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A novel ubiquinone reductase activity in rat cytosol

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Ubiquinone (UQ) reductase activity which reduces UQ to ubiquinol (UQH_2) in rat tissues was roughly proportional to the UQH_2/total UQ ratio in respective tissues. The highest activity was found in the liver, showing the highest UQH_2/total UQ ratio. A greater part of liver UQ reductase activity was located in the cytosol. Within a week, the liver UQ reductase activity decreased by 80% even at −20°C. The DT-diaphorase activity was stable. UQ reductase required NADPH as the hydrogen donor and was not inhibited by a less than 1 μM concentration of dicoumarol. There was no stimulation of UQ reductase in the presence of bovine serum albumin nor in Triton X-100. Yet, both stimulated DT-diaphorase. As a result, UQ reductase appeared to be a novel NADPH-UQ oxidoreductase and responsible for the UQ redox state in liver.

NAD(P)H; DT-diaphorase; Cytosol (rat liver); Ubiquinone reductase; Subcellular organelle

1. INTRODUCTION

It is well-known that ubiquinone (UQ) functions as one of the indispensable electron carriers in the mitochondrial respiratory chain [1]. However, UQ is found not only in the mitochondria but also in almost all subcellular fractions [2,3]. In addition, some non-mitochondrial organelles can synthesize UQ de novo [3,4]. Recently Åberg et al. [5] reported that a greater part of UQ in rat and human tissues existed as the reduced hydroquinone form, ubiquinol (UQH_2). We also observed that 20 to 80% of total UQ found in non-mitochondrial fractions of rat tissues was UQH_2 (unpublished data). Furthermore, UQ administered orally or injected intraperitoneally in rats was also found mainly in the reduced form [6,7]. These findings in non-mitochondrial UQ and UQH_2 suggest that UQH_2 must play a physiologically important role outside the mitochondria and that the non-mitochondrial organelles must possess their own machineries for keeping UQH_2 levels. However, it is unknown where and how UQ in non-mitochondrial organelles is reduced to UQH_2.

In this paper we have surveyed activities to reduce UQ to UQH_2 in rat tissues and their subcellular fractions. We found that rat liver cytosol has a novel UQ reductase different from DT-diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2) and mitochondrial respiratory enzymes.

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2. MATERIALS AND METHODS

UQ-9 and UQ-10 were donated by the Eisai Co., Tokyo. All other chemicals used were of the highest commercial grade available. UQ-9 and UQ-10 were dissolved in ethanol and used for experiments. Specific pathogen-free, male Wistar rats (8 weeks old, 180–200 g in body weight) were purchased from SLC Co., Shizuoka, Japan and fed on MF, a commercial diet (Oriental Yeast Co., Tokyo, Japan) until experiments began.

For cell fractionation, each rat was anesthetized with diethylether, injected with 0.2 ml of heparin solution (1,000 U/ml) through the femoral vein, and then dissected. First, blood was drawn from the abdominal aorta into a heparinized vacutainer. Next, the liver was perfused in situ with 20 ml of chilled isotonic saline through the hepatic portal vein to the inferior vena cava and then all organs were removed for UQ analysis. The enucleated organs were homogenized immediately with 4 vols. of chilled 0.25 M sucrose containing 50 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA in a Potter–Elvehjem glass-Teflon homogenizer. Fractions of nuclei, mitochondria, lysosomes, crude microsomes (containing Golgi complex) and cytosol were prepared stepwise from these tissue homogenates by differential centrifugations of 600 × *g* for 10 min, 6,500 × *g* for 20 min, 10,000 × *g* for 15 min, and 105,000 × *g* for 60 min, respectively [8].

Contaminants of other subcellular fractions in each fraction isolated above were estimated to be less than 5% on the basis of measurements of marker enzyme activities described below.

NADPH-cytochrome *c* reductase activity (microsomal marker) was determined by the reduction of ferricytochrome *c* in the presence of NADPH. Briefly, 0.30 ml of 0.2 M potassium phosphate buffer (pH 7.5), 0.15 ml of 1% ferricytochrome *c*, 0.15 ml of 0.1 M NaN_3 and 0.6 ml of the subcellular fractions were mixed in a 1 ml cuvette at 37°C. The reaction was started with the addition of 0.2 ml of 0.05% NADPH, and then the absorbance increase at 550 nm was followed for 3 min. Succinate-cytochrome *c* reductase activity (mitochondrial marker) was determined by Tisdale's method [9]. Acid phosphatase activity (lysosomal marker) was measured by the method of Walter and Schutt [10] and lactate dehydrogenase activity (cytosolic marker) by the method of Bergmeyer and Bernt [11].

UQ reductase activity (pmol/min/mg protein) was determined with the amount of UQH_2-10 formed by incubating 0.05 mM UQ-10 with tissue homogenates (0.05–0.01 mg protein) or their subcellular fractions in 250 μl of a reaction mixture. Two hundred-fifty μl of the reaction mixture contained 0.5 mM 2-mercaptoethanol, 0.08% Triton

X-100 and 0.2 mM NADPH in a 50 mM Tris-HCl buffer (pH 7.4) as well as UQ-10 and enzyme samples. The enzyme reaction was performed at 37°C for 10 min, and stopped with 3.5 ml of ethanol/*n*-hexane (2:5). Then, the UQH_2-10 formed in the reaction mixture was determined by the HPLC method which will be described later.

DT-diaphorase (NAD(P)H reductase(quinone), EC 1.6.99.2) activity was determined by the reduction of 2,6-dichlorophenolindophenol (DCIP) by enzyme samples in the presence of NAD(P)H as described by Ernster [12]. Protein content of subcellular fractions was determined by the method of Lowry et al. [13].

Reduced and oxidized forms of UQ homologues in tissues, subcellular fractions and the enzyme reaction mixture were extracted with ethanol and *n*-hexane and determined by HPLC with an electrochemical detector as previously described [14].

3. RESULTS

The obtained percentages of UQH_2-9 of the total UQ-9 in various tissue homogenates and UQ reductase activities of their 105,000 × *g* supernatants (cytosol) prepared as described above are listed in Table I. UQH_2-9 represented 74.6% of the total UQ-9 in the liver, but 10–45% in most of the other tissues. Among the tissues tested, UQ reductase activity was also highest in liver. In general, it was observed that the greater the UQ reductase activity in the cytosol fraction of a tissue, the higher the level of reduced form of UQ. In addition, when compared within the liver, specific activities (means ± S.E. pmol/min/mg protein, $n = 3$) of UQ reductase in nuclei, mitochondria, lysosomes, microsomes and cytosol were 94.6 ± 27.6, 39.3 ± 2.7, 24.0 ± 4.4, 171 ± 19 and 474 ± 31, respectively. Total activity of UQ reductase in the cytosol explained about 62~70% of that in the tissue homogenate, according to protein distribution among subcellular fractions. The results suggest that UQ reductase activity in cytosol may be responsible for the redox state of UQ in the whole tissue. In plasma, the rate of UQH_2-9 amounted to 80.8% of total UQ-9. However, the UQ reductase activity was negligible. This exceptional case in the relation between UQ reductase activity and the redox state of UQ-9 can easily be accounted for, if plasma UQ was supplied by the liver as Kålen et al. suggested [3].

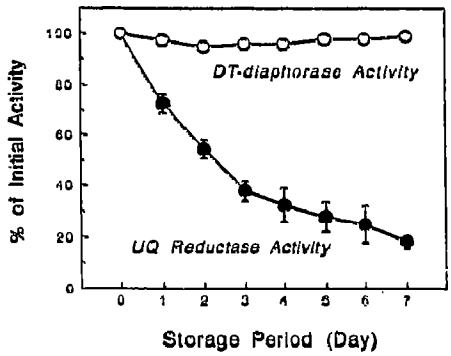

Fig. 1. Stabilities of DT-diaphorase and UQ reductase activities in rat liver cytosol. The cytosol was freshly prepared from rat liver and immediately stored at −20°C. Then, its DT-diaphorase and UQ reductase activities were determined every day and expressed as a percent of the initial activities of day 0. The initial activities of DT-diaphorase and UQ reductase were 46.2 ± 6.2 nmol/min/mg protein and 347 ± 16 pmol/min/mg protein, respectively. Values are the means ± S.E. of three experiments.

It is known that liver cytosol has a potent DT-diaphorase activity, which can reduce naphthoquinones to naphthoquinols and UQ to UQH_2 [15]. The optimum pH for UQ reductase activity was 7.4, different from the pH 5 of rat liver DT-diaphorase activity [16]. Then, in order to ascertain whether or not the UQ reductase activity found in liver cytosol is due to DT-diaphorase, some enzymatic properties of the UQ reductase activity were compared with those of DT-diaphorase.

First, we compared the stabilities of UQ reductase activity and DT-diaphorase activity in the same cytosol during storage at −20°C. As shown in Fig. 1, DT-diaphorase activity was quite stable during the seven days of storage, yet the UQ reductase activity promptly decreased, dropping to about 20% of the original activity by the seventh day.

Table I

Distribution of ubiquinone-9 and ubiquinone reductase activity in rat tissues

Tissues	UQ-9 content (ng/mg protein)		UQH_2-9	UQ reducing activity
	Total UQ-9*	UQH_2-9	(%**)	(pmol/min/mg protein)
Liver	341 ± 43	256 ± 56	74.6 ± 8.9	259 ± 12
Heart	1,270 ± 320	110 ± 30	10.2 ± 4.7	18.4 ± 13.6
Kidney	755 ± 162	261 ± 98	33.3 ± 7.8	57.3 ± 17.1
Testis	163 ± 54	76 ± 31	45.4 ± 4.8	31.5 ± 30.3
Lung	200 ± 10	51 ± 23	25.4 ± 11.2	8.7 ± 3.6
Spleen	125 ± 26	15 ± 5	11.4 ± 1.9	4.8 ± 3.6
Brain	283 ± 43	43 ± 3	14.3 ± 1.2	4.5 ± 3.6
Skeletal muscle	150 ± 21	19 ± 9	12.3 ± 1.2	8.4 ± 1.8
Plasma	0.87 ± 0.49	0.69 ± 0.37	80.8 ± 2.7	0

Reduced and oxidized forms of UQ-9 were extracted from plasma or each tissue homogenate, and determined by HPLC as described previously [20]. UQ reductase activities were determined by the formation of UQH_2-10 at 37°C as described in the text. Values are means ± S.E. of three rats. *Total UQ-9 is the sum of both oxidized and reduced forms of UQ-9. **(UQH_2-9/Total UQ-9) × 100.

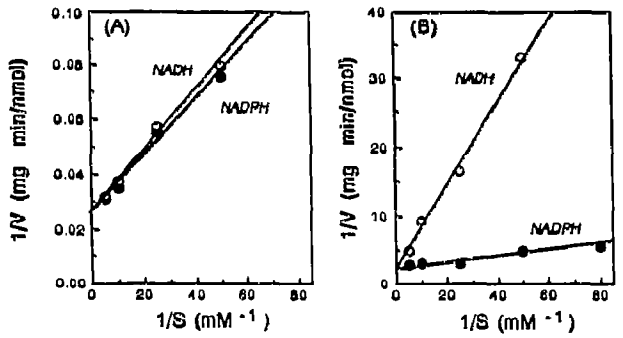

Fig. 2. Effect of NADH and NADPH as the electron donor on DT-diaphorase and UQ reductase activities in rat liver cytosol. The enzyme reactions were conducted at 37°C. V_{max} and K_m values of DT-diaphorase (A) and UQ reductase (B) to NADH (○) and NADPH (●) were extrapolated from Lineweaver–Burk double reciprocal plots by least-squares linear regression. DT-diaphorase: K_m(NADH) = 38.0 μM, V_{max}(NADH) = 36.2 nmol/min/mg protein; K_m(NADPH) = 36.3 μM, V_{max}(NADPH) = 36.0 nmol/min/mg protein. UQ reductase: K_m(NADH) = 307 μM, V_{max}(NADH) = 489 pmol/min/mg protein; K_m(NADPH) = 19.2 μM, V_{max}(NADPH) = 487 nmol/min/mg protein.

Next, specificity of the UQ reductase to hydrogen donors was compared to that of the DT-diaphorase. Lineweaver–Burk double reciprocal plots of these two enzyme activities in the same cytosol to NADH and NADPH are shown in Fig. 2. DT-diaphorase showed about the same K_m and V_{max} values in NADH and NADPH. On the other hand, the UQ reductase had quite different K_m values: 19.2×10^{-6} M for NADPH and 307×10^{-6} M for NADH. However, their V_{max} values were almost the same: 489 and 487 pmol/min/mg protein for NADH and NADPH, respectively. The UQ reductase appeared to be a type of NADPH-dependent UQ reductase.

Then, it was determined whether this NADPH-dependent UQ reductase activity is stimulated in the presence of bovine serum albumin or a surfactant, both of

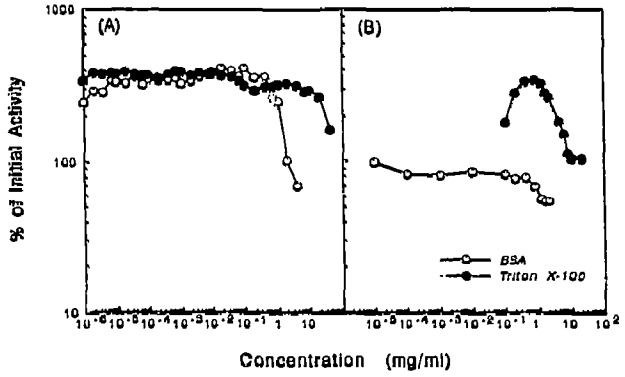

Fig. 3. Effect of bovine serum albumin and Triton X-100 on DT-diaphorase and UQ reductase activities in the same rat liver cytosol. DT-diaphorase (A) and UQ reductase activities (B) were determined in the presence of the indicated concentrations of bovine serum albumin (○) and Triton X-100 (●). Values are a percent of the original activities in the absence of both albumin and Triton X-100.

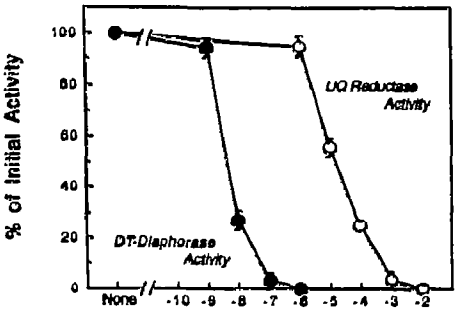

Fig. 4. Inhibition of DT-diaphorase and UQ reductase activities by dicoumarol. DT-diaphorase activity (●) and UQ reductase activity (○) were determined in the presence of the indicated concentrations of dicoumarol. Both enzyme activities were induced as a percent of the specific activities in the absence of dicoumarol. Values are the means ± S.E. of three experiments.

which are known to activate DT-diaphorase activity [17]. As shown in Fig. 3, DT-diaphorase activity was enhanced by 300–400% in the presence of more than 2×10^{-6} mg/ml of either bovine serum albumin or Triton X-100. However, UQ reductase activity was not stimulated but slightly depressed by bovine serum albumin. This activity was also stimulated by only a narrow concentration span of Triton X-100 in the high range of 0.1–5 mg/ml.

From the results on enzymatic properties observed above, the UQ reductase was deduced to be a different enzyme from DT-diaphorase. We then investigated whether or not dicoumarol, a specific inhibitor of DT-diaphorase, inhibits UQ reductase activity in liver cytosol. UQ reductase activity, which was detected using NADPH as hydrogen donor and UQ-10 as electron acceptor, was inhibited only at concentrations higher than 1 μM dicoumarol (Fig. 4).

On the other hand, DT-diaphorase activity (NAD(P)H-DCIP reductase activity), which was detected using NADH as hydrogen donor and DCIP as electron acceptor, was inhibited at concentrations lower than 1 μM of dicoumarol. Consequently, UQ reductase appears to be a novel NADPH-UQ oxidoreductase different from DT-diaphorase.

4. DISCUSSION

Some papers have pointed out that animal tissues contain high levels of UQ in subcellular fractions other than mitochondria [3]. Recently we observed that 70–80% of the total UQ amount in the liver and plasma and 20–30% of that in other tissues exist in the reduced hydroquinone form (unpublished data). Mitochondrial respiratory enzymes and DT-diaphorase were expected to be possible sites for UQ reduction in cells. In addition, microsomal electron transport systems might be involved in UQ reduction. UQ is an essential redox

carrier in mitochondria, but it is not easy to account for how UQH_2 formed in mitochondria is transferred to other subcellular organelles. DT-diaphorase is mainly located in the cytosol, but it is also distributed in microsomes and mitochondria [17]. However, UQ, especially one with a long isoprenoid side-chain is not a preferable substrate for DT-diaphorase. From these points, it is an interesting problem where and how UQ existing in the cell is hydrogenated to UQH_2.

The present results revealed that in rat tissues the cytosol had the greatest ability of UQ reduction among subcellular fractions and that the novel NADPH-dependent UQ reductase described here may be the central device responsible for UQ reduction. The facts that liver cytosol possesses an extremely high activity of the UQ reductase and that blood plasma lacked it, agree with the line of thought as Kålen et al. [3] which is that UQ is synthesized and reduced to UQH_2 in the liver and then secreted to the plasma as lipoproteins through the Golgi complex. It is presently under study whether this enzyme can reduce UQ built into such biomembranes as microsomes and the Golgi complex.

Dallner and his colleagues [13] suggested that most of the subcellular organelles, as well as mitochondria, have their own system for UQ biosynthesis. Our present results, as well as their findings strongly suggest that UQH_2 in organelles, other than mitochondria, also plays a certain important role. Recently, it has been suggested that non-mitochondrial UQ is involved in anti-oxidation, growth control, calcium transport and so on [19–21]. Further study is necessary to elucidate the relation between this UQ reductase activity and such physiological roles of UQ and UQH_2 in subcellular organelles, other than mitochondria.

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