reduced at 89% and remained at a similar redox state even after 30 minutes of warm ischaemia (Figure 6g, S5a). To explore whether the tissue collection time from cervical dislocation to clamp-freezing has an effect on CoQ redox state, we next assessed the level of CoQ redox state in hearts obtained from terminally anaesthetized mice. In these mice the heart was excised directly after chest opening. This enabled a still-beating heart to be rapidly frozen and clamped. This gave a CoQH$_2$ % reduction of 85% (Fig S5b), similar to that from hearts excised from animals after cervical dislocation. The redox state of the CoQ$_{10}$ pool was also similar indicating that, as mentioned above, the two CoQ pools are bioenergetically equivalent (Fig 6g, S5a,b). The CoQ pool can be fully oxidised by homogenizing the heart in buffer and incubation at 37˚C for 1 hr (Figure 6h, S5c). We also determined the CoQ redox state in freshly excised and ischaemic liver and found it to be highly reduced at 92% (Figure 6i, S5d). As in the heart we also did not observe any significant changes in the CoQ redox state during ischaemia in liver (Figure 6i, S5d). For all tested tissues the LC-MS/MS traces were clean and the retention times of the individual CoQ species matched those of pure CoQ (Figure S5e).
3. CONCLUSIONS

Changes in the CoQ redox state have broad implications in the regulation of metabolic processes, respiration and mitochondrial ROS production. The difficulty in determining the CoQ redox state in biological samples and in vivo have been a significant impediment for the characterisation of CoQ function within mitochondria under physiological and pathophysiological conditions. Here we have developed a sensitive and comparable approach to assess the CoQ redox state by LC-MS in isolated mitochondria, cells and tissues. Applying this approach, we are able to detect CoQ and CoQH$_2$ at equal sensitivity, allowing relative quantification without the need for a range of internal standards, an improvement on previous methods, which used standard curves for both CoQ and CoQH$_2$. In parallel, the total CoQ pool size can be determined using only a standard curve of oxidised CoQ. In contrast to related approaches [18], we found that while the redox states of CoQ$_9$ and CoQ$_{10}$ can be manipulated with inhibitors and uncouplers they were essentially the same during normal conditions in a range of systems. Furthermore, we found that the CoQ redox state is highly (~90%) reduced in tissues in vivo, something that was not found in other studies, perhaps due to oxidation of the CoQ pool during extraction and processing [18]. Thus our method enables the redox state of the CoQ pool in vivo to be determined more accurately than hitherto. Our protocol opens up new possibilities to unravel the role of the CoQ redox state in vivo to understand the interplay between metabolism and bioenergetics in physiological and pathophysiological processes.

4. MATERIALS AND METHODS

4.1 Materials

All chemicals were purchased from Sigma unless otherwise stated. LC-MS grade Methanol was purchased from Fisher. All buffers, except LC-MS buffers, were stored over Chelex-100. Acidified solvents were prepared by addition of 0.1% (w/v) HCl.

4.2 Synthesis of $d_6$-CoQ$_{10}$

Sodium hydroxide (400 µL, 1 M in CD$_3$OD) was added to a solution of CoQ$_{10}$ (400 mg, 0.46 mmol, 1.0 eq) in hexane (5 mL) and $d_4$-MeOD (4 mL). The solution was stirred at RT for 6 h then quenched with acetic acid. The solution was extracted into diethyl ether (~20 mL), washed with brine (2 × 75 mL), dried over sodium sulfate and concentrated under vacuum.
The residue was purified by automated column chromatography using a 25g SNAP ultra
cartridge on a Biotage Isolera using gradient elution from CH$_2$Cl$_2$:Hexane (0:100) to (80:20).
The product was recrystallised from isopropanol-diethyl ether to give the labelled CoQ$_{10}$ as
an orange solid (162 mg, 40%). $\delta^H$ (400 MHz: CDCl$_3$): 5.13-5.04 (9H, m, 9 × CH=), 4.94
(1H, tq, $J = 7.1 + 1.4$ Hz, CH=), 3.18 (2H, d, $J = 7.0$ Hz, CH$_2$), 2.10-2.03 (16H, m, 8 × CH$_2$),
2.01 (3H, s, CH$_3$), 2.00-1.93 (20H, m, 10 × CH$_2$), 1.74 (3H, s, CH$_3$), 1.68 (3H, s, CH$_3$), 1.60
(21H, broad s, 7 × CH$_3$), 1.58 (3H, s, CH$_3$). m/z (ESI): Found: 891.7120. C$_{59}$H$_{84}$D$_6$NaO$_4$
requires (M+Na)$^+$, 891.7108.

4.3 Calculation of CoQ reduction potential
The CoQ reduction potential was calculated using the following equation, assuming a matrix
pH of 7.7 [24]. $E_h$ for the CoQ/CoQH$_2$ couple in mV is:
$$E_h \left( \frac{\text{CoQ}}{\text{CoQH}_2} \right) = -38 + 30.5 \log_{10} \left( \frac{\text{CoQ}}{\text{CoQH}_2} \right)$$

4.4 Preparation of mitochondria and mitochondrial membranes
Bovine heart mitochondrial membranes were kindly provided by Dr Hiran Prag and Prof
Judy Hirst (MRC MBU) and were prepared as described previously [27]. Female Wistar rats
(Charles River) of 10-12 weeks of age were killed by stunning, followed by cervical
dislocation. Rat hearts were homogenized in STEB (250 mM sucrose, 5 mM Tris-HCl, 1 mM
EGTA, 0.1% fatty acid free BSA, pH 7.4, 4°C). Mitochondria were isolated by differential
centrifugation (2x 700 x g for 5 min, 2 x 5.500 x g for 10 min) at 4°C and the protein content
was determined by the bicinchoninic acid assay using the Pierce™ BCA Protein assay kit
with bovine serum albumin as a standard.

4.5 Mouse heart and liver experiments
All mouse experiments were carried out in accordance with the UK Animals (Scientific
Procedures) Act 1986 and the University of Cambridge Animal Welfare policy (Project
license 70/8238). Male mice (8-10 weeks; C57BL/6J; Charles River Laboratories) were
terminally anaesthetized with sodium pentobarbital injected intraperitoneally and ventilated
with O$_2$. Core temperature was monitored continuously via a rectal probe. Alternatively, mice
were culled by cervical dislocation. A sternal thoracotomy was performed (heart) or the
abdomen was opened (liver) and the organ was rapidly excised and then frozen with a
Wollenberger clamp cooled in liquid nitrogen followed by storage at -70°C. Ischaemia was
induced by excising the organ and keeping it in the abdomen or chest of the warmed mouse
for indicated lengths. Organs were then frozen and stored as described.

4.6 Reduction of ubiquinone
Ubiquinones (CoQ₉, CoQ₁₀ and d₆-CoQ₁₀) were reduced to ubiquinols using sodium
borohydride. All glassware was rinsed in hexane and dried under nitrogen before use. CoQ₉
or CoQ₁₀ were made up to 10 mM in hexane, then diluted 1:10 in hexane. Solid sodium
borohydride was added (2 mg/100 µl) to the CoQ dilution followed by addition of methanol
(to 5% v/v), vortexing for 3 min and incubation in the dark for 5 min. The reaction was
stopped by addition of 1 vol. acidified H₂O with vortexing for 1 min followed by
centrifugation (1500 x g, 5 min at 4°C). The CoQ-containing upper hexane layer was
transferred to a glass vial, overlaid with argon and stored at -20°C. UV scanning spectra
confirmed reduction and enabled the concentration to be calculated: ε₂₉₀ = 4.1 mM⁻¹ cm⁻¹
(CoQ₉H₂), 4.0 mM⁻¹ cm⁻¹ (CoQ₁₀H₂); ε₂₇₅ = 14.7 mM⁻¹ cm⁻¹ (CoQ₉), 14.6 mM⁻¹ cm⁻¹ (CoQ₁₀)
[28].

4.7 Mass spectrometry
The mass spectrometric fragmentation patterns for the ammonium adducts of each CoQ
CoQH₂ compound were determined by direct infusion of 10 µM of the compound in 2 mM
ammonium formate in methanol at 2 µl/min into a triple quadrupole mass spectrometer
(Waters Xevo TQ-S). Electrospray ionisation in positive ion mode was used as published
previously with the following settings [29]: capillary voltage – 1.7 kV; cone voltage – 30 V;
ion source temperature – 100°C; collision energy – 22 V. Nitrogen and argon were used as
the curtain and the collision gases, respectively.

4.8 Liquid chromatography tandem mass spectrometry
LC-MS/MS analyses were carried out using an I-Class Acquity LC system attached to a
Xevo TQ-S triple quadrupole mass spectrometer (Waters) using published parameters [29].
Samples were kept at 8°C prior to injection by the autosampler of 2-10 µl into a 15 µl flow-
through needle and RP-HPLC at 45°C using an Acquity C18 column (2.1x50 mm, 1.7 µM;
Waters). Mobile phase was isocratic 2 mM ammonium formate in methanol run at 0.8 ml/min
over 5 min. For MS analysis, electrospray ionization in positive ion mode was used as
described above. Multiple reaction monitoring in positive ion mode was used for compound
Transitions used for quantification were: CoQ₉, 812.9 > 197.2; CoQ₁₀, 880.9 > 197.2; CoQ₉H₂, 814.9 > 197.2; CoQ₁₀H₂, 882.9 > 197.2; d₆-CoQ₁₀, 887.0 > 203.1; d₆-
CoQ₁₀H₂, 889.0 > 203.1. Standard curves were prepared using known amounts of CoQ, which were spiked with d₆-CoQ₁₀ (IS). Standards and samples were quantified using
MassLynx 4.1 software to determine the peak area for CoQ₉, CoQ₁₀, CoQ₉H₂, CoQ₁₀H₂, d₆-
CoQ₁₀ and d₆-CoQ₁₀H₂, and the standard curves used to determine the total amount of CoQ present in samples.

4.9 Mitochondrial Incubations
Mitochondrial membrane or rat heart mitochondria were incubated at 15 µg protein in 100 µl KPi buffer (50 mM KPi, pH 7.8) or KCl buffer (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2), respectively. Substrates and inhibitors were added at the following concentrations where indicated: 1 mM NADH, 1 mM succinate, 1 mM glutamate/malate, 0.5 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 0.5 µM rotenone, 1 mM malonate, 1 mM KCN. Samples were incubated with shaking for 5 min or 20 min at 37°C.

4.10 Cell incubations
C2C12 mouse myoblast cells (ATCC, UK) were cultured at 37°C under a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM/4.5 g/l glucose + GlutaMAX, Gibco, UK) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100mg/ml streptomycin. Cells (100,000) were plated 24 hrs prior to experiment in 6-well plates with 2 ml medium. For experiments inhibitors were added at the following concentrations: 1 µM rotenone, 1 mM theonoyltrifluoroacetone (TTFA), 5 µM FCCP, 5 µM Antimycin A. Cells were then incubated for 5 min at 37°C, washed with PBS and scraped in 200 µl fresh PBS on ice prior to CoQ extraction.

4.11 CoQ extraction
To extract CoQ from mitochondrial membranes and mitochondria, incubations of 100 µl containing 15 µg protein were added to ice cold extraction solution (200 µl acidified methanol and 200 µl hexane) followed by vortexing. To extract CoQ from cells, the cell suspension in 200 µl PBS was transferred to ice cold extraction solution (200 µl acidified methanol and 300 µl of hexane) followed by vortexing. To extract CoQ from tissues, 5 mg frozen tissue was weighed into cooled lysis tubes (Precellys, CK-14) on dry ice. Then a mixture of 250 µl ice-cold acidified methanol and 250 µl hexane was added, and tissue was
homogenised in the bullet blender (Bertin Instruments, Precellys 24) at setting 6500 during 15 seconds (CK14 (1.4 mm) beads). The homogenate was transferred to fresh 1.5 ml Eppendorf tubes. In all cases the upper, CoQ-containing hexane layer was separated by centrifugation (5 min, 17,000 x g, 4°C) and then dried down in 1 ml glass mass spectrometry vials (186005663CV, Waters) under a stream of N₂. Dried samples were then resuspended in methanol containing 2 mM ammonium formate and diluted (heart 1:10, liver not dilution: CoQ₁₀ or 1:3 dilution CoQ₉), overlaid with argon and stored at -20°C until analysis. The extracts were stable under storage for up to at least 15 days.

4.12 CoQ extraction for CoQ pool size determination
To extract CoQ from tissues, 5 mg frozen tissue was weighed into cooled lysis tubes (Precellys, CK-14) on dry ice. Then 150 µl KPi buffer (50 mM KPi, pH 7.8) was added and tissue was homogenised in the bullet blender (Bertin Instruments, Precellys 24) at setting 6500 during 15 seconds (CK14 (1.4 mm) beads). CoQ was extracted from 100 µl of the homogenate by adding a mixture of 250 µl ice-cold acidified methanol and 250 µl hexane and vortexing. The upper, CoQ-containing hexane layer was separated by centrifugation (5 min, 17,000 x g, 4°C) and then dried down in 1 ml glass mass spectrometry vials (186005663CV, Waters) under a stream of N₂. Dried samples were then resuspended in methanol containing 2 mM ammonium formate. Heart tissue was diluted 1:10 (20 µl in 170 µl methanol containing 2 mM ammonium formate and 10 µl of 5 µM d₆-CoQ₁₀ internal standard), while liver homogenate was either not diluted (CoQ₁₀ analysis) or diluted 1:3 (CoQ₉ analysis). Samples were overlaid with argon and stored at -20°C until analysis.

ACKNOWLEDGEMENTS

Work in the MPM laboratory was supported by the Medical Research Council UK (MC_U105663142) and by a Wellcome Trust Investigator award (110159/Z/15/Z) to MPM. Work in the RCH lab laboratory was supported by a Wellcome Trust Investigator award (110158/Z/15/Z). AM received funding from the Swedish Research Council.

APPENDIX A. Supplementary data

ABBREVIATIONS
\( \Delta p \)  
mitochondrial protonmotive force

CoQ\(_{10}\)  
coenzyme Q\(_{10}\)

CoQ\(_{10}H_2\)  
reduced Q\(_{10}\)

CoQ\(_9\)  
coenzyme Q\(_9\)

CoQ\(_9H_2\)  
reduced Q\(_9\)

ETC  
electron transfer chain

IS  
internal standard

LC-MS/MS  
liquid chromatography tandem mass spectrometry

MRM  
multiple reaction monitoring

RET  
reverse electron transfer

ROS  
reactive oxygen species

---

**REFERENCES**


FIGURE LEGENDS

Figure 1: The CoQ pool in the mitochondrial inner membrane as essential link between metabolic pathways and cellular energy supply.

Various mitochondrial enzymes of diverse metabolic function feed electrons into the CoQ pool of the inner mitochondrial membrane. The electrons are then relayed to complex III of the ETC and thereby coupled to the maintenance of ∆p and ATP production. a Complex I (NADH:Ubiquinone oxidoreductase) and complex II (succinate dehydrogenase) of the ETC chain are the canonical enzymes feeding electrons into the CoQ pool under normal conditions. The electrons are shuttled to complex III (ubiquinol:cytochrome c oxidoreductase) to be transferred onto cytochrome c and finally oxidised by complex IV (cytochrome c oxidase). By pumping protons from the matrix into the intermembrane space, complexes I, III & IV establish the mitochondrial protonmotive force (∆p), which is used for ATP synthesis by complex V (ATP-synthase) and various transport processes across the inner membrane. b The two electron carrier ubiquinone is a 1,4-benzoquinone, linked to an isoprene tail which in mammals consists of 9-10 isoprenyl subunits. Ubiquinone is reduced to ubiquinol by two electrons and acts as electron shuttle and antioxidant. c Numerous other mitochondrial oxidoreductases, such as SQR (hydrogen sulfide:ubiquinone oxidoreductase, catabolism of hydrogen sulphide), DHODH (dihydroorotate dehydrogenase, pyrimidine biosynthesis), CHDH (choline dehydrogenase, choline oxidation), G3PDH (glycerol 3-phosphate dehydrogenase, glycerol-3-phosphate shuttle), ETF-QO (electron-transferring-flavoprotein dehydrogenase, fatty acid oxidation) and PRODH (proline dehydrogenase, catabolism of proline) feed electrons into the mitochondrial CoQ pool. The electrons are funnelled by CoQ via complex III and cytochrome c to complex IV to reduce O2 to H2O.

Figure 2: Method development for the detection of CoQ9/10 and CoQ9/10H2 by LC-MS/MS

a The absorbance CoQ9 and CoQ10 were characterised by spectral scanning before and after reduction with NaBrH4. Upon reduction, the characteristic absorption maximum shifts from 275 to 290 nm. b Representative precursor and product MS scans of CoQ9 and CoQ10 stocks and their reduced forms as well as the deuterated d6-CoQ10 internal standard. Precursor scans of the characteristic fragment products were performed. The product scans were performed by fragmenting the ammonium adduct precursors. Upon fragmentation the CoQ precursor loses its isoprene side chain and forms a tropylium ion. c Representative LC-MS/MS chromatograms showing the m/z transitions measured simultaneously for 0.5 pmol of CoQ9,
CoQ<sub>10</sub>, CoQ<sub>9</sub>H<sub>2</sub>, CoQ<sub>10</sub>H<sub>2</sub> and <sup>6</sup>CoQ<sub>10</sub>. Traces are normalised to the highest peak for each sample. CoQ<sub>9</sub> and CoQ<sub>10</sub> show bleed through into the transitions for CoQ<sub>9</sub>H<sub>2</sub> and CoQ<sub>10</sub>H<sub>2</sub>, respectively. Bleed through of the CoQ<sub>9</sub> and CoQ<sub>10</sub> signals has been projected in red onto the CoQ<sub>9</sub>H<sub>2</sub> and CoQ<sub>10</sub>H<sub>2</sub> traces to show they are separated by LC.

Figure 3: Determining and quantifying the ratio of CoQ and CoQH<sub>2</sub> by LC-MS/MS

To validate the quantification of oxidised and reduced CoQ, CoQ<sub>9/10</sub> and borohydride reduced CoQ<sub>9/10</sub>H<sub>2</sub> stocks were mixed in predetermined ratios and measured by LC-MS/MS. a Detected peak areas of increasing amounts of CoQH<sub>2</sub> and CoQ mixed in a 1:1 ratio. b The detected CoQH<sub>2</sub>/CoQ ratios were blotted against the expected theoretical CoQH<sub>2</sub>/CoQ ratios. c The proportion of CoQH<sub>2</sub> was blotted against the expected theoretical CoQH<sub>2</sub>/CoQ ratios. d The redox potential of CoQH<sub>2</sub>/CoQ mixtures was calculated based on the detected CoQH<sub>2</sub>/CoQ ratios and was blotted against the expected theoretical CoQH<sub>2</sub>/CoQ ratios. The calculations are outlined in the methods section. Data are mean ± S.D. of 3 replicates.

Figure 4: Standard curve for the quantification of the CoQ pool size by LC-MS/MS

Representative standard curve for oxidised CoQ<sub>9</sub> (a) and CoQ<sub>10</sub> (b). The MS response of increasing concentrations of CoQ<sub>9</sub> and CoQ<sub>10</sub> was compared to 0.5 pmol of <sup>6</sup>CoQ<sub>10</sub> (internal standard, IS). Data are mean ± S.D. of 3 replicates. The grey-shaded section of the standard curve on the left is expanded and replotted in the right panel.

Figure 5: Extracting CoQ from biological samples and modifying the CoQ redox state in mitochondrial membranes, mitochondria and cells

a A two-phase extraction with acidified methanol and hexane was used to extract CoQ from bovine mitochondrial membranes, mitochondria and cells. CoQ from incubations of biological samples was extracted by vortexing in extraction solution, phase separation by centrifugation and resuspending the extract in MS sample buffer (methanol with 2 mM ammonium formate). b Various inhibitors can be used to manipulate the mitochondrial CoQ redox state in biological samples. c-h Bovine heart mitochondrial membranes (BHMM), rat heart mitochondria (RHM) and C2C12 cells were incubated in KPi (BHMM), KCl (RHM) buffer or DMEM (cells) and different combinations of substrates and inhibitors were added before CoQ extraction and LC-MS/MS analysis of the CoQ redox state. All incubations were performed for 5 minutes at 37°C except otherwise indicated. CoQ redox state of: c BHMM
incubated with NADH or NADH combined with rotenone or KCN. d BHMM incubated with
succinate or succinate combined with malonate or KCN. e BHMM incubated for 20 min at
37°C. NADH or rotenone + NADH was added to indicated samples after 15 minutes. f RHM
incubated with glutamate + malate (GM) or GM combined with rotenone or KCN. g RHM
incubated with succinate or succinate combined with malonate, FCCP or KCN. h C2C12
cells incubated in standard DMEM with FCCP, rotenone, TTFA or Antimycin A.
For all experiments, data are represented as mean ± S.D. of 3 replicates. The proportion of
CoQH$_2$ is shown for the indicated most prevalent CoQ species.

Figure 6: Extracting CoQ from tissues and determining the CoQ redox state
a A two-phase extraction with acidified methanol and hexane was used to extract CoQ from
tissues. CoQ from clamp frozen tissue samples was extracted by homogenising in extraction
solution, phase separation by centrifugation and resuspending the extract in MS sample
buffer (methanol with 2 mM ammonium formate). b CoQ was extracted from mouse heart
and liver tissue homogenates and the tissue homogenate was then reextracted. The peak areas
for CoQ and CoQH$_2$ were combined and the cumulative proportion combined with all
previous extractions of the total is shown. Data are shown as mean ± S.D. of 6 different
samples from 3 different animals. c The CoQ pool size of CoQ$_9$ and CoQ$_{10}$ in mouse heart
and liver. Data are shown as mean ± S.D. of 6 different samples from 3 different animals. d
The CoQ$_{9}$/CoQ$_{10}$ ratio in mouse heart and liver. Data are shown as mean ± S.D. of 6 different
samples from 3 different animals. e CoQ redox state changes over time (at 8˚C) during
several consecutive LC-MS runs (~80 min per run per sample set; final run at 24 hours) in
CoQ extracts of control and ischaemic mouse hearts. The proportion of CoQH$_2$ is represented
as mean ± S.D. of 3 different hearts. f CoQ redox state changes during long term storage for
up to 65 days (at -20˚C) in CoQ extracts of control mouse hearts. The proportion of CoQH$_2$ is
represented as mean ± S.D. of 3 different hearts. g CoQ redox state of control and ischaemic
mouse heart. The proportion of CoQH$_2$ is represented as mean ± S.D. of 3 different hearts. h
CoQ redox state of mouse heart tissue and tissue homogenates of the same hearts, after
oxidising the CoQ pool in KPi buffer for 1 hr at 37˚C. The proportion of CoQH$_2$ is
represented as mean ± S.D. of 3 different hearts. i CoQ redox state of control and ischaemic
(10 and 30 min) mouse liver. The proportion of CoQH$_2$ is represented as mean ± S.D. of 3
different livers.
Highlights

- Sensitive LC-MS/MS assay to quantify CoQ redox state
- Changes in CoQ redox state can be measured in mitochondria, cells and in vivo
- CoQ redox state is stable during extraction and analysis