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Characterization of NADPH-Dependent Ubiquinone Reductase Activity in Rat Liver Cytosol: Effect of Various Factors on Ubiquinone-Reducing Activity and Discrimination from Other Quinone Reductases[1]

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Cytosolic NADPH-dependent ubiquinone reductase (NADPH-UQ reductase) accounted for about 68% of the total ubiquinone (UQ) reductase activity in rat liver homogenate [Takahashi, T. *et al.* (1995) *Biochem. J.* 309, 883-890]. We investigated the effects of various factors on this enzyme activity in rat liver cytosol with the aim of elucidating its physiological roles. The NADPH-UQ reductase in rat liver cytosol catalyzed the reduction of UQ to UQH_2 with concomitant oxidation of equimolar NADPH. The optimal pH was around 7.4, and the optimal temperatures were about 28°C for NADH and about 37°C for NADPH. NADH, deamino NADH, and deamino NADPH were much less active hydrogen donors than NADPH, whereas reduced nicotinamide mononucleotide, ascorbate, erythorbate, reduced glutathione, and cysteine were inactive. As the hydrogen acceptor, UQ-9 had the highest V_{max}/K_m among the long-chain UQ homologues tested. FAD and FMN stimulated the activity. Anionic detergents, Mg^{2+} and Sr^{2+} also enhanced the activity. Rotenone, malonic acid, antimycin A, and KCN, which inhibit mitochondrial and microsomal electron transfer enzymes, superoxide dismutase, and acetylated cytochrome *c* had no effect on the NADPH-UQ reductase activity. These results indicated that the NADPH-UQ reductase in rat liver cytosol is a flavoprotein that reduces UQ-10 by a two-electron reduction mechanism and is distinguishable from known microsomal and mitochondrial enzymes, as well as DT-diaphorase [EC 1.6.99.2].

Key words: cytosol, liver, NADPH ubiquinone reductase, ubiquinol, ubiquinone.

Ubiquinone (UQ) is an indispensable factor in the mitochondrial respiratory chain (*1*). However, UQ is found not only in the mitochondria, but also in almost all intracellular organelles (*2, 3*) and extracellular fluids (*4*), and a portion of the UQ in these organelles is in the form of ubiquinol (UQH_2), the reduced form of UQ (*5*). In addition, some intracellular organelles other than mitochondria can synthesize UQ *de novo* (*3, 6, 7*). These observations suggest that UQ plays a physiologically important role in addition to that of electron transport in the mitochondrial respiratory chain. It has been suggested that UQ acts as an endogenous lipid-soluble antioxidant (*8-10*), serves as an electron carrier in the Golgi apparatus (*2*) and plasma membranes (*11*), and contributes to the fluidity of the phospholipid bilayer membranes (*12, 13*). Above all, the notion that UQH_2 functions as a regenerative lipid-soluble antioxidant in biomembranes (*8*) and plasma lipoproteins (*9, 10*), is persuasive, as UQH_2 is a physiologically active hydrogen donor. However, it is necessary to determine whether or not the reduction system specific for UQ, by which UQH_2 in organelles other than mitochondria is regenerated from UQ promptly enough to efficiently quench free radicals or peroxides, is present in tissues.

Some enzymes, which may contribute to the regeneration of intracellular UQH_2, have been found in endomembranes and the plasma membranes. For example, NADH-UQ reductase activities are located in the Golgi apparatus (*2*) and in the plasma membranes (*11*), as well as in mitochondria. There are several UQ reductases, such as NADH dehydrogenase complex (complex I, EC 1.6.99.3) in the mitochondria. NADH-cytochrome b_5 reductase [EC 1.6.2.2] and NADPH-cytochrome reductase [EC 1.6.2.4] in the endoplasmic reticulum may also reduce UQ to UQH_2 in the presence of NADH and NADPH, respectively (*14*). However, most of these enzymes reduce UQ to UQH_2 by one-electron transfer, that is, through the intermediate of ubisemiquinone, which can reduce oxygen to form superoxide anions (*15*). Therefore, it remains unclear whether or not some of these UQ reducing enzymes help reduce UQ to UQH_2 in the intracellular membranes in which they reside.

We found that the redox ratios of $UQH_2/(UQ+UQH_2)$ in various intracellular fractions from rat tissues were similar

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Abbreviations: 2′,5′-ADP, adenosine 2′,5′-diphosphate; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; NEM, *N*-ethylmaleimide; NMN, nicotinamide mononucleotide; NMNH, reduced NMN; PCMB, *p*-chloromercuribenzoic acid; SOD, superoxide dismutase; UQ, ubiquinone; UQH_2, ubiquinol; tUQ, total amount of UQ and UQH_2.

and that they depended on the UQ reducing activity in the cytosol from these tissues (*5, 16*). In addition, we revealed that rat tissues, especially their cytosols, have an NADPH-dependent UQ reductase (NADPH-UQ reductase) activity which is not accounted for by NAD(P)H: (quinone-acceptor) oxidoreductase (=DT-diaphorase, EC 1.6.99.2) (*17*). In the rat liver, the NADPH-UQ reductase activity accounts for 68% of total UQ reductive activity in the tissue homogenate (*16*).

Furthermore, the NADPH-UQ reductase reduced UQ to UQH_2 in lecithin liposomes and in rat liver microsomes in the presence of dicoumarol, an inhibitor of DT-diaphorase, and inhibited 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced lipid peroxidation of UQ-10-containing lecithin liposomes (*16*).

These findings suggested that the NADPH-UQ reductase activity in cytosol plays a central role in maintaining the intracellular steady-state redox levels of UQ, and thereby acts as an endogenous antioxidant in protecting intracellular membranes from lipid peroxidation. However, it is still obscure whether this enzyme activity is derived from a novel enzyme specific for UQ reduction, or is based upon side reactions by other reductases.

In this study, the effects of various factors on this cytosolic NADPH-UQ reductase activity were investigated using rat liver cytosol, to characterize the enzyme activity and to distinguish it from those of other reductases.

MATERIALS AND METHODS

Chemicals and Animals—UQ-9 and UQ-10 were donated by Eisai (Tokyo). Other homologues were donated by Dr. Karl Folkers (University of Texas at Austin), Eisai, and Nisshin Flour Milling (Tokyo). Glucose-6-phosphate dehydrogenase from yeast [EC 1.1.1.49] was purchased from Oriental Yeast (Tokyo). Acetylated cytochrome *c* was prepared according to Kakinuma and Minakami (*18*). All other chemicals were of the highest grade commercially available. Specific pathogen-free, male Wistar rats (8 weeks old, 180-200 g body weight) were purchased from SLC (Shizuoka) and fed on Lavo MR Stock, a commercial feed (SLC) until used in experiments.

Cell Fractionation—Intracellular fractions of livers were prepared as described (*5*). In brief, fractions of nuclei, mitochondria, crude lysosomes (containing peroxisomes), crude microsomes (containing Golgi complex), and cytosol were prepared serially from rat liver homogenates by differential centrifugation at $600\times g$ for 10 min, $6{,}500\times g$ for 20 min, $10{,}000\times g$ for 15 min, and $105{,}000\times g$ for 60 min, respectively. The purity of these fractions was determined by measuring their marker enzyme activities (*5*).

Determination of UQH_2 and tUQ—The UQH_2 and tUQ homologues in the tissues and subcellular fractions were extracted with ethanol and *n*-hexane in a nitrogen atmosphere and determined by using electrochemical detection as described (*19*).

Enzyme Assays—NADPH-UQ reductase activity (pmol/min/mg protein) was determined by measuring the amount of UQH_2-10 formed after incubating UQ-10 with rat liver cytosol in the presence of NADPH at 37°C. Two hundred and fifty microliters of the reaction mixture contained 0.5 mM 2-mercaptoethanol (for protection of UQH_2-10 formed against re-oxidation) and 0.08% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.4), 0.05 mM UQ-10, and 0.25 mg of cytosolic protein. UQ-10 was dissolved in ethanol to a final concentration of 2.5 mM, then added to the reaction mixture. The enzyme reaction was started by adding 0.2 mM NADPH, and stopped with 3.5 ml of ethanol/*n*-hexane (2 : 5, v/v). The UQH_2-10 formed in the reaction mixture was extracted with *n*-hexane and determined by HPLC as described (*19*). The protein content of intracellular fractions was determined by the method of Lowry *et al.* (*20*).

Determination of $NADP^+$—To measure the amount of $NADP^+$ formed in the assay mixture containing NADPH-UQ reductase activity, an aliquot (1 ml) of the assay mixture was deproteinized with 0.2 ml of 3 N $HClO_4$, then centrifuged by $1{,}800\times g$ for 10 min. The protein-free supernatant (1 ml) was mixed with 0.2 ml of 1 M K_2HPO_4, adjusted to pH 7.2 with KOH and chilled on ice for 20 min to crystallize $KClO_4$. The neutralized protein-free supernatant was centrifuged at $1{,}800\times g$ for 10 min to remove crystalline $KClO_4$ and used as the $NADP^+$-sample. The $NADP^+$ content in the sample was quantified (*21*). The reaction mixture consisted of 10 mM $MgSO_4$, 10 mM glucose-6-phosphate, and the $NADP^+$-sample. After incubation in a cuvette at 37°C for 15 min, 1.5 units of glucose-6-phosphate dehydrogenase per ml of reaction mixture was added to the cuvette and the absorbance at 340 nm was continuously monitored until a plateau was reached (about 10-20 min later). The $NADP^+$ content was estimated from the difference in the absorbance at 340 nm between reaction mixtures with and without glucose-6-phosphate dehydrogenase, using the molecular extinction coefficient of NADPH ($6.22\times10^3\ cm^{-1}\cdot M^{-1}$).

RESULTS

Basal Assay Conditions for NADPH-UQ Reductase Activity—The UQ reductase activity of rat liver cytosol was determined in the presence of UQ-10 as the substrate and either NADH or NADPH as the hydrogen donor. The UQ-10 reduction by rat liver cytosol was not observed when heat-denatured or trypsin- or pronase-digested cytosol was added instead of the native one to the reaction mixture (data not shown). NADPH was better than NADH as a hydrogen donor by a factor of about 2 (Table I).

The reduction rates of UQ-10 by cytosol in air were 75 to 80% of those under nitrogen gas, due to the reoxidation of formed UQH_2-10 (Table I). The addition of 0.5 mM 2-mercaptoethanol to the reaction mixture completely prevented the reoxidation of UQH_2-10 (up to 24 h), and 2-mercaptoethanol itself did not reduce UQ-10. Therefore, 0.5 mM 2-mercaptoethanol was added to the reaction mixtures in the following experiments.

The reduction of UQ-10 by rat liver cytosol with NAD(P)H linearly increased depending on the incubation time up to 30 min and on the amounts of cytosolic protein up to 4 mg/ml reaction mixture (Fig. 1). The optimum temperatures in the reaction at pH 7.4 (Tris-HCl buffer) were 28-30°C for NADH and 37-40°C for NADPH (Fig. 2A). However, an additive effect of NADH and NADPH was not observed either at 28 or 37°C (data not shown). The pH optimum of the enzyme reaction was in the range of 7-7.5, irrespective of whether the hydrogen donor was NADH or NADPH (Fig. 2B). NADPH, but not NADH, was a better hydrogen donor for UQ-10 reduction by the cytosol at

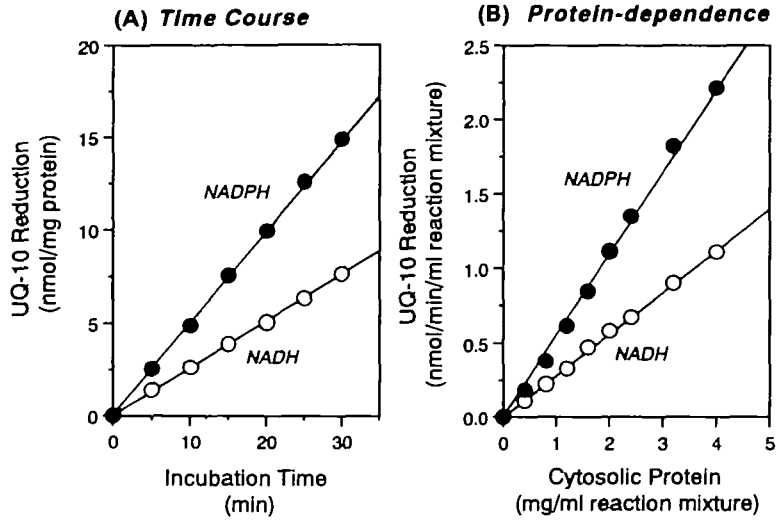

Fig. 1. **UQ-10 reduction by rat liver cytosol with NAD(P)H as the hydrogen donor.** (A) Time course of UQ-10 reduction. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.5 mM 2-mercaptoethanol, 0.25 mg of cytosolic protein, and either 0.2 mM NADH (○) or 0.2 mM NADPH (●). The reaction mixture was incubated at 37°C for the indicated periods. (B) Effect of various concentrations of cytosolic protein on UQ-10 reduction. The reaction mixture was the same as in (A), except for the indicated quantities of cytosolic protein. The reaction proceeded at 37°C for 10 min. The amount of formed UQH_2-10 was analyzed by HPLC as described in the text.

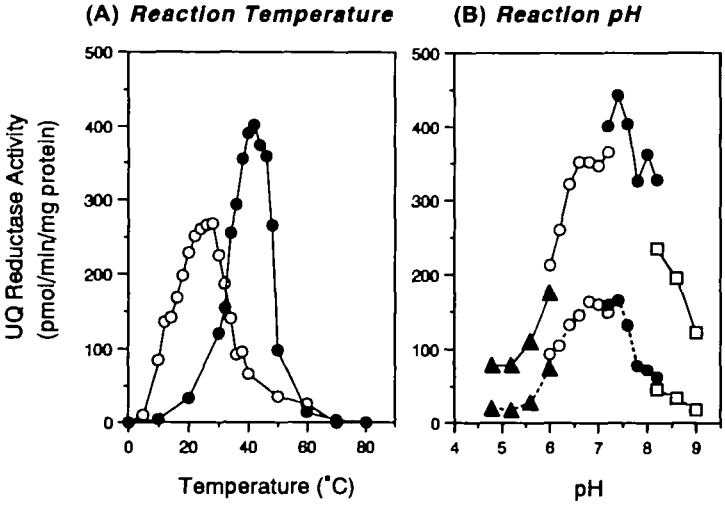

Fig. 2. **Effect of reaction temperature and pH on UQ-10 reduction by rat liver cytosol.** (A) Effect of reaction temperature on UQ-10 reduction by rat liver cytosol. The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM NADH (○) or NADPH (●), 0.5 mM 2-mercaptoethanol, and 0.25 mg of cytosolic protein. The mixture was incubated at the indicated temperatures for 10 min. (B) Effect of the reaction pH upon UQ-10 reduction by rat liver cytosol. The reaction mixture (250 μl) consisted of various buffers adjusted to the indicated pH: ▲, 50 mM citrate buffer (pH 4.8-6.0); ○, 50 mM phosphate buffer (pH 6.0-7.2); ●, 50 mM Tris-HCl buffer (pH 7.2-8.2); □, 50 mM borate buffer (pH 8.2-9.0), containing 0.08% Triton X-100, 50 μM UQ-10, 0.5 mM 2-mercaptoethanol, 0.2 mM NADH (dotted lines) or NADPH (solid lines), and 0.25 mg of cytosolic protein. The reaction mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text.

physiological pH and temperature.

Stability of NADPH-UQ Reductase—The stability of NADPH-UQ reductase activity in rat liver cytosol stored at various pH values and temperatures and incubated for various periods, is shown in Fig. 3. In the physiological pH range, the enzyme activity was stable for 1 h at 0°C (Fig. 3A), but it was lost rapidly at 30°C or higher temperatures (Fig. 3B). In the presence of 0.2 mM NAD(P)H, however, the enzyme activity was stable for more than 180 min at 37°C. In the absence of NAD(P)H, the enzyme activity decreased rapidly to 60 to 70% of the initial activity during incubation for 30 min at 37°C.

Hydrogen Donor for NADPH-UQ Reductase—We examined whether or not deamino NAD(P)H and some sulfhydryl agents could substitute for NAD(P)H as a hydrogen donor (Table II). The activities of deamino NADH and deamino NADPH were 36 and 90% of that of NADPH, respectively. Reduced nicotinamide mononucleotide (NMNH) did not function as a hydrogen donor. Ascorbate, erythorbate, reduced glutathione, and cysteine as well as 2-mercaptoethanol were also inactive as hydrogen donors (data not shown). Dithiothreitol and dithioerythritol reduced UQ-10 non-enzymatically (data not shown).

TABLE I. **Effect of 2-mercaptoethanol on UQ-10 reduction by rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM NADPH or NADH, and 0.25 mg of cytosolic protein in the presence or absence of 0.5 mM 2-mercaptoethanol. The reaction mixture was incubated at 37°C for 10 min under air or N_2 gas. The formed UQH_2-10 was analyzed by HPLC as described in the text. The values are the means ± SD of three experiments.

Conditions	UQ reductase activity (pmol/min/mg protein)	
	+2-Mercaptoethanol	−2-Mercaptoethanol
NADPH		
Air	450±32	359±27*
N_2	447±27	443±20
NADH		
Air	202±2	151±1*
N_2	202±6	209±9

*Significant differences ($p<0.05$) as compared with the presence of 2-mercaptoethanol under the same conditions, using Student's unpaired *t* test.

Effects of Some NAD(P)H-Related Adenine Nucleotides—The effects of $NAD(P)^+$ and related adenine nucleotides on the reduction of UQ-10 by cytosol with

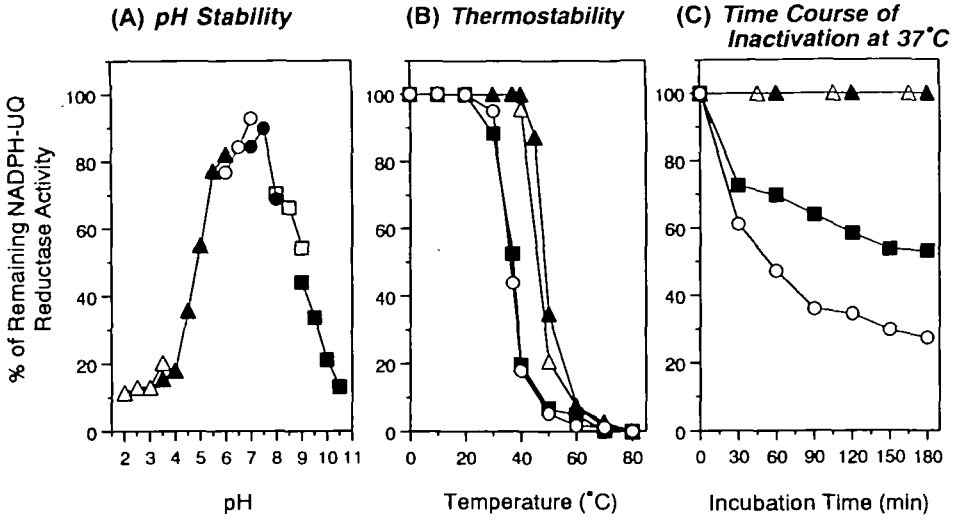

Fig. 3. **Stability of NADPH-UQ reductase in rat liver cytosol.** The reaction mixture (250 μl) for assay of NADPH-UQ reductase activity consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, and 0.25 mg of treated cytosolic protein. The mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text. (A) The pH stability of NADPH-UQ reductase in rat liver cytosol. Cytosolic protein (1 mg/ml) was incubated with various buffers at the indicated pH: △, 10 mM glycine-HCl buffer (pH 2.0-3.5); ▲, 10 mM citrate buffer (pH 3.5-6.0); ○, 10 mM phosphate buffer (pH 6.0-7.0); ●, 10 mM Tris-HCl buffer (pH 7.0-8.0); □, 10 mM borate buffer (pH 8.0-9.0); ■, 10 mM glycine-NaOH buffer (pH 9.0-10.5). After a 1 h incubation, the NADPH-UQ reductase activities were assayed. (B) The thermostability of NADPH-UQ reductase in rat liver cytosol. Cytosolic protein (1 mg/ml of 50 mM Tris-HCl buffer, pH 7.4), was incubated at the indicated temperatures without (○) or with 0.2 mM NADH (△), 0.2 mM NADPH (▲), or 50 μM UQ-10 (■) for 1 h. Thereafter, NADPH-UQ reductase activities were assayed. (C) Time course of NADPH-UQ reductase inactivation in rat liver cytosol. Cytosolic protein (1 mg/ml of 50 mM Tris-HCl buffer, pH 7.4) was incubated at 37°C for the indicated periods without (○) or with 0.2 mM NADH (△), 0.2 mM NADPH (▲), or 50 μM UQ-10 (■).

TABLE II. **Effect of reduced forms of pyridine nucleotides on UQ-10 reduction by rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM pyridine nucleotides as electron donors, 0.5 mM 2-mercaptoethanol, and 0.25 mg of cytosolic protein. The K_m and V_{max} values were determined from Lineweaver-Burk plots generated by using a range of 0.05-1 mM pyridine nucleotides as the electron donor. The mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text. The values are the means±SD of three experiments.

Addition	UQ reductase activity (pmol/min/mg)	K_m (μM)	V_{max} (pmol/min/mg)
None	0	–	–
NADPH	450±20	14.4±2.5	461±28
NADH	209±9	259 ±22	463±28
Deamino NADPH	413±68	31.7±2.5	459±16
Deamino NADH	160±31	557 ±46	463±17
NMNH	0	–	–

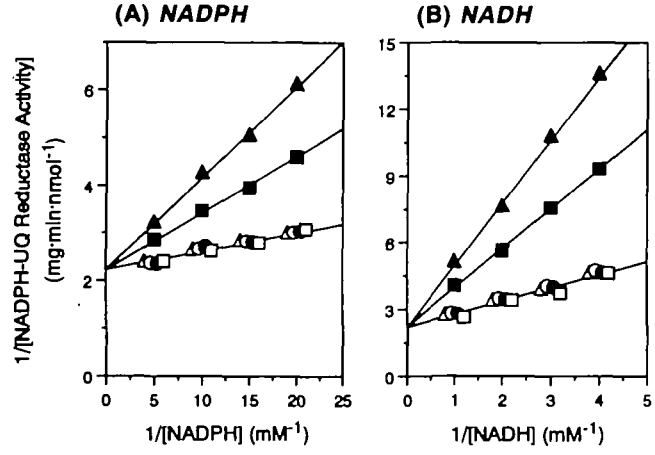

Fig. 4. **Effect of adenine nucleotides on UQ-10 reduction by rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.25 mg of cytosolic protein, the indicated concentrations of NADPH (A) or NADH (B), 0.5 mM 2-mercaptoethanol, and one of the following adenine nucleotides (○, none; △, 1 mM NAD^+; ▲, 0.2 mM $NADP^+$; ●, 1 mM NMN; □, 1 mM AMP; ■, 0.2 mM 2′,5′-ADP). The reaction mixtures were incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text.

NAD(P)H are shown in Fig. 4. $NADP^+$ inhibited the enzyme activity in the presence of either NADPH or NADH as the hydrogen donor, and adenosine 2′,5′-diphosphate (2′,5′-ADP) was also inhibitory to some extent. However, fivefold molar concentrations of NAD^+, nicotinamide mononucleotide (NMN) and AMP did not affect the activity. These observations indicate a rigid structural specificity of the enzyme protein for NADPH itself, or at least the phosphate group at position 2′ of the ribose moiety.

Effects of FAD and FMN—The effects of FAD and FMN on the reduction of UQ-10 by cytosol with NADPH are shown in Fig. 5. The additions of 200 μM FAD and FMN to the reaction mixture containing NADPH significantly stimulated the activity about 2.5- and 3.6-fold, respectively. On the other hand, the addition of chlorpromazine, a flavin antagonist, inhibited the enzyme activity to some extent (Table V). These results suggested that the NADPH-UQ reductase is a flavin enzyme.

Effect of Detergents—The effects of ionic and non-ionic detergents on the enzyme activity are listed in Table III. Cationic detergents and 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), which is likely to be present as a cation in the reaction mixture at pH 7.4, stimulated the enzyme activity. Some non-ionic detergents having a phenyl skeleton, such as Triton X-100, Triton X-114, and Triton N-101 also potently stimulated the activity, but it is uncertain whether or not this was due to a physical affinity with UQ or the enzyme protein. However, Triton X-100 decreased the K_m value for UQ-10 of the NADPH-UQ reductase activity, but did not affect the

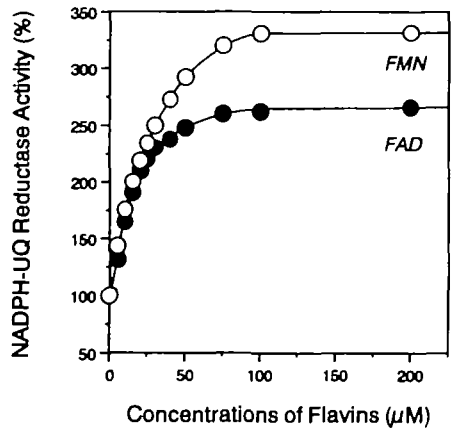

Fig. 5. **Effect of flavin nucleotides upon NADPH-UQ reductase activity in rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, 0.25 mg of cytosolic protein, and the indicated concentrations of either FAD (●) or FMN (○). The mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text.

TABLE III. **Effect of detergents on NADPH-UQ reductase activity in rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 50 μM UQ-10, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, 0.25 mg of cytosolic proteins, and 0.08% of detergent or BSA. The reaction mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text. The values are the means±SD of three experiments.

Detergents		NADPH-UQ reductase activity (%)
None		100[a]
BSA		94±11
Anionic detergents:	SDS	19±0
	Sodium deoxycholate	76±3
Cationic detergents:	Benzalkonium chloride	553±37
	Cetyltrimethylammonium bromide	190±11
Amphoteric detergents:	CHAPS	123±6
Non-ionic detergents:	Triton X-100	553±24
	Triton X-114	545±17
	Triton N-101	485±16
	Tween 20	312±16
	Tween 60	61±2
	Tween 80	61±2
	Brij-35	46±3
	Brij-56	44±2
	HCO-60	42±11

[a]In the absence of detergents and BSA, NADPH-UQ reductase activity was 79.2±3.9 pmol/min/mg protein and this was defined as 100%.

K_m value for NADPH (data not shown). Only Tween 20 from among the Tween series detergents enhanced the activity threefold. Other non-ionic detergents such as the Brij series detergents and HCO-60 inhibited the activity.

Effects of Metal Ions—NAD(P)H-UQ reductase in rat liver microsomes is reportedly activated 1.5-fold in the presence of Mg^{2+} (*22*). We therefore tested the effect of various metal ions at a concentration of 10 mM on the NADPH-UQ reductase activity in rat liver cytosol. The results are listed in Table IV. EDTA (1 mM) did not inhibit the enzyme activity, but high concentrations of Mg^{2+} and Sr^{2+} stimulated the enzyme activity by 1.49- and 1.33-fold, respectively. These metal ions increased the V_{max} values for UQ-10 and NADPH of the NADPH-UQ reductase activity, but did not affect the K_m values for UQ-10 and NADPH (data not shown). $CdCl_2$, $CeCl_3$, $CoCl_2$, CuCl, $CuCl_2$, $HgCl_2$, $LaCl_3$, $TiCl_4$, and $ZnCl_2$ depressed UQ-10 reduction by more than 60% as compared to the control without metal ions. LiCl, NaCl, KCl, $CaCl_2$, $FeCl_2$, $FeCl_3$, $MnCl_2$, and $NiCl_2$ neither inhibited nor enhanced the

TABLE IV. **Effect of metal ions on NADPH-UQ reductase activity in rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, 0.25 mg of cytosolic proteins, and 1 mM EDTA or 10 mM metal ions. The mixture was incubated at 37°C for 10 min. The formed UQH_2-10 was analyzed by HPLC as described in the text. The values are the means±SD of 3-12 experiments.

Addition	NADPH-UQ reductase activity (%)
None	100[a]
EDTA	99.6±2.4
$CdCl_2$	7.1±1.3[c]
$CeCl_3$	8.4±0.7[c]
$CoCl_2$	40.8±2.7[c]
CuCl	0[c]
$CuCl_2$	0[c]
$HgCl_2$	0[c]
$LaCl_3$	13.5±1.8[c]
$MgCl_2$	149±5[b]
$SrCl_2$	133±13[b]
$TiCl_4$	0[c]
$ZnCl_2$	19.5±1.8[c]

[a]In the absence of EDTA and metal ions, NADPH-UQ reductase activity was 450±9 pmol/min/mg protein and this was defined as 100%. [b,c]Stimulation and inhibition of UQ-10 reduction (significant differences of $p<0.001$, respectively, as compared with no addition, using Student's unpaired t test). LiCl, NaCl, KCl, $CaCl_2$, $FeCl_2$, $FeCl_3$, $MnCl_2$, and $NiCl_2$ exhibited no effect on the activity.

TABLE V. **Effect of various inhibitors on NADPH-UQ reductase-catalyzed UQ-10 reduction.** The concentrations of various inhibitors required for 50% inhibition of the UQ-10 reduction (IC_{50}), except for *p*-chloromercuribenzoic acid (PCMB) and *N*-ethylmaleimide (NEM), were determined using the standard assay mixture described in the text containing 0.001-10 mM inhibitors. The K_i values were determined from Dixon plots generated by using 2, 10, or 50 μM UQ-10 as the electron acceptor in the presence of 0.001-10 mM inhibitors except for PCMB and NEM. For determination of the IC_{50} and K_i values for PCMB and NEM, each assay mixture was modified by omitting 2-mercaptoethanol. The reaction mixture was incubated at 37°C for 10 min. The formed UQH_2-10 were analyzed by HPLC as described in the text. The IC_{50} values are the means±SD of three experiments. N.D., not determined.

Inhibitor	IC_{50} (μM)	K_i (μM)
Dicoumarol	16.0±0.6	12.4
Warfarin	>7,500	N.D.
Chlorpromazine	2,500±60	136
2,4-Dinitrophenol	990±20	1,000
p-Chloromercuribenzoic acid	80±8	74.7
N-Ethylmaleimide	160±23	N.D.
Insulin	No effect	
Glucagon	No effect	
Rotenone	No effect	
KCN	No effect	
NaN_3	No effect	

TABLE VI. **Reduction of UQ homologues by NADPH-UQ reductase in rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 2-10 μM UQ homologues as the electron acceptor, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, and 0.25 mg of cytosolic protein. The K_m and V_{max} values were determined from Lineweaver-Burk plots. The concentrations of UQ homologues required for 50% inhibition of the UQ-10 reduction (IC_{50}) were determined using the standard assay mixture described in the text containing 10-100 μM UQ homologues other than UQ-10. The K_i values were determined from Dixon plots generated by using 2, 10, or 50 μM UQ-10 as the electron acceptor in the presence of 1-10 μM UQ homologues other than UQ-10. The reaction mixture was incubated at 37°C for 10 min. The formed UQH_2 homologues were analyzed by HPLC as described in the text. The values (except for K_i) are the means±SD of three experiments. N.D., not determined.

UQ homologue	Kinetic parameters			Inhibition of UQ-10 reduction	
	K_m (μM)	V_{max} (pmol/min/mg)	K_m/V_{max}	IC_{50} (μM)	K_i (μM)
UQ-4	N.D.	N.D.	N.D.	14.6±1.8	2.01
UQ-7	2.35±0.12	433±7	182±14	29.6±1.6	2.51
UQ-8	2.53±0.31	465±10	191±36	27.8±6.6	2.54
UQ-9	2.66±0.48	487±14	200±37	44.0±8.5	2.61
UQ-10	3.27±0.14	461±20	136±28	N.D.	N.D.
UQ-11	4.45±0.42	437±36	110±26	84.0±12.5	3.82

TABLE VII. **Effects of Cu^{2+},Zn^{2+}-SOD and its inhibitor on superoxide anion generation and NADPH-UQ reductase activity of rat liver cytosol.** The reaction mixture (250 μl) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.08% Triton X-100, 50 μM UQ-10, 0.5 mM 2-mercaptoethanol, 0.2 mM NADPH, 20 μM acetylated cytochrome *c*, and 0.25 mg of cytosolic protein. The mixture was incubated at 37°C in a cuvette and the absorption change at 550 nm was continuously monitored for 10 min. The rate of acetylated cytochrome *c* reduction was estimated from the absorption coefficient of the reduced form of acetylated cytochrome *c* at 550 nm, $19.1\times10^3\ M^{-1}\cdot cm^{-1}$. After the incubation, the amount of UQH_2-10 formed in the reaction mixture was analyzed by HPLC as described in the text. The values are the means±SD of three experiments. n.d., not detected.

	Acetylated cytochrome *c* reduction (nmol/min/mg)	NADPH-UQ reductase activity (pmol/min/mg)
Complete	0.48±0.11	581±63
+100 units Cu^{2+},Zn^{2+}-SOD	0.48±0.12	592±56
+1 mM KCN	0.44±0.03	596±18
−UQ-10	0.47±0.10	n.d.
−Acetylated cytochrome *c*	n.d.	586±59

activity.

Effects of Various Enzyme Inhibitors—The effects of some enzyme inhibitors on NADPH-UQ reductase activity are listed in Table V. Sulfhydryl blockers, such as *p*-chloromercuribenzoic acid (PCMB) and *N*-ethylmaleimide (NEM), chlorpromazine, and 2,4-dinitrophenol inhibited the enzyme activity to some extent. Dicoumarol inhibited the enzyme only at a 3,000-fold higher concentration than that required to inhibit DT-diaphorase, and warfarin had little effect. Rotenone (*23*) and insulin (*24*), inhibitors of NADH dehydrogenases in mitochondria and plasma membranes, respectively, exhibited no effect on cytosolic NADPH-UQ reductase activity. Other mitochondrial electron transport inhibitors, such as antimycin A, KCN, NaN_3, and malonic acid, also had no effect on the cytosolic NADPH-UQ reductase activity (data not shown). Glucagon (*24*) and KCN (*2*), stimulators of NADH dehydrogenases in plasma membranes and Golgi apparatus, respectively, exhibited no effect on the cytosolic NADPH-UQ reductase activity.

Effects of UQ Homologues on Reduction of UQ-10—All UQ homologues tested were reduced by the NADPH-UQ reductase and gave similar K_m and V_{max} values. However, the V_{max}/K_m values of UQ-9 were the highest among those of the long-chain UQ homologues. In addition, UQ homologues with isoprenoid chains longer than that of UQ-4 competitively inhibited the reduction of UQ-10 by the enzyme (Table VI). However, short homologues of UQ-0, -1, and -3 stimulated the UQ-10 reduction, but this stimulation was strongly inhibited by dicoumarol, a potent inhibitor of DT-diaphorase, or cytochrome *c* (data not shown). Therefore, we suppose that the stimulation of UQ-10 reduction by these short-chain UQ homologues is due to DT-diaphorase in rat liver cytosol, since it was reported that short-chain quinones, such as menadione, mediated the electron flow from NADPH to UQ-10 by DT-diaphorase (*25*). This possibility is now under study.

Effect of Superoxide Dismutase on UQ-10 Reduction—The effect of superoxide dismutase (SOD) [EC 1.15.1.1] on the reduction of acetylated cytochrome *c* and UQ-10 in the presence of cytosol and NADPH is shown in Table VII. If the reduction of UQ-10 to UQH_2-10 by cytosol were achieved through one-electron transfer, ubisemiquinone radicals would be formed as an intermediate, and under aerobic conditions, the superoxide anion may also be concomitantly formed, as it was in the reductions of menadione by microsomal enzymes (*26*) and of UQ by mitochondrial respiratory enzymes (*27*). However, the reduction of UQ-10 by cytosol and NADPH was not accompanied with reduction of acetylated cytochrome *c*. Furthermore, the addition of SOD and KCN, an inhibitor of Cu^{2+},Zn^{2+}-SOD, did not affect the reductions of acetylated cytochrome *c* and UQ-10. Moreover, the reduction of UQ-10 to UQH_2-10 was accompanied with equimolar oxidation of NADPH to $NADP^+$ and the regression line between $NADP^+$ (Y) formed and UQH_2-10 (X) formed was expressed as $Y=1.013X-0.047$ (data not shown). From these results, the NADPH-UQ reductase in liver cytosol seems to be a two-electron transfer catalyst like DT-diaphorase.

DISCUSSION

If UQH_2 functioned as an endogenous antioxidant in cells, the enzymatic system(s) for cyclic reduction from UQ, formed by releasing the reducing equivalent for the antioxidant action, back to UQH_2, would also be present. Some enzymes having quinone reductase activities, as well as mitochondrial respiratory enzymes such as NADH dehydrogenase complex (complex I) and succinate dehydrogenase complex (complex II, EC 1.3.99.1), are present in cells. DT-diaphorase in cytosol can reduce a variety of quinone compounds through a two-electron transfer reaction in the presence of NAD(P)H. It has been reported that a kind of NADH-UQ reductase is also present in the Golgi apparatus (*2*) and in the plasma membranes (*11*). In addition, NADH-cytochrome b_5 reductase and NADPH-cytochrome reductase in microsomes may also help reduce UQ to UQH_2 *via* the formation of ubisemiquinone radicals by a one-electron transfer reaction in the presence of NADH and

NADPH, respectively (*14*).

We found that the soluble fraction of rat tissue homogenate after centrifugation at 105,000×*g* for 1 h, contained a UQ-10 reducing enzyme (so-called NADPH-UQ reductase), which was distinguished from DT-diaphorase according to the following criteria. NADPH-UQ reductase preferred NADPH to NADH as a hydrogen donor and was not inhibited by low concentrations (below 5 μM) of dicoumarol (*17*). We also reported that the NADPH-UQ reductase was separated from DT-diaphorase located in rat liver cytosol by means of Cibacron blue-binding agarose gel chromatography (*16*). The NADPH-UQ reductase has an optimal pH of about 7.4, an optimal temperature of about 37°C for NADPH and about 28°C for NADH, and it preferred UQ-9 among long chain homologues of UQ-7 to UQ-11 as a hydrogen acceptor. In addition, the enzyme was activated by bivalent metal ions such as Mg^{2+} and Sr^{2+}, flavin nucleotides of FAD and FMN, and cationic detergents. Antimycin A, an inhibitor of mitochondrial UQ-cytochrome *c* oxidoreductase (cytochrome bc_1 complex), also had no effect on the formation of UQH_2 by the enzyme. Furthermore, it was not inhibited by rotenone and malonic acid, inhibitors of mitochondrial NADH-UQ reductase and succinate dehydrogenase, respectively, or by KCN, an inhibitor of Cu^{2+},Zn^{2+}-SOD, which catalyzed the reduction from semiquinone radicals to the corresponding quinols with superoxide anion (*28*). In addition, the reduction of UQ by NADPH-UQ reductase in cytosol was not accompanied with the formation of superoxide anion, as indicated by a lack of the reduction of acetylated cytochrome *c*. Moreover, UQ-10 reduction by the NADPH-UQ reductase was followed by equimolar NADPH oxidation ($NADP^+$ formation). These observations support the notion that the NADPH-UQ reductase catalyzed the reduction from UQ to UQH_2 by a two-electron transfer reaction.

Some quinone reductases preferring NADPH to NADH as a hydrogen donor are present in liver. Shigemura *et al.* reported that NAD(P)H-UQ reductase in rat liver microsomes was able to reduce UQ-10 to UQH_2-10 in the presence of NAD(P)H (*22*). The NAD(P)H-UQ reductase in microsomes preferred NADPH to NADH as a hydrogen donor in the same manner as the NADPH-UQ reductase in cytosol, and the K_m values for NAD(P)H of the microsomal enzyme were similar to those of the cytosolic enzyme. However, the K_m value for UQ-10 of the microsomal enzyme was about 7 times greater than that of the cytosolic enzyme. In addition, $NADP^+$, which exhibited no effect on the microsomal enzyme activity, inhibited the cytosolic NADPH-UQ reductase activity. These results indicated that the NADPH-UQ reductase in cytosol differed from the NAD(P)H-UQ reductase in microsomes. Furthermore, we found that the properties of NADPH:(quinone-acceptor) oxidoreductase [EC 1.6.99.6] in hog liver cytosol were similar to those of the NADPH-UQ reductase in rat liver cytosol (*29*). However, it has been reported that the hog liver enzyme could not reduce UQ to UQH_2 and was not affected by 2,4-dinitrophenol and FMN (*29*), which inhibited and stimulated the NADPH-UQ reductase activity in rat liver cytosol, respectively.

The above enzymatic properties indicated that the NADPH-UQ reductase activity obtained here was derived from a UQ-reducing flavoenzyme. The enzymatic properties also indicated that the NADPH-UQ reductase was different from the known NADH-dependent enzymes in mitochondria, Golgi apparatus, microsomes, and plasma membranes. At least the possibility of UQ reduction by enzymes released from these organelles during the preparation of the cytosol from rat liver could be disregarded. We showed that 68% of the total UQ-reducing activity in rat liver homogenate is present in cytosol and the greater part of the cytosolic activity could be explained by the action of this NADPH-UQ reductase (*16*). These result indicate that the NADPH-UQ reductase obtained here is a novel UQ-reducing flavoprotein in the cytosol and is distinct from known quinone reductases.

In rat liver cytosol, the concentration of NADPH and the ratio of $NADP^+$/NADPH were reportedly 97.1±13 μM (*30*) and 0.014 (*31*), respectively. Furthermore, UQ-9 concentrations in rat liver homogenate and cytosol were 132±3 and 26.8±7.2 μM, respectively, as calculated from our published data (*5*). These values are much higher than the K_m values of 14.4±2.5 μM for NADPH and 2.66±0.48 μM for UQ-9 of NADPH-UQ reductase activity in rat liver cytosol. From these results, we propose that the cytosolic NADPH-UQ reductase described here is the enzyme responsible for the cyclic reduction of intracellular UQ to UQH_2. Purification of this enzyme, which is a very unstable protein, is now under way.

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