SOME PROPERTIES OF KIDNEY CORTEX AND SPLENIC MICROSOMAL NADPH-CYTOCHROME *C* REDUCTASE

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1. Introduction

An NADPH-cytochrome *c* reductase activity was observed in microsome particles from several mammalian tissues [1–5], anaerobically grown yeast [6] and housefly (Larvae) [7]. Hepatic microsomal flavoprotein, NADPH-cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase, EC 1.6.99.2), which contains one to two molecules of FAD per molecule of enzyme has been purified [1,8,9]. However, the reductase from rabbit or pig microsomes was re-examined as flavoprotein containing one mole each of FAD and of FMN per mole enzyme [10] and the two flavines have nonidentical properties from the data of the oxidation–reduction reactions [10,11]. The active mixed-function oxidase system has been reconstituted from microsomal fractions containing NADPH-cytochrome *c* reductase and cytochrome *P*-450 [12,13]. No evidence for the existence of an iron-sulfur protein of adrendoxin and putidaredoxin or non-heme iron protein of rubredoxin type has been obtained in the hepatic microsomes [14,15]. A possible functional role of each flavine in hepatic enzyme has been discussed [11]. It is, therefore, important to compare the characteristics of kidney cortex and splenic microsomal NADPH-cytochrome *c* reductase with those of hepatic NADPH-cytochrome *c* reductase (NADPH-cytochrome *P*-450 reductase).

The present paper provides evidence that a purified NADPH-cytochrome *c* reductase prepared by trypsin solubilization and chromatography from kidney cortex and splenic microsomes contained one mole each of FAD and of FMN per protein molecule. O_2-stable semiquinone with distinctive absorption spectrum was obtained by reduction with NADPH in the presence of oxygen and not completely reduced by excess NADPH. These results obtained with kidney and splenic enzyme were very similar to those of hepatic NADPH-cytochrome *c* reductase [10], and suggest that kidney cortex and splenic microsomal NADPH-cytochrome *c* reductase have properties and functions similar to those of hepatic enzyme.

2. Materials and methods

Fresh pig kidney cortex and spleen were homogenized for a total of 5 min in four volumes of ice-cold 0.15 M KCl and fractionated by differential centrifugation [10]. Pig kidney cortex and splenic NADPH-cytochrome *c* reductase were prepared by the same method described elsewhere [10]. Yield of the purified reductase was about 10–20% of microsomal activity. NADH- and NADPH-cytochrome *c* reductase activities were determined by the method of Omura and Takesue [9]. Flavin content was determined by the method of Iyanagi and Mason [10], using phosphodiesterase and apoflavodoxin. Enzyme concentration expressed as flavine concentration was determined spectrophotometrically, using ϵ 455 = 10.7 mM^{-1} cm^{-1} [10]. All chemicals and reagents were of standard reagent grades obtained from commercial sources.

3. Results

The molecular weight of trypsin-solubilized NADPH-cytochrome *c* reductase from pig kidney cortex and splenic microsomes has been estimated to be 66 000,

Table 1
FMN and FAD of kidney cortex and splenic microsomal NADPH-cytochrome *c* reductase, estimated with phosphodiesterase and Apo-flavodoxin

Sample	Initial fluorimetric reading at 520 nm	Reading after phosphodiesterase addition (0.8 mg/ml)	Reading after apo-flavodoxin addition (2×10^{-6} M)
D-Amino acid oxidase (1.0×10^{-6} M)	7.2	77.0	1.8
Kidney NADPH-cyt.*c.* reductase (0.95×10^{-6} M)	38.2 37.8	71.0 Not treated	1.3 4.7
Splenic NADPH-cyt.*c* reductase (1.0×10^{-6} M)	37.7	76.2	2.3

Flavine solutions from D-amino acid oxidase and NADPH-cytochrome *c* reductase were prepared by the method of Mayhew and Massey [16]. Fluorescence was measured at 520 nm (emission) and 445 nm (excitation) in 0.02 M Na-acetate buffer, pH 6.0 (containing 0.2 M NaCl).

by using the gel filtration method with Sephadex G-100. The pH optimum of NADPH-cytochrome *c* reductase activity was in the range of pH 8–9. The K_m values for NADPH and NADH were 3.3×10^{-6} M, 4×10^{-2} M (kidney) and 4.7×10^{-6} M, 5×10^{-2} M (spleen), respectively. The K_m values for cytochrome *c* were 9.5×10^{-6} M (kidney), 6.0×10^{-6} M (spleen).

The flavin of purified NADPH-cytochrome *c* reductase was analyzed using phosphodiesterase and apoflavodoxin. The addition of snake venom phosphodiesterase to total flavine released by the method of Mayhew and Massey [16] from NADPH-cytochrome *c* reductase resulted in increase of 1.86 (kidney) and 2.0 (spleen) in fluorescence at 520 nm; that is, fluorescence due to FMN upon hydrolysis of FAD. Furthermore, addition of 2-fold excess of apoflavodoxin to the phosphodiesterase-treated flavines resulted in decrease of about 50-fold in fluorescence at 520 nm. These results (table 1) as well as the results obtained in hepatic NADPH-cytochrome *c* reductase indicate that kidney cortex and splenic microsomal NADPH-cytochrome *c* reductase contained FAD and FMN in about equimolar quantities.

Fig. 1 depicts the visible absorption spectrum of purified kidney NADPH-cytochrome *c* reductase. The oxidized enzyme has absorption peaks at 455 nm and 3.80 nm. The absorption spectrum of the stable semiquionone was obtained at the NADPH addition in the presence of air. This distinctive spectrum (fig. 1: curve B) is similar to that obtained with hepatic reductase [10]. The stable semiquinone form was not

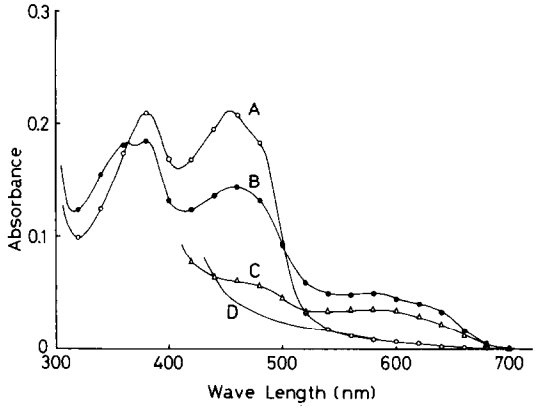

Fig. 1. Absorption spectra of purified kidney NADPH-cytochrome *c* reductase. The stable semiquinone was prepared by adding NADPH (66 μM, final concentration) in the presence of air to purified enzyme (20 μM) in 0.1 M potassium phosphate buffer (pH 7.7): curve A, oxidized: curve B, stable semiquinone form: curve C, after NADPH (660 μM, final concentration) under anaerobic conditions: curve D, hydrosulfite reduced form.

completely reduced by excess NADPH (fig. 1: curve C). Almost identical results were also obtained in splenic NADPH-cytochrome *c* reductase.

4. Discussion

The electron transfer system of the hepatic microsomal cytochrome *P*-450 mixed function oxidase differs from *Pseudomonas putida* and adrenal mitochondria containing the reductase, non-heme iron protein and *P*-450; namely, iron–sulfur protein with a $g=1.94$ signal is not detected in hepatic microsomes [14,15]. In addition, differences in oxidation-reduction reactions between microsomal NADPH-cytochrome *c* reductase and adrenodoxin reductase or putidaredoxin reductase have been reported, e.g., hepatic microsomal reductase forms the stable semiquinone form during the titration of the oxidized enzyme by NADPH or sodium dithionite [11] but adrenodoxin reductase does not form a stable semiquione [17]. Adrenodoxin and putidaredoxin reductase [17,18], which contain FAD as a prosthetic group, do not react with cytochrome *c* but react with it only in the presence of non-heme iron protein [17]. These reductases play a similar role in electron transfer, as follows [21,22],

NAD(P)H → fp (FAD) → non-heme iron protein → *P*-450

The results of the reconstitution of mixed function oxidase show that the hepatic NADPH-cytochrome *c* reductase fraction can replace kidney reductase [2] or yeast reductase fractions [19]. Furthermore, NADPH-cytochrome *c* reductase activity in spleen microsomes is inhibited by liver anti-NADPH-cytochrome *c* reductase γ-globulin [3]. These results suggest that these reductases have very similar properties in structure and function.

Electron transfer components in microsomes are summarized in table 2. This table suggests that the same cytochrome *P*-450 reductase system operates in microsomes. Adrenodoxin [20,21] and putidaredoxin [22] are components of the electron transfer system containing *P*-450. However, microsomal system, which does not contain the non-heme iron protein [26], may contain FMN instead of adrenodoxin and putidaredoxin. If microsomal NADPH-cytochrome *c* reductase is directly involved in the microsomal reduction of cytochrome *P*-450 during the mixed function oxidase cycle, it appears likely that the electron transfer sequence can be the following [11].

NADPH → fp (FAD ⇌ FMN) → *P*-450

In this case, each flavine in hepatic, kidney and splenic NADPH-cytochrome *c* reductase may have an individual function, that is, one flavine accepts two reducing equivalents from NADPH and the other acts as a one-electron carrier in the electron transfer from NADPH to cytochrome *P*-450. The low potential state (−371 mV) [11] of flavin in the microsomal NADPH-cytochrome *c* reductase may act as non-heme protein

Table 2
The electron transfer components in microsomes

Source of microsomes	NADPH-cyt. *c* reductase activity (nmoles/min per mg of protein)	Prosthetic group of NADPH-Cyt.*c* reductase	*P*-450	Cyt. b_5
Liver	209 [10]	FAD, FMN [10,11]	+	+
Kidney	30–38 [24]	FAD, FMN [This paper]	+	+
Spleen	3.3 [3]	FAD, FMN [This paper]	+	(+)
Thyroid	4.7 [25]	?	Peroxidase**	+
Yeast	+ [19]	(FAD, FMN)*	+	+
Housefly (Larvae)	53.3 [7]	?	+	+

* Although the localization within the cell is not clear, the isolation of NADPH-cytochrome *c* reductase from yeast is reported [23].

** The existence of cytochrome *P*-450 is not reported, but contain peroxidase [4].

in the microsomal electron transport system. That is, it probably functions as a univalent carrier operating between the semiquinone and fully reduced forms [16].

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