ISOLATION AND PROPERTIES OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-HEPATOREDOXIN REDUCTASE OF RABBIT LIVER MITOCHONDRIA*

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SUMMARY: An NADPH-hepatoredoxin reductase was purified from mitochondria of rabbit hepatocytes. The optical absorption spectrum showed a typical flavoprotein. The NADPH-hepatoredoxin reductase has an FAD as a coenzyme and the molecular weight of the NADPH-hepatoredoxin reductase was estimated to be 51000 by SDS-polyacrylamide gel electrophoresis. The NADPH-hepatoredoxin reductase was immunochemically similar to NADPH-adrenodoxin reductase of bovine and pig adrenocortical mitochondria, but not NADPH-cytochrome P-450 reductase of rabbit liver microsomes. The NADPH-cytochrome c reductase activity of the NADPH-hepatoredoxin reductase and hepatoredoxin complex, unlike NADPH-cytochrome P-450 reductase, was decreased by increasing ionic strength.

INTRODUCTION: Cytochrome P-450-linked mixed function oxidase exists as a microsomal and a mitochondrial type. NADPH-dependent cytochrome P-450-linked mixed function oxidase system of the mitochondrial type is composed of NADPH-ferredoxin reductase, ferredoxin and cytochrome P-450 and may exist in adrenocortical and renal mitochondria(1-3). Recently, Pedersen et al. reported that hepatic ferredoxin(hepatoredoxin)was purified from bovine hepatic mitochondria(3). We could purify the hepatoredoxin, cytochrome P-450 and NADPH-hepatoredoxin reductase from rabbit liver mitochondria. This communication reports on the isolation and properties of NADPH-hepatoredoxin reductase from the mitochondria of rabbit hepatocytes.

MATERIALS AND METHODS: Male New Zealand white rabbits weighing 2.0 to 2.5 Kg were fed a standard commercial diet for a week before the beginning of the experiments. They were killed by air embolism. The livers were quickly removed, perfused with ice-cold isotonic saline to remove blood, freed of connective tissue, fat and the principal blood vessels, then finely divided with a blade and homogenized in 5 volume of ice-cold 0.25 M sucrose adjusted with 0.1 M Tris, with a loosely fitting Teflon Potter-Elvehjem homogenizer. The homogenate was used to prepare the liver mitochondria by the method of Hogeboom and Schneider (4). Contamination of the mitochondria with microsomes was less than 2% on a protein basis, as estimated from glucose 6-phosphatase activity. The mitochondria were used to isolate the NADPH-hepatoredoxin reductase as a material source. Hepatoredoxin was purified from rabbit liver mitochondria by the method of Pedersen et al.(3) with some modification. NADPH-cytochrome c reductase and NADPH-ferricyanide reductase activities of NADPH-hepatoredoxin reductase were assayed in the presence and absence of 0.2 mM KCN by the method of Hiwatashi

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Table 1. Purification of NADPH-hepatoredoxin reductase from rabbit liver mitochondria

Preparation	Total volume (ml)	Total protein (mg)	Total activity (*)	Purification (fold)	Specific activity (**)	Yield (%)
Mitochondria	712	9875	36.5	1	3.7	100
Mitochondrial extract	520	3242	31.8	3	9.8	87
1st DEAE-cellulose	320	200	14.6	20	73.4	40
2nd DEAE-cellulose	115	30	6.0	54	199.3	16
2'5'-ADP-Sepharose 4B	4	2.8	2.3	220	813.0	6.3

* μmoles/min; ** nmoles/min/mg protein.
These activities were determined with NADPH-ferricyanide reductase activity.

et al.(1) Glucose 6-phosphatase activity was assayed by the method of Swanson(5). NADH, NADPH and their oxidized forms and cytochrome c (type III) were obtained from Sigma Chemical Co. Molar coefficients of NADH and NADPH used were 6.30 x 10^3 $M^{-1}cm^{-1}$ at 340 nm (pH 7.6 and 25°C). This value was obtained according to McComb et al.(6) and Ziegenhorn et al.(7).

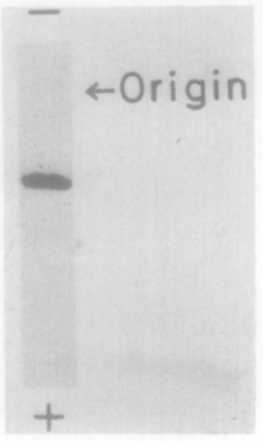
Freund's complete adjuvant was a product of Difco, and Ouchterlony double diffusion agar plates were obtained from Nakarai Chemical Co.

Antibodies were prepared and inoculated to immunize the rabbits. Five mg protein of bovine NADPH-adrenodoxin reductase in 0.5 ml of 0.01 M K-phosphate buffer, pH 7.4, was mixed with 5 ml of Freund's complete adjuvant and injected into the foot pad area. After 2 weeks, booster injections of 5 mg protein of the NADPH-adrenodoxin reductase were administered. The rabbits were bled from the Arteria carotis to collect 150ml of blood. The blood was used as a sourse of bovine NADPH-adrenodoxin reductase antibody. The antibody was purified by the method of Kekwick(8) with modification. NADPH-adrenodoxin reductase was purified from bovine adrenocortical mitochondria by the method of Hiwatashi et al.(1).

The amount and species of flavin in NADPH-hepatoredoxin reductase were measured. To release the flavin, NADPH-hepatoredoxin reductase was digested with 0.2%(w/v) protease for 30 min at 37°C, then the hepatoredoxin reductase solution was heated for 10 min at 80°C, rapidly cooled to 0°C, and centrifuged to remove the denatured protein(9). The species and amounts of flavin were determined by paper chromatography with three different solvent systems [1-butanol-acetic acid-H_2O (4:1:5), pyridine-H_2O (2:1) and 5% Na_2HPO_4] (10) and by activation of the apoenzyme of D-amino acid oxidase, which was prepared by the method of Negelein and Brömel(11). D-Amino acid oxidase activity was measured by the method of Fonda and Anderson(12). Protein content was determined by the method of Lowry et al. (13) and the biuret reaction(14). 2'5'-ADP-Sepharose 4B(Sepharose 4B-bound N^6-(6-aminohexyl)-adenosine 2'5'-bisphosphate) was synthesized by the method of Brodelius et al.(15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the methods of Weber and Osborn(16) and O'Farrell (17).

RESULTS: NADPH-hepatoredoxin reductase was purified from rabbit liver mitochondria. The isolation procedures were carried out at 0°C to 4°C. The mitochondrial fraction(9,875 mg protein) was suspended in 10 mM Na-phosphate buffer, pH 7.4, and the solution(14 mg protein/ml x 700 ml) was homogenized twice for 4 min using a Matsushita homogenizer, Model MX-140S, because the NADPH-hepatoredoxin reductase was solubilized more from the mitochondria with Na-phosphate



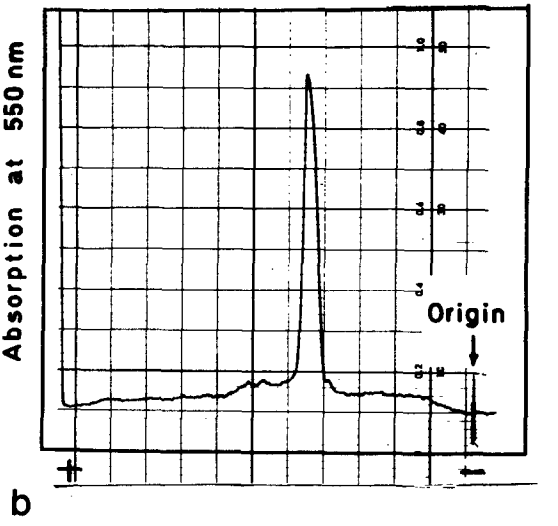


Figure 1(a, b).
a) Electrophoretogram of rabbit NADPH-hepatoredoxin reductase on SDS-polyacrylamide gel. Five μg protein of the hepatoredoxin reductase was used on the gel.
b) Figure 1(b) is a densitometric scan of the gel of Fig.1(a).

buffer, pH 7.4, than K-phosphate buffer, pH 7.4. The homogenized mitochondrial fraction was centrifuged at 65,000 x g for 60 min in a refrigerated Hitachi 55p ultracentrifuge with a refrigerated barrel rotor. The brown supernatant was applied to a DEAE-cellulose column(6 x 40 cm) preequilibrated with 10 mM K-phosphate buffer, pH 7.4. The column was washed with 1 liter of 10 mM K-phosphate buffer, pH 7.4. Next, the yellow-brown band of NADPH-hepatoredoxin reductase was eluted with 30 mM K-phosphate buffer, pH 7.4, while the brown pigment, hepatoredoxin, remained at the top of the column. The NADPH-hepatoredoxin reductase was fractionated between 30 and 60% saturation of ammonium sulfate. The precipitate was dissolved in 10 mM K-phosphate buffer, pH 7.4, and dialyzed against 10 liters of the same buffer for 20 hrs using a magnetic stirrer; the outer medium was changed three times. The dialyzed solution was centrifuged at 9,000 x g for 90 min and the supernatant was applied to a DEAE-cellulose column(3 x 35 cm) preequilibrated with 10 mM K-phosphate buffer, pH 7.4. The column was washed with 300 ml of 10 mM K-phosphate buffer, pH 7.4, then the crude NADPH-hepatoredoxin reductase solution was eluted with 30 mM K-phosphate buffer, pH 7.4. The crude NADPH-hepatoredoxin reductase was fractionated between 35% and 55% saturation of ammonium sulfate. The precipitate obtained was dissolved in 10 mM K-phosphate buffer, pH 7.4, and dialyzed against 10 liters of the same buffer for 12 hrs. The dialyzed solution was applied to 2'5'-ADP-Sepharose 4B column

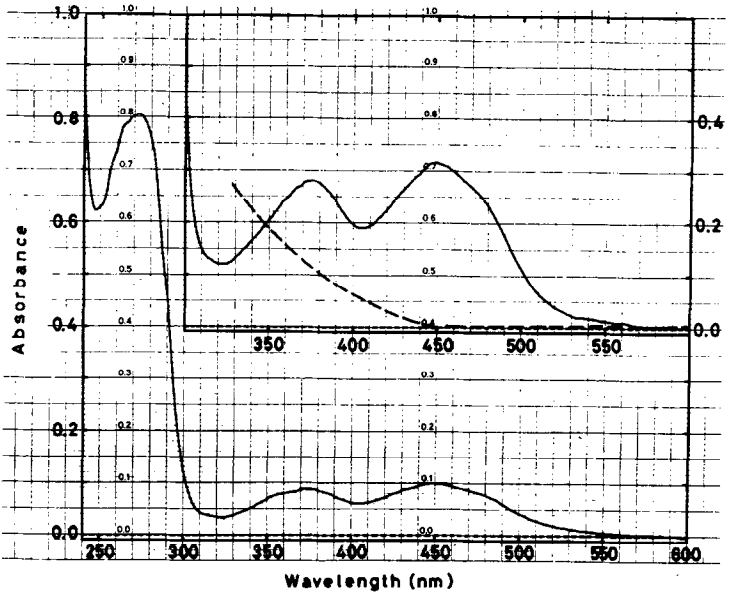


Figure 2.
Optical absorption spectra of rabbit NADPH-hepatoredoxin reductase. The cuvette contained 0.5 mg protein/ml (1.5 mg protein/ml for magnified spectra in the visible region) in 0.1 M K-phosphate buffer, pH 7.4 and 25°C. ————, Oxidized form; — — — , dithionite-reduced form; ------, base line.

Table 2. Activities of NADPH-cytochrome c reductase of complex of rabbit NADPH-hepatoredoxin reductase with various ferredoxins

Electron carrier	NADPH-cytochrome c reductase activity (nmoles/min/mg protein)
None	10.7 ± 4.7
Rabbit hepatoredoxin	627.3 ± 35.2
Bovine adrenodoxin	298.7 ± 29.5
Pig adrenodoxin	321.7 ± 20.2
Spinach ferredoxin	24.7 ± 5.5

Values are averages of five estimations ± standard deviations. NADPH-cytochrome c reductase activity was determined in the presence of 2 μM superoxide dismutase by the method of Hiwatashi et al. (1).

(2 x 7 cm) preequilibrated with 50 mM K-phosphate buffer, pH 7.4. The column was washed with five column volumes of the 50 mM K-phosphate buffer, pH 7.4, then the NADPH-hepatoredoxin reductase was specifically eluted with 0.5 mM $NADP^+$ in the buffer. Table 1 shows a typical purification scheme for NADPH-hepatoredoxin reductase of rabbit liver mitochondria.

In SDS-polyacrylamide gel electrophoresis, the isolated NADPH-hepatoredoxin reductase was a single band of protein. It was electrophoretically homogeneous

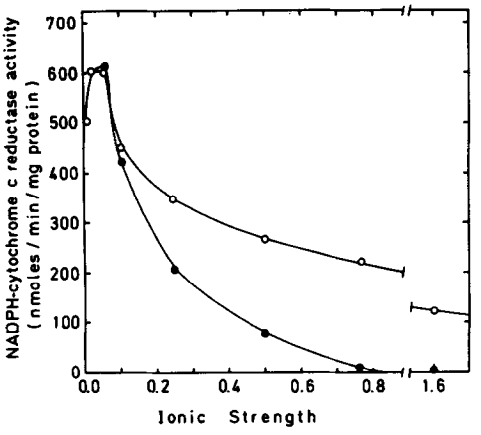


Figure 3.
Effect of ionic strength on the NADPH-cytochrome c reductase activity of the NADPH-hepatoredoxin reductase and hepatoredoxin complex. Ionic strength was adjusted by adding NaCl in 10 mM K-phosphate buffer, pH 7.4(●) and various concentrations of K-phosphate buffer, pH 7.4(O). The activity was measured at 25°C.

and highly pure, as shown in Figure 1. The specific activity of NADPH-cytochrome c reductase of hepatoredoxin and the NADPH-hepatoredoxin reductase complex was 0.637 ± 0.035 μmoles/min/mg protein (25°C). Hepatoredoxin could be replaced by bovine and pig adrenodoxin and spinach ferredoxin. The NADPH-cytochrome c reductase activity of NADPH-hepatoredoxin reductase and various ferredoxins was summarized in Table 2. The NADPH-cytochrome c reductase activity of the NADPH-hepatoredoxin reductase and ferredoxin complex showed the most activity with use of the hepatoredoxin of the same animal rather than that of another.

Figure 2 shows optical absorption spectra of NADPH-hepatoredoxin reductase in the oxidized and reduced forms. The absorption peaks were at 273, 376, and 447 nm with shoulders at 290, 386, and 480 nm. This oxidized absorption was a typical flavoprotein. We found that NADPH-hepatoredoxin reductase accepts two electrons from NADPH. The coenzyme of the flavoprotein was an FAD per mole of the NADPH-hepatoredoxin reductase. This was determined by the activation of apo-D-amino acid oxidase and paper chromatography. Flavin mononucleotide(FMN) and metals such as iron, copper and manganese were not detectable in the hepatoredoxin reductase. The molecular weight of the NADPH-hepatoredoxin reductase was estimated to be 51000 by SDS-polyacrylamide gel electrophoresis. This value was similar to the molecular weights of bovine and pig NADPH-adrenodoxin reductases, but not as large as that of microsomal NADPH-cytochrome P-450 reductase (18).

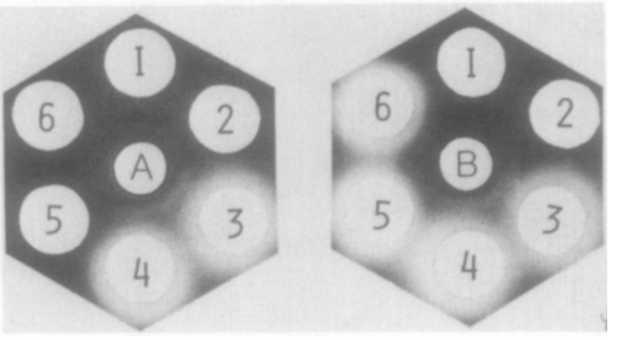


Figure 4(A, B).
Ouchterlony double diffusion analysis of bovine NADPH-adrenodoxin reductase antibody against NADPH-hepatoredoxin reductaes of rabbit and bovine hepatic mitochondria, bovine NADPH-adrenodoxin reductase, NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase of rabbit microsomes.
A) Figure 4A shows the interactions between the bovine NADPH-adrenodoxin reductase antibody in the center well and bovine NADPH-adrenodoxin reductase(1, 2), rabbit NADPH-hepatoredoxin reductase(3, 4), rabbit NADPH-cytochrome P-450 reductase(5), rabbit NADH-cytochrome b_5 reductase(6).
B) Figure 4B shows the interactions between the bovine NADPH-adrenodoxin reductase antibody in the center well and bovine NADPH-adrenodoxin reductase(1, 2), rabbit lung mitochondria(3), rabbit kidney mitochondria(4), rat liver mitochondria(5), chicken liver mitochondria(6). These mitochondria were sonicated and they were used for the immunochemical reactions.

Figure 3 shows the effects of ionic strength on NADPH-cytochrome c reductase activity of the complex of NADPH-hepatoredoxin reductase and hepatoredoxin. In the case of NADPH-cytochrome c reductase activity of the NADPH-hepatoredoxin reductase and hepatoredoxin complex, the maximal activity was attained at an ionic strength of 0.06. The minimal activity was attained above an ionic strength of 0.80. The activity decreased with increasing ionic strength. The decrease was reversible by dilution of the test solution from one of high ionic strength to one of low ionic strength. This may be due to dissociation of the complex into the hepatoredoxin and the NADPH-hepatoredoxin reductase. The effect of ionic strength on NADPH-cytochrome c reductase activity is very different from that on NADPH-cytochrome c reductase activity of microsomal NADPH-cytochrome P-450 reductase(19).

Figure 4 demonstrates interactions of antibody of bovine NADPH-adrenodoxin reductase to NADPH-hepatoredoxin reductase on Ouchterlony double diffusion agar plates. The precipitin lines indicate partial immunochemical identity between bovine NADPH-adrenodoxin reductase and rabbit NADPH-hepatoredoxin reductase. NADPH-hepatoredoxin reductase was also found in the livers of pig, chicken, rat and guinea pig. Similar ferredoxin reductases were also found in the mitochondria of kidney and lung of the animals. This figure shows that bovine NADPH-adreno-

doxin reductase antibody did not interact with NADH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase. The result shows that NADPH-hepatoredoxin reductase in rabbit liver mitochondria is immunochemically similar to bovine and pig NADPH-adrenodoxin reductases.

DISCUSSION: NADPH-hepatoredoxin reductase was isolated from rabbit liver mitochondria. NADPH was the physiological reductant for the hepatoredoxin reductase and not NADH. Hepatic mitochondrial hydroxylase of cholesterol was confirmed to consist of three components, NADPH-hepatoredoxin reductase, hepatoredoxin and cytochrome P-450, judging from the cholesterol hydroxylation activity after reconstitution. NADPH-hepatoredoxin reductase was an essential component of the hydroxylase system of cholesterol. NADPH-cytochrome c reductase activity of the complex of NADPH-hepatoredoxin reductase and hepatoredoxin showed that hepatoredoxin was specific for NADPH-hepatoredoxin reductase. The effect of ionic strength on this complex was investigated; it was released at an ionic strength above 0.8. This result differs from the dissociation at 0.25 ionic strength of the complex of bovine NADPH-adrenodoxin reductase and adrenodoxin(20). This indicates that NADPH-hepatoredoxin reductase may be bound more tightly with hepatoredoxin. NADPH-cytochrome P-450 reductase of rabbit liver microsomes could not reduce hepatoredoxin of rabbit liver mitochondria. Even in the case of the same animal, the NADPH-ferredoxin reductase was immunochemically different in different tissues. Unexpectedly, NADPH-ferredoxin reductases in bovine livers and adrenocortices did not fuse and spur formation was observed.

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