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STUDIES ON REDUCED AND OXIDIZED COENZYME Q (UBIQUINONES)

II. THE DETERMINATION OF OXIDATION-REDUCTION LEVELS OF COENZYME Q IN MITOCHONDRIA, MICROSOMES AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Reduced and oxidized coenzyme Q_{10} ($Q_{10}H_2$ and Q_{10}) in guinea-pig liver mitochondria were rapidly extracted and determined by high-performance liquid chromatography (HPLC). The percentages of $Q_{10}H_2$ as compared to the total (sum of Q_{10} and $Q_{10}H_2$) were increased by the addition of respiratory substrates such as succinate, malate and β-hydroxybutyrate (State 4). The levels of $Q_{10}H_2$ in State 4 were increased more extensively with electron-transport inhibitors such as KCN, NaN_3 and antimycin A. These results indicate that the method for determining $Q_{10}H_2$ and Q_{10} by HPLC is quite useful for investigation of the physiological function of coenzyme Q in mitochondria and other organelles. The reduced and oxidized coenzyme Q levels of rat liver mitochondria, which contain both coenzyme Q_9 and coenzyme Q_{10}, were measured simultaneously. The results suggest that coenzymes Q_9 and Q_{10} play a similar role as an electron carriers. The liver microsomes of guinea-pig contained approx. 133 nmol total coenzyme Q_{10} per g protein. The $Q_{10}H_2$ levels of microsomes were increased from 46.5 to 67.5 and 64.8% with NADH and NADPH, respectively. The plasma levels of total coenzyme Q were 0.92 μg/ml for man, 0.35 μg/ml for guinea-pig and 0.27 μg/ml for rat. The reduced coenzyme Q were also present in those plasma samples. The levels of reduced coenzyme Q were 51.1, 48.9 and 65.3%, respectively.

Introduction

A function of coenzyme Q in mitochondria which is well known is its behavior as a mobile carrier in the respiratory chain which shuttles reducing equivalents from the dehydrogenase complexes to the cytochrome b-c_1 complex [1]. It has been suggested that reduced coenzyme Q might act as an anti-oxidant of lipid peroxidation in bovine heart mitochondria [2]. While the physiological significance of mitochondrial coenzyme Q is well characterized [3,4], no comparison of the role of coenzyme Q homologues has been made. It would be interesting to know whether coenzyme Q_9 and coenzyme Q_{10} in rat mitochondria show similar reduction levels.

Coenzyme Q is also a constituent of microsomes in which oxidative and hydroxylative enzymes such as NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 are present [5,6]. Reduction of endogenous coenzyme Q in microsomes has not yet been investigated, since the content of coenzyme Q in microsomes are quite low compared with those of mitochondria, and suitable methods for the determination

Abbreviations: Q, the general term for oxidized coenzyme Q homologues; QH_2, the general term for reduced coenzyme Q homologues; Q_9, oxidized coenzyme Q_9; Q_{10}, oxidized coenzyme Q_{10}; Q_9H_2, reduced coenzyme Q_9; $Q_{10}H_2$, reduced coenzyme Q_{10}; total Q_9, the sum of Q_9 and Q_9H_2; total Q_{10}, the sum of Q_{10} and $Q_{10}H_2$; HPLC, high-performance liquid chromatography.

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of reduced and oxidized coenzyme Q are lacking.

In previous papers [7,8], simultaneous determination of reduced and oxidized coenzyme Q in tissues and mitochondria by HPLC has been described. The present paper deals with some applications of the HPLC method to biological samples as follows: (1) reduced levels of coenzyme Q and its homologues in mitochondria; (2) reduced levels of coenzyme Q in microsomes; (3) reduced levels of coenzyme Q in plasma.

Materials and Methods

Chemicals

Coenzyme Q_9 and coenzyme Q_{10} were synthesized by our company and Nisshin Chemical Co., respectively. Reduced coenzyme Q_9 and coenzyme Q_{10} (Q_9H_2 and $Q_{10}H_2$) were obtained by the reduction of Q_9 and Q_{10} with sodium borohydride. ADP, antimycin A, sodium β-hydroxybutyrate, rotenone, NADH and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Malonic acid disodium salt (Tokyo Kasei Kogyo) sodium azide (Iwai Kagaku) and potassium cyanide (Kanto Chemicals) were used. Other reagents used were of analytical grade.

Preparation and incubation of mitochondria

Mitochondria were isolated from livers of male guinea-pigs and rats by differential centrifugation in 0.25 M sucrose using the method of Johnson et al. [9]. The isolated mitochondria were suspended in 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose at a protein concentration of 0.54–4.2 mg/ml which was determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

The mitochondrial suspensions were incubated at 25°C for predetermined time with various combinations of 2–5 mM succinate, 18.9 mM phosphate buffer (pH 7.4), 2.5 mM $MgCl_2$ or 0.26–0.73 mM ADP. In inhibition experiments, the mitochondrial suspensions were incubated with a substrate for 3 min, and then with 1.9 mM KCN, 1.0 mM NaN_3 or 0.44 μg antimycin A/mg protein. In the case of rotenone (4.0 μM) and malonate (4.0 mM), these inhibitors were first added to the suspensions and then a substrate was added.

Preparation and incubation of microsomes

The liver from a male guinea-pig was homogenized in ice-cold 10 mM Tris-HCl (pH 7.4)/0.25 M sucrose/5 mM $MgCl_2$/0.1 mM EDTA. The microsomal fraction was obtained as a precipitate after the centrifugation (105 000 × *g* for 60 min) of the 15 000 × *g* (15 min) supernatant of the homogenate. Succinate-cytochrome *c* reductase activity could not be detected in the microsomal fraction by the method of Green et al. [11]. The microsomes were suspended in the buffer described above at a protein concentration of 2.7 mg/ml. In experiments to reduce coenzyme Q, the microsomal suspensions were incubated at 37°C for 15 min with 0.5 mM NADH or NADPH. In order to check the possibility of the reduction of coenzyme Q due to contaminated mitochondria, 4.0 μM rotenone was first added to the suspensions, and then a reductant was added.

Extraction and analysis of Q and QH_2

1 ml of the mitochondrial or microsomal suspensions was rapidly denatured by adding 7.0 ml of a mixture of ethanol/*n*-hexane (2 : 5), and the tube was then rapidly shaken for 10 min to extract Q and QH_2. This extraction was repeated three times. The combined *n*-hexane phase was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml ethanol or isopropanol and then subjected to the HPLC described below. In experiments with plasma, 1.0 ml portions of plasma samples which had been freshly and quickly prepared were diluted with 1.0 ml H_2O, and then 14 ml of the ethanol/*n*-hexane mixture were added to the diluted plasma. Subsequent procedures were as described for mitochondria, above.

HPLC analysis

HPLC was carried out as described previously [7,8]. The HPLC system was a YANACO L-2000 pump (Yanagimoto Manufactory Co., Ltd. Japan) with a Rheodyne loop injector. The ultraviolet and electrochemical detectors were a JASCO UVIDEC 100 ultraviolet detector (Japan Spectroscopic Co., Japan) and a YANACO VMD 101 electrochemical detector, respectively. The electochemical detector was connected to the outlet of the ultraviolet detector. Reverse-phase chromatography was carried out with a Nucleosil C-18 column (15 cm × 4.0 mm i.d., Machery-Nagel Co., 5 μm). The mobile phase was prepared by dissolving 7.0 g $NaClO_4 \cdot H_2O$ in 1000

ml ethanol/methanol/70% $HClO_4$ (700 : 300 : 1). The flow rate was 1.2 ml/min. The HPLC measurements were performed at 30 ± 0.1°C. In order to prevent oxidation by dissolved oxygen during column separation, the mobile phase was deaerated by bubbling nitrogen gas.

Measurement of oxygen consumption rate

The mitochondrial suspensions (10.8 mg/3 ml) was added into a 3.0 ml chamber and incubated at 25°C. The oxygen consumption rate in the suspensions was measured using an oxygen electrode with a reecorder (Yanagimoto).

Results

Reduced levels of coenzyme Q in mitochondria

For evaluation of the HPLC method to determine Q and QH_2 in biological samples, experiments were carried out using the mitochondria of guinea-pig, since the mitochondrial electron transfer system with Q has been well characterized.

Table I shows the reduced levels of Q_{10} in the liver mitochondria 3 min after adding succinate, and then 30 s after adding ADP. In this paper, the reduced level of Q and the level of QH_2 mean the percentage of QH_2 as compared to the sum of Q and QH_2. The levels of $Q_{10}H_2$ increased to 56.9% with succinate, then decreased to 26.2% with ADP. The levels increased again 3 min after adding ADP, since the ADP was exhausted. The levels were increased more extensively 15 min after adding ADP, since the oxygen was exhausted.

The metabolic states of mitochondria can be characterized by oxygen consumption rates which are measured by an oxygen electrode. It is important to compare levels of $Q_{10}H_2$ given by the HPLC analysis to states characterized by an oxygen electrode. Fig. 1 shows the changes in the oxygen consumption rate and of the levels of $Q_{10}H_2$. After the addition of succinate, State 4 was established by a gradual decrease of oxygen in the incubation medium. The level of $Q_{10}H_2$ at Stage 4 was high. The subsequent addition of ADP initiated State 3. This state showed a rapid decrease of oxygen in the medium, which corresponded to oxidation of $Q_{10}H_2$. After a certain period, when ADP was exhausted, the rate of oxygen consumption decreased slowly, and the mitochondria attained a new State 4. At the new State 4, the $Q_{10}H_2$ levels became higher than those at State 3. It is clear that the contents of $Q_{10}H_2$ and Q_{10} analyzed by HPLC are correlated with the various metabolic states in mitochondria as monitored by an oxygen electrode.

Table II shows effects of inhibitors in electron transport on $Q_{10}H_2$ levels in the liver mitochondria. The inhibitors used were KCN, NaN_3, antimycin A, malonate and rotenone. Since cyanide is powerful reversible inhibitor of cytochrome *c* oxidase [12], Q_{10} is expected to be greatly reduced by substrates such as succinate, malate and β-hydroxybutyrate. As shown in Table II, Q_{10} was greatly reduced and the

TABLE I

THE REDUCED LEVELS OF COENZYME Q_{10} IN GUINEA-PIG LIVER MITOCHONDRIA

The mitochondrial concentration was 0.54 mg/ml. Final concentrations of additives were succinate, 2 mM; ADP, 0.26 mM. The total amount of coenzyme Q_{10} was 1.55 μmol/g protein. Each value is represented as the average of two independent determinations.

Metabolic state	Addition	Time after adding ADP	$Q_{10}H_2$/total Q_{10} [a] (%)
2	none	–	18.3
4	succinate	–	56.9
3	succinate + ADP	30 s	26.2
4	succinate + ADP	3 min	51.2
5	succinate + ADP	15 min	84.7

[a] Total Q_{10} is the sum of $Q_{10}H_2$ and Q_{10}.

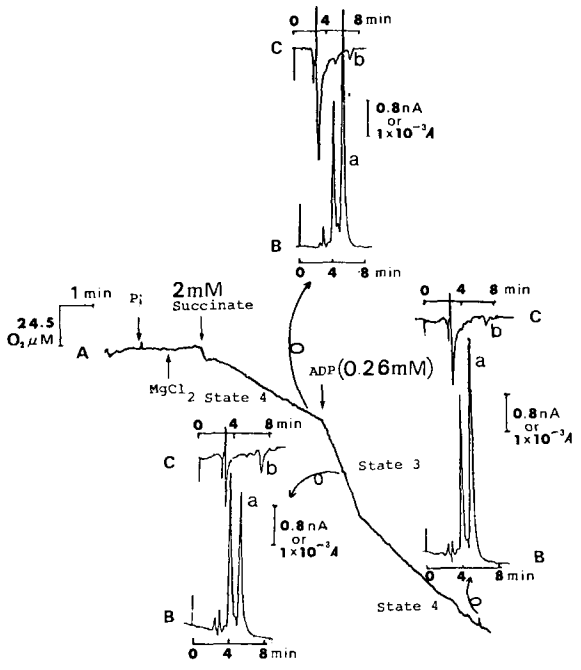

Fig. 1. Chromatograms of the extracts from mitochondria and oxygen concentration in the mitochondrial suspensions. The mitochondrial concentration was 10.8 mg/3 ml. A, oxygen concentration; B, HPLC-electrochemical detector (0.7 V vs. Ag|AgCl); C, HPLC-ultraviolet detector (275 nm); a, $Q_{10}H_2$; b, Q_{10}.

levels of $Q_{10}H_2$ reached approx. 85% in the presence of KCN. Antimycin A is known to inhibit the respiratory chain between cytochromes *b* and c_1. $Q_{10}H_2$ generated by succinate was increased by the addition of antimycin A to an extent similar to that found with cyanide. Malonate could not inhibit the reduction of Q_{10} with malate and β-hydroxyburyrate as NAD-linked substrates. Rotenone did not influence the reduction of Q_{10} with succinate, but it inhibited that of Q_{10} with malate and β-hydroxybutyrate.

TABLE II

EFFECT OF RESPIRATORY INHIBITORS ON THE LEVELS OF REDUCED COENZYME Q_{10} IN LIVER MITOCHONDRIA

Each value is represented as the average of two independent determinations. The mitochondrial concentration was 0.54 mg/ml.

Condition		$Q_{10}H_2$/total Q_{10} [a] (%)
Inhibitor	Substrate	
None	none	18.9
	succinate (2 mM)	57.9
	malate (2 mM)	49.1
	β-hydroxybutyrate (4.5 mM)	60.3
KCN	succinate	85.2
	malate	82.2
	β-hydroxybutyrate	84.1
NaN_3	succinate	80.0
Antimycin A	succinate	74.2
Rotenone	succinate	54.2
	malate	15.9
	β-hydroxybutyrate	12.4
Malonate	succinate	12.5
	malate	47.3
	β-hydroxybutyrate	58.1

[a] Total Q_{10} is the sum of $Q_{10}H_2$ and Q_{10}.

TABLE III

THE REDUCED LEVELS OF COENZYME Q_9 AND COENZYME Q_{10} IN RAT LIVER MITOCHONDRIA

The mitochondrial concentration was 4.2 mg/ml. Final concentrations of additives were: succinate, 5 mM; ADP, 0.73 mM; KCN, 1.9 mM. The total amounts of coenzyme Q_9 and coenzyme Q_{10} were 1.74 and 0.29 μmol/g protein, respectively. Each value is represented as the average of two independent determinations

Addition	Q_9H_2/ total Q_9 [a] (%)	$Q_{10}H_2$/ total Q_{10} [b] (%)
None	57.8	67.5
Succinate	74.9	81.3
Succinate + ADP	51.4	58.3
Succinate + KCN	83.3	89.7

[a] Total Q_9 is the sum of Q_9H_2 and Q_9.
[b] Total Q_{10} is the sum of $Q_{10}H_2$ and Q_{10}.

Reduced levels of coenzyme Q homologues in mitochondria of rat liver

In order to compare reduced levels of coenzyme Q homologues in mitochondrial respiratory chain, rat liver mitochondria, which contain both Q_9 and Q_{10}, were examined to determine levels of reduced quinone, Q_9H_2 and $Q_{10}H_2$. The contents of Q_9 and Q_{10} in mitochondria were 1.74 and 0.29 μmol/g protein, respectively. As shown in Table III, the levels of Q_9H_2 and $Q_{10}H_2$ increased to 74.9% and 81.3% 3 min after adding succinate, and then decreased to 51.4% and 58.3% with ADP, respectively. When KCN was added, most of the Q_9 and Q_{10} was reduced, and the levels of Q_9H_2 and $Q_{10}H_2$ reached 83.3% and 89.7%, respectively.

TABLE IV

REDUCTION OF MICROSOMAL COENZYME Q_{10}

Each value is represented as the average of two independent determinations.

Reductant	Addition	$Q_{10}H_2$/total Q_{10} [a] (%)
None	none	46.5
NADH	none	67.5
	rotenone	65.0
NADPH	none	64.8
	rotenone	66.2

[a] Total Q_{10} is the sum of $Q_{10}H_2$ and Q_{10}.

Reduction of coenzyme Q in microsomes

The liver microsomes of guinea-pig contained approx. 133 nmol Q_{10} per g protein. This value was 1/11 as compared with the level of liver mitochondria. Table IV shows the levels of $Q_{10}H_2$ in the liver microsomes 15 min after adding NADH or NADPH. The $Q_{10}H_2$ levels increased to 67.5% and 64.8% with NADH and NADPH, respectively. Rotenone was added to the incubation medium in order to prevent the reduction of Q_{10} which originated from mitochondria. As shown in Table IV, the reduction of Q_{10} with NADH or NADPH was not inhibited by rotenone.

TABLE V

PLASMA LEVELS OF REDUCED COENZYME Q

Q and QH_2 in rat and guinea-pig plasma are separated by the mobile phase prepared by dissolving 7.0 g $NaClO_4 \cdot H_2O$ in 1000 ml ethanol/methanol/H_2O/70 % $HClO_4$ (500 : 500 : 10 : 1). Each value is represented as the mean ± S.E. of the numbers of animals in parentheses. n.d., not detected.

Animals	$Q_{10}H_2$ (%)	Q_9H_2 (%)	Content (μg/ml)	
			Total Q_{10} [a]	Total Q_9 [b]
Man (6)	51.1 ± 4.2	n.d.	0.92 ± 0.12	n.d.
Guinea-pig (4)	48.9 ± 3.1	n.d.	0.35 ± 0.02	n.d.
Rat (4)	trace	65.3 ± 3.6	trace	0.27 ± 0.03

[a] Total Q_{10} is the sum of $Q_{10}H_2$ and Q_{10}.
[b] Total Q_9 is the sum of Q_9H_2 and Q_9.

Plasma levels of reduced and oxidized coenzyme Q

The extractability of Q_{10} and $Q_{10}H_2$ from human plasma was constant in the range of 92–98%. Similar results were obtained with plasma samples from guinea-pig and rat. The QH_2 in the residue after evaporation of the *n*-hexane extract was stable for 5 h when kept under N_2 gas. Once the residue was dissolved in isopropanol, however, the solution had to be injected into the HPLC column as soon as possible (within 30 min), since the level of QH_2 in the solution gradually decreased and was several percent lower 12 h after dissolution.

Table V shows the plasma levels of QH_2 in man, guinea-pig and rat. These levels were 51.1, 48.9 and 65.3%, respectively. The level of plasma in guinea-pig was similar to that of liver microsomes (46.5%, as shown in Table IV).

Discussion

The method of extracting Q and QH_2 followed by the HPLC analysis was evaluated using the liver mitochondria of guinea-pig. As shown in Table I, the $Q_{10}H_2$ levels increased after addition of succinate and decreased after addition of ADP. These changes of $Q_{10}H_2$ levels with succinate and ADP were clearly correlated with the mitochondrial metabolic states which were characterized by measuring the oxygen consumption rates as shown in Fig. 1. The $Q_{10}H_2$ levels measured by the HPLC method are good agreement with those analyzed by Klingenberg et al. [13] and Redfearn et al. [14]. These are many reagents which affect mitochondrial electron transport. Some of them are known to inhibit the electron transport at specific sites. The sites of inhibition of KCN, antimycin A, rotenone and malonate are well characterized, and therefore those inhibitors are used in these experiments. As shown in Table II, the inhibitors affected the mitochondrial $Q_{10}H_2$ levels in manners to be expected from their sites of action. All of the aforementioned results using the mitochondria of guinea-pig indicate that the method of extracting Q and QH_2, followed by HPLC analysis is useful and reliable in investigating the reduced levels of Q in the biological sample.

The Q_9, Q_{10}, Q_9H_2 and $Q_{10}H_2$ levels of the liver mitochondria of rats were simultaneously measured. As shown in Table III, the changes in $Q_{10}H_2$ levels were similar to those in Q_9H_2 levels, even though Q_{10} is a minor component of Q as compared with Q_9 in the rat mitochondria. This result provides the first evidence that Q_{10} plays the same role as Q_9 in liver mitochondrial electron transport.

Ramasarma et al. [15] reported that $[^{14}C]Q_{10}$ was detected in the liver microsomes after the injection of $[^{14}C]Q_{10}$ in rats, and $[^{14}C]Q_{10}$ in the microsomes was reduced with NADH or NADPH. As shown in Table IV, the $Q_{10}H_2$ level of the isolated microsomes was 46.5%, and the level increased in the presence of NADH or NADPH. This result indicates that endogenous Q_{10} in microsomes is reduced with NADH or NADPH. Microsomal electron transport system is well characterized, and coenzyme Q is not involved in the transport system [16]. However, coenzyme Q function has been proposed in electron transport in Golgi membranes, which contain most of the microsomal coenzyme Q in rat liver [17].

The QH_2 levels of plasma were measured and are shown in Table V. The human plasma contained 0.92 μg Q_{10} per ml, and 51.1% of the Q_{10} was in the reduced form. The $Q_{10}H_2$ levels of guinea-pig were 48.9% of those of the Q_{10} in the plasma. This value of $Q_{10}H_2$ in the plasma of guinea-pig is similar to that (46.5%) in the microsomes (Table IV). The plasma Q_{10} and $Q_{10}H_2$ in guinea-pig were located in low-density and high-density lipoprotein [18] which are synthesized in liver and secreted as very-low-density lipoprotein [19]. Therefore, it is suggested that microsomal Q_{10} and $Q_{10}H_2$ might be incorporated into very-low-density lipoprotein through the biosynthesis of the lipoprotein. In addition, lipoprotein secretion is through Golgi membranes, which may also contribute to the high coenzyme Q content in lipoprotein in plasma.

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