MICROSOMAL DPNH CYTOCHROME *C* REDUCTASE*

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Liver homogenates have been shown by STRITTMATTER AND BALL and others[1-4] to contain a non-mitochondrial particulate hemoprotein with DPNH§ cytochrome *c* reductase activity. The heme of this particle has been characterized as cytochrome b_5[2]. CHANCE AND WILLIAMS[5] have studied the kinetics of the reduction of the particle-bound heme by DPNH. Recently STRITTMATTER AND VELICK[3] isolated from rabbit liver a highly purified and soluble cytochrome b_5 which was prepared by treating a particulate suspension of the cytochrome with pancreatic lipase. Subsequently they liberated a soluble flavoprotein from the hemoprotein particle and showed that the isolated flavoprotein catalyzed the oxidation of DPNH by cytochrome b_5 but not by cytochrome *c*[6,7].

In the present communication the purification and properties of a particulate DPNH cytochrome *c* reductase from beef liver are described. This enzyme is found in association with the microsomal fraction. The purified enzyme contains flavin, cytochrome b_5 and lipide, and is approximately 80 to 100 fold more active than the original microsomal preparations in catalyzing the oxidation of DPNH by ferricyanide or cytochrome *c*.

METHODS AND REAGENTS

Assays of enzymic activity were carried out spectrophotometrically as described previously[8]. Azide (0.01 *M*) was used instead of cyanide to inhibit reoxidation in the cytochrome *c* reductase assay. Flavin (total and acid-extractable), iron and protein were determined by previously published procedures[9,10]. Heme was determined by the methods of CHANCE[11] and BASFORD *et al.*[12]. Lipide was extracted from the particle with a 1:3 mixture (v/v) of ethyl ether and ethanol. The lipide fraction was then reextracted with petroleum ether before gravimetric estimation. We are indebted to Doctor HELMUT BEINERT of the Institute for Enzyme Research for the iron and lipide determinations reported in the present communication. Ribonucleic acid was determined by the method of LITTLEFIELD *et al.*[13].

The sources of the following reagents are indicated in parentheses: DPNH and cytochrome *c* (Sigma Chemical Company), FMN§ (Hoffman-La Roche), and FAD§ (California Foundation for Biochemical Research).

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§ The following abbreviations have been used: DPNH, dihydrodiphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

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PREPARATION OF ENZYME

(a) A microsomal preparation was obtained from fresh beef liver by the following method. All procedures were carried out in the temperature range of 0 to 5°. Beef liver was quickly ground in an electric meat grinder. To each 500 g of liver, 1500 ml of a solution containing dibasic potassium phosphate (0.01 *M*) and sucrose (0.25 *M*) were added along with 2 ml of 6 *N* potassium hydroxide. The mixture was blended at moderate speed for 20 sec, and was centrifuged for 15 min at 1300 *g*. The supernatant fluid (volume 7 to 8 l) was decanted through cheese cloth, and 2 to 3 l of 0.25 *M* sucrose solution were added to the filtrate. The filtrate was spun in the refrigerated Sharples centrifuge at full speed (flow rate of 300 ml/min), and the effluent was collected and diluted with an equal volume of 0.9% potassium chloride solution. This mixture was again spun at full speed in the Sharples centrifuge but at a reduced flow rate of 100 ml/min. The precipitate (microsomal fraction) was collected and suspended in an equal volume of a 0.25 *M* solution of sucrose.

(b) Stage I enzyme: Suspensions of microsomes were diluted with 0.2 *M* Tris buffer of pH 8.0 to a final protein concentration of 20 mg/ml. A neutral solution (10%) of deoxycholate was added in sufficient amount so that the final concentration of deoxycholate/mg of protein was between 0.2 and 0.3 mg. (The exact amount of deoxycholate for best yield of enzyme should be determined for each preparation of microsomes.) The mixture was centrifuged in the 30 rotor of the Spinco preparative ultracentrifuge for 90 min at 30,000 r.p.m., and the supernatant fluid was dialyzed for 24 h against 30 volumes of 0.02 *M* phosphate buffer of pH 7.5. The dialyzed fraction was centrifuged in the 30 rotor of the Spinco for 90 min at 30,000 r.p.m., and the loosely packed residue (Stage I) was suspended in a solution containing sucrose (0.25 *M*) and phosphate buffer (0.02 *M*, pH 7.5). The protein concentration should be about 20 mg/ml. The residue fraction, which will be referred to hereafter as Stage I, contained 70 to 80% of the total DPNH cytochrome *c* reductase activity of the original microsome fraction and was approximately 8 times as active per mg of protein (*cf.* Table I). The final supernatant fraction which was discarded contained a large amount of diaphorase and some 20 to 30% of the original cytochrome *c* reductase activity. A proportionate amount of cytochrome b_5 could be detected in the supernatant fraction. By repeated dialysis and centrifugation of the supernatant fraction, partial recovery of the residual cytochrome *c* reductase could be achieved.

(c) Stage II enzyme: Suspensions of the particulate enzyme (Stage I) were mixed with 0.25 volumes of saturated ammonium sulfate (neutralized) and an equal volume of *n*-butanol. The mixture was quickly and thoroughly homogenized and then centrifuged in the 30 rotor of the Spinco. As soon as a speed of 30,000 r.p.m. was attained with maximum acceleration, the centrifuge was turned off. The water and butanol layers were poured off and discarded. The interface layer of solid material (between the butanol and water layers) was carefully separated from the packed denatured material at the bottom of the tubes and suspended in approximately 200 ml of 0.02 *M* phosphate buffer of pH 7.5. The suspension was homogenized and centrifuged in the 30 rotor of the Spinco for 20 min at 30,000 r.p.m. The supernatant fluid (*s*) was set aside while the residue was homogenized in 0.02 *M* phosphate buffer of pH 7.5. The suspension was then centrifuged in the 30 rotor at 4000 r.p.m. for 15 min. The supernatant fluid was combined with (s), and the mixture was centrifuged in the 30 rotor

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for 65 min at 30,000. The supernatant was discarded, and the residue (Stage II) was suspended in 0.02 *M* phosphate buffer of pH 7.5. Stage II suspensions contained 40 to 50% of the total DPNH cytochrome *c* reductase activity of the starting suspensions of microsomes and were 80 to 100 times more active per mg of protein (*cf.* Table I). The preparations slowly lost activity when stored at 0° or frozen at −20°, approximately 50% of the activity being lost over a period of 1 to 2 weeks. Stage II particle suspensions did not show TPNH cytochrome *c* reductase, aldehyde oxidase, xanthine oxidase or succinic dehydrogenase activity. The ratio of DPNH-ferricyanide to DPNH cytochrome *c* activity* was as low as 1.0 in occasional fresh preparations at Stage II. But more often the ratio of activities had a value between 1.5 and 2.0. With handling of the suspension *e.g.* by alternate freezing and thawing, the DPNH-cytochrome *c* reductase activity declined relative to the DPNH-ferricyanide activity. Preincubation of the enzyme suspension with cholate (1 mg cholate per mg enzyme protein) led to an increase in both activities, but the increase in DPNH-ferricyanide activity was proportionately greater (ratio of 1.8 to 2.0).

TABLE I

COMPOSITION OF MICROSOMAL DPNH CYTOCHROME *c* REDUCTASE AT VARIOUS STAGES OF PURITY

*Preparation**	*Flavin*		*Heme*	*Non-heme iron*	*Lipide*	*Activity*	
	TCA extracted	*Total trypsin digested*				*Cyt. c*	*Fe(CN)*$_1$
	mμmole/mg protein		*mμmole/mg protein*	*mμmole/mg protein*	*% dry wt.*	*μmole DPNH/mg/min*	
Original microsomes	0.30	0.60	0.30	—	—	0.35	2.7
Stage I enzyme	0.31	0.85	1.9	7.0	71.0	4.7	9.8
Stage II enzyme	1.6	2.1	5.3	2.6	71.0	23.0	34.0

* Each value represents the average of determinations on 4 or more preparations.

RESULTS

Spectroscopic and enzymic properties

When Stage II preparations were reduced with excess DPNH or with dithionite and the light absorption of the reduced form was measured against that of the oxidized form, difference spectra as shown in Fig. 1 were obtained. The absorption maxima at 556, 526 and 425 mμ are characteristic of cytochrome b_5, while a minimum at about 450 mμ indicates the presence of flavin. Approximately 85% of the cytochrome b_5 present in the preparation was reduced by DPNH.

The purified enzyme catalyzed the oxidation of DPNH by cytochrome *c*, ferricyanide or 2,6-dichlorophenolindophenol. The rate of oxidation of DPNH by 2,6-dichlorophenolindophenol was about one fourth that with ferricyanide. TPNH could not replace DPNH as substrate. Stage II preparations of the highest purity catalyzed the oxidation of 25 to 30 μmoles of DPNH per min/mg of protein with cytochrome *c* as acceptor, and 40 to 50 μmoles of DPNH with ferricyanide as acceptor. The ratio of ferricyanide activity to cytochrome *c* activity (as defined above) was consistently between 1.5 and 2.0. Phosphate ions were not required for enzymic activity, the reaction proceeding equally well in Tris buffer. In phosphate buffer, activity was maximal over the pH range 6.5 to 8.0.

* *μM* DPNH oxidized by ferricyanide/min: *μM* DPNH oxidized by cytochrome *c* per min.

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The K_m values of the enzyme preparation for DPNH and cytochrome *c* were estimated to be $5.8 \cdot 10^{-6}$ and $3 \cdot 10^{-6}$ *M* respectively from the experimental data.

Antimycin A at high levels (100 γ/mg enzyme protein) and amytal ($4 \cdot 10^{-3}$ *M*) did not inhibit the oxidation of DPNH by cytochrome *c*. Versene ($1 \cdot 10^{-3}$ *M*), Versene EDG ($1 \cdot 10^{-3}$ *M*), *o*-phenanthroline ($1 \cdot 10^{-3}$ *M*) and acetyl acetone ($5 \cdot 10^{-4}$ *M*) were without effect on the activity of the enzyme. Quinacrine ($1 \cdot 10^{-4}$ *M*) did not inhibit activity with ferricyanide, 2,6-dichlorophenolindophenol or cytochrome *c* (rechecked at $6 \cdot 10^{-4}$ *M* quinacrine) as terminal acceptors. *p*-Chloromercuribenzoate ($1 \cdot 10^{-4}$ *M*) inhibited both cytochrome *c* and ferricyanide reductase activities 100%. In all inhibition studies, the enzyme was preincubated with the inhibitor for 3 min at 0° before the reaction was begun.

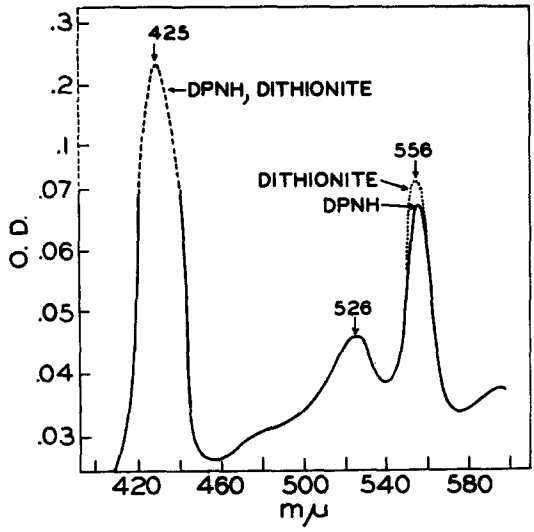

Fig. 1. Difference spectra (reduced minus oxidized) of microsomal DPNH cytochrome *c* reductase (Stage II). The enzyme suspension was reduced with dithionite (0.2 mg) or DPNH (1 mg). The sample contained 0.4 mg of enzyme protein in 1 ml of 0.02 *M* phosphate buffer (pH 7.5).

Composition of enzyme preparations

The composition and activities of enzyme preparations at varying degrees of purity are shown in Table I. The heme concentration per mg of protein (cytochrome b_5) and the concentration of acid-extractable flavin increased with increasing activity of the preparations. Flavin was removed in large amounts during both stages in the purification procedure (particularly Stage I). This loss of flavin could in part be accounted for by a diaphorase present in discarded fractions. The small loss of heme during purification may have been due to denaturation of small amounts of enzyme by the procedures employed. Flavin was approximately 80% extractable with 7.5% TCA in the Stage II preparations. As yet the identity of the flavin has not been established. When Stage II preparations were treated by the method of DE BERNARD[14], which has been used successfully in releasing DPNH cytochrome *c* reductase from heart particles, only free flavin was liberated. Lipoprotein lipase or snake venom (*Crotalus adamanteus*) failed to liberate flavoprotein or flavin from the particle. Lipide

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constituted a major proportion, 71%, of the dry weight of the preparations at all levels of purification. Stage I preparations contained less than 1% ribonucleic acid as compared with 4 to 6% in the original microsomes. Non-heme iron was continuously removed during purification of the enzyme, but it was always found to be present in Stage II preparations. Prolonged dialysis of the preparations against 0.01 *M* solutions of Versene at pH 8.0 did not remove non-heme iron.

DISCUSSION

The observations reported above suggest that a particulate enzyme containing flavin, cytochrome b_5 and lipide is responsible for the DPNH cytochrome *c* reductase activity of beef liver microsomes. No evidence was found for a soluble form of DPNH cytochrome *c* reductase in our preparations, nor could a soluble flavoprotein (with cytochrome *c* reductase or diaphorase activity) be prepared from our highly purified, particulate DPNH cytochrome *c* reductase by the methods described. Although the flavin and cytochrome b_5 present in the purified enzyme preparations were reducible by DPNH, no additional evidence for their participation in the enzymic catalysis has as yet been obtained. The mechanism of the activation by cholate has not been established.

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SUMMARY

A particulate DPNH cytochrome *c* reductase has been isolated from beef liver microsomes in highly purified form. Preparations of the enzyme contained per mg protein an average of $2.1 \cdot 10^{-3}$ μM flavin, $5.3 \cdot 10^{-3}$ μM cytochrome b_5, and $2.6 \cdot 10^{-3}$ μM non-heme iron. Lipide accounted for 71% of the total dry weight. The enzyme catalyzed the oxidation of 50 μmoles of DPNH per min/mg enzyme protein by ferricyanide and 30 μmoles per min/mg by cytochrome *c*. Enzymic activity was inhibited by *p*-chloromercuribenzoate, but was unaffected by antimycin A, amytal and chelating agents.

REFERENCES

1 E. KUN, *Proc. Soc. Exptl. Biol. Med.*, 77 (1951) 441.
2 C. F. STRITTMATTER AND E. G. BALL, *J. Cellular Comp. Physiol.*, 43 (1954) 57.
3 P. STRITTMATTER AND S. F. VELICK, *J. Biol. Chem.*, 221 (1956) 253.
4 D. GARFINKEL, *Biochim. Biophys. Acta*, 21 (1956) 199.
5 B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 209 (1954) 945.
6 P. STRITTMATTER AND S. F. VELICK, *J. Biol. Chem.*, 221 (1956) 277.
7 P. STRITTMATTER AND S. F. VELICK, *Biochim. Biophys. Acta*, 25 (1957) 228.
8 B. MACKLER AND D. E. GREEN, *Biochim. Biophys. Acta*, 21 (1956) 1.
9 F. L. CRANE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
10 D. E. GREEN, S. MII AND P. M. KOHOUT, *J. Biol. Chem.*, 217 (1955) 551.
11 B. CHANCE, *Nature*, 169 (1952) 215.
12 R. E. BASFORD, H. D. TISDALE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 24 (1957) 107.
13 J. W. LITTLEFIELD, E. B. KELLER, J. GROSS AND P. C. ZAMECNIK, *J. Biol. Chem.*, 217 (1955) 111.
14 B. DE BERNARD, *Biochim. Biophys. Acta*, 23 (1957) 510.

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