STUDIES ON THE TERMINAL ELECTRON TRANSPORT SYSTEM

V. EXTRACTION OF A SOLUBLE DPNH CYTOCHROME *C* REDUCTASE FROM THE ELECTRON TRANSPORT PARTICLE

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In a previous communication of this series by CRANE, GLENN AND GREEN[1] the properties and isolation of the electron transport particle (ETP) of beef heart mitochondria were described. This sub-mitochondrial particle catalyzes the oxidation of both succinate and DPNH by molecular oxygen through the intermediation of various oxidation-reduction groups including cytochromes *b*, c_1, and *a*. The present communication deals with the extraction of a soluble DPNH dehydrogenase from ETP which can react with cytochrome *c* as terminal electron acceptor. In a separate communication BASFORD *et al.*[2] will describe the extraction of a soluble succinic dehydrogenase from ETP.

MATERIAL AND METHODS

Reagents

DPNH and TPNH (enzymically reduced) were products of the Sigma Chemical Company. The samples of cytochrome *c* obtained from the Sigma Chemical Company were about 90% pure as determined by the concentration of heme (550 mμ peak) per mg protein. Crystalline bovine serum albumin was a product of Armour and Company. Glycylglycine and tris(hydroxymethyl)-aminomethane were commercial samples that were used directly for preparation of buffers, whereas Diol (2-amino-2-methyl-1,3-propanediol) was recrystallized before use.

Analytical methods

FAD was estimated in the D-amino acid apooxidase assay system[3] in which the oxidation with 2,6-dichlorophenolindophenol as electron acceptor was followed spectrophotometrically. The identification of flavin as FAD was further established by chromatography in two systems (K_2HPO_4–amyl alcohol[4] and *tert.*-butanol–water[5]). Total flavin was determined spectrophotometrically by the decrease in absorbance at 450 mμ which followed reduction with dithionite[6]. The difference between the molecular coefficients of the oxidized and reduced forms of flavin was assumed to be $10.3 \cdot 10^6$ cm^2/mole[7].

Iron was estimated colorimetrically as the *o*-phenanthroline complex. The sample to be determined was dialyzed for 24–48 hours first against 0.01 *M* Tris acetate of pH 7.8 and then against 0.001 *M* EDG Versene with several changes of buffer. The dialyzed sample was then wet ashed with concentrated nitric acid[8].

Protein was determined colorimetrically by the biuret method[9] with bovine plasma albumin as primary standard.

Total heme was determined by the pyridine hemochromogen method[10] or directly from the absorption bands of the enzyme solution. The molecular extinction coefficient at 550 mμ of the enzyme reduced with $Na_2S_2O_4$ was taken to be $19.1 \cdot 10^6$ cm$^2 \times$ mole^{-1}.[11]

Assay of enzymic activity

Diaphorase and cytochrome *c* reductase activities of the enzyme were both measured spectrophotometrically—the first by the rate of oxidation of DPNH by ferricyanide[12] and the second by the rate of reduction of cytochrome *c* by DPNH[13]. The decrease in optical density at 410 mμ

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per min per mg of protein in 1.0 ml total volume at 38° was taken as a measure of specific diaphorase activity. Correspondingly, specific reductase activity is expressed in terms of the increase in optical density at 550 mμ.

In the assay for diaphorase activity the final concentrations of reactants were as follows: ferricyanide 0.5 μmole and potassium phosphate (pH 7.4) 40 μmoles. A correction was made routinely for the slow non-enzymic reduction of ferricyanide by DPNH. The extinction coefficient for ferricyanide was taken to be $1.0 \cdot 10^6$ cm^2 × mole^{-1} at 410 mμ.

In the assay of DPNH cytochrome *c* reductase activity the experimental cuvette contained 20 μmoles of glycylglycine (pH 8.7), 0.05 μmole of oxidized cytochrome *c*, 0.29 μmole of DPNH, 0.2–0.4 μg of enzyme protein and water to 1.0 ml. The control cuvette contained 0.05 μmole of oxidized cytochrome *c* and 2.0 μmoles of glycylglycine in 1 ml.

RESULTS

Preparation of the enzyme

Suspensions of ETP (0.25 *M* sucrose) were prepared from beef heart mitochondria according to the procedure described by CRANE *et al.*[1]. These suspensions were kept frozen at —10°. In general more active extracts are obtained from fresh suspensions of ETP than from suspensions stored for extended periods at —10°.

The following procedure was carried out between 0 and 4° unless otherwise stated. The sucrose suspension of ETP was centrifuged and the particulate residue was washed with distilled water, and finally suspended evenly (by homogenization) in distilled water. The protein concentration was then adjusted to 30 mg/ml. The conditions for the extraction of a soluble DPNH cytochrome *c* reductase from ETP which proved most satisfactory are similar to those described by EDELHOCH *et al.*[13] and MAHLER *et al.*[14,15] for the preparation of the enzyme from pig heart mitochondria. The water suspension of ETP was acidified to pH 4.8 by addition of dilute acetic acid. Then absolute alcohol was added dropwise with gentle stirring to a final concentration of 9%. The suspension was then immediately placed in a bath maintained at 44° and kept at that temperature for 15 minutes from the time that the internal temperature reached 43°. Then the suspension was cooled to 0° and the pH was brought back to 7.0 by addition of alkali. The suspension was then centrifuged at 59,000 × *g* for 15 minutes in the Spinco preparative ultracentrifuge. All the activity is found in the clear yellow supernatant fluid which contains about 0.5 mg protein per ml. The extraction of activity would appear to be essentially quantitative.

The enzyme in the form of a lyophilized powder is very stable when stored at —12°. A solution of the enzyme is stable for several hours at 0° but the activity decreases rapidly when the enzyme solution is exposed to temperatures of >20°.

Enzymic activity of the extract

The diaphorase activity of the soluble extract, assayed by the ferricyanide method is about 30 μmoles DPNH/mg protein/min at 38°, whereas the cytochrome *c* reductase activity is 10 μmoles DPNH/mg protein/min. The pH activity curve of diaphorase had an optimum at pH 7.4 while that of the reductase had a maximum at pH 8.7.

TPNH would not replace DPNH as electron donor. Similarly succinate was inactive either with ferricyanide or cytochrome *c* as electron acceptor.

Inhibitors of enzymic activity

Table I summarizes the effects of pyrophosphate, phosphate, citrate, *p*-chloromercuribenzoate, antimycin A, and amytal on diaphorase and reductase activities respec-

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TABLE I

EFFECT OF VARIOUS INHIBITORS ON DPNH CYTOCHROME *c* REDUCTASE ACTIVITY AND ON DIAPHORASE ACTIVITY OF THE ENZYME

Inhibitor	*Concentration*	*% Inhibition Reductase activity*	*% Inhibition Diaphorase activity*
Pyrophosphate	$1 \cdot 10^{-2} M$	90	0
Phosphate	$1 \cdot 10^{-2} M$	65	0
Citrate	$4 \cdot 10^{-2} M$	65	0
Antimycin A	1–5 γ/ml	0	0
Amytal	$1.25 \cdot 10^{-3} M$	0	0
p-Chloromercuribenzoate	$1 \cdot 10^{-4} M$	100	100

tively. These results follow closely those reported by MAHLER *et al.*[14] for the corresponding enzyme isolated from pig heart mitochondria.

Absorption spectrum

The enzyme solution is non-fluorescent and shows a 4-banded spectrum. Three of these peaks with maxima at 450, 360 and 270 mμ respectively are characteristic of a flavoprotein spectrum. The fourth band has a maximum in the Soret region at 410 mμ and can be assigned to a heme component (*cf.* Fig. 1). The enzyme solution can be reduced by dithionite or sodium borohydride. Under anaerobic conditions the enzyme is rapidly reducible by DPNH (*cf.* Fig. 2). Reduction by any of these three reducing agents leads to a shift of the Soret band from 410 mμ to 420 mμ. If the enzyme solution is sufficiently concentrated reduction also leads to the appearance of a new band at 550 mμ which is probably the α band of the reduced heme component.

Flavin component of the prosthetic group

The flavin content of the enzyme was determined by two independent methods. (1) The enzyme was reduced with dithionite and the decrease in optical density at 450 mμ was used as a measure of flavin. The molecular extinction of flavin was assumed to be $10.3 \cdot 10^6$ cm^2 $\times$ mole^{-1}. (2) The enzyme solution was acidified with perchloric acid (0.6%) and the denatured protein was centrifuged off. The supernatant fluid was neutralized and clarified again by centrifugation. The change in optical density at 450 mμ on addition of dithionite again served as the basis for estimation of flavin. From the measurements by both these methods the flavin content of the enzyme was estimated to be $0.7 \cdot 10^{-2}$ μmole per mg of protein.

Table II shows that the flavin content of the soluble enzyme is per mg protein about 14 times as high as that of ETP and that the flavin associated with the soluble DPNH cytochrome *c* reductase extractable from ETP accounts for 45% of the total flavin originally present in ETP. This last result is in line with the assumption that ETP contains equal amounts of flavin associated with DPNH cytochrome *c* reductase and flavin associated with succinic dehydrogenase. Only the former is extracted by the procedure described above.

The flavin liberated from the enzyme by acid treatment is only slightly active in the D-amino acid apooxidase assay system for FAD, and behaves differently from a known sample of FAD when subjected to paper chromatography and paper electro-

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phoresis. MAHLER *et al.*[14] have reported in extenso on the non-identity of the flavin component of DPNH cytochrome *c* reductase with FAD.

TABLE II

PERCENTAGE OF TOTAL FLAVIN EXTRACTED FROM ETP

Material	*Total protein mg*	*Flavin/mg of protein μmoles*	*Total flavin μmoles*	% *Extracted flavin*
ETP	1078.35	$0.44 \cdot 10^{-3}$	0.474	
Enzyme	33.97	$0.62 \cdot 10^{-2}$	0.210	44
Residue	890.65	$0.29 \cdot 10^{-3}$	0.258	

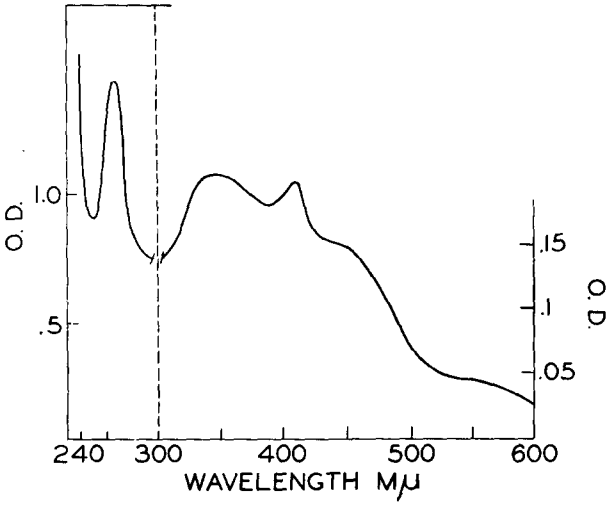

Fig. 1

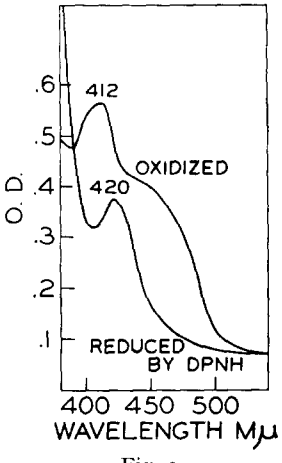

Fig. 2

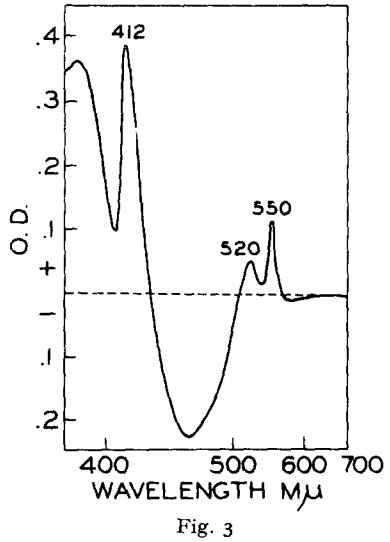

Fig. 3

Fig. 1. Absorption spectrum of the soluble DPNH-cytochrome *c* reductase. Spectrum was recorded with a Beckman DK spectrophotometer.

Fig. 2. Spectrum of the oxidized enzyme and of the enzyme reduced anaerobically with DPNH. Experimental points (not shown) were taken at 5 mμ intervals, except near maxima or minima (2.5 mμ). Spectra were recorded anaerobically with a Beckman DU spectrophotometer at 0°. The experimental cuvettes ($d = 1$ cm) contained 20 μmoles of glycylglycine (pH 8.7); 2.88 mg of enzyme protein and 0.6 μmole of DPNH (where indicated) in a final volume of 1 ml. DPNH was a Sigma product.

Fig. 3. Difference spectrum of the pyridine hemochromogen of the soluble DPNH-cytochrome *c* reductase. The spectrum was recorded with a Beckman DK spectrophotometer. 16.4 mg of enzyme protein were used for the preparation of the pyridine hemochromogen. Dithionite was the reducing agent.

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Heme component

The absorption bands of the heme component present in the solution of the reductase correspond exactly with those of cytochrome *c*. When the heme is converted to the form of its pyridine hemochromogen the absorption spectrum (*cf.* Fig. 3) corresponds very closely with that of the hemochromogen of cytochrome *c*. Various preparations of the reductase prepared by the standard procedure described above have an average value of $0.3 \cdot 10^{-3}$ μmole heme per mg of protein whereas the flavin content is 23 times as high.

All attempts thus far to eliminate the heme component from solutions of the enzyme have been unsuccessful. Fractionation with ammonium sulfate, electrophoresis on a starch column or in the Tiselius apparatus, adsorption and elution from Al c γ and calcium phosphate gels or from an Amberlite 50 ARC column, and finally fractionation with a zinc salt and ethyl alcohol were ineffective in eliminating the heme component from the enzyme.

The possibility that the soluble enzyme may bind cytochrome *c* liberated from ETP during the extraction procedure has been checked by incubating cytochrome *c* with the enzyme at neutral and acid pH in presence and absence of ethanol. The enzyme after precipitation with ammonium sulfate did not show increased amounts of the heme component *i.e.* $> 0.3 \cdot 10^{-3}$ μmole per mg.

Metal content

Non-heme iron in the standard enzyme preparation is present to the extent of about 1.4 μatoms $\times 10^{-2}$ per mg of protein (*cf.* Table III). Since the flavin content is about $0.7 \cdot 10^{-2}$ μmole per mg it would appear that there are two iron atoms for each molecule of flavin.

TABLE III

RATIO OF FLAVIN: IRON IN THE SOLUBLE DPNH CYTOCHROME *c* REDUCTASE EXTRACTED FROM ETP

Prep. No.	*μmoles of flavin/mg of protein* $\times 10^{-2}$	*μatoms of iron/mg of protein* $\times 10^{-2}$	*Ratio* $\frac{flavin}{iron}$
I	0.63	1.30	
II	0.75	1.52	
III	0.80	1.60	
IV	0.63	1.20	
V	0.62	1.40	
Average	0.70	1.40	1:2

DISCUSSION

The soluble DPNH cytochrome *c* reductase extracted from ETP in general shows properties which closely follow those of the enzyme prepared in soluble form from pig heart muscle by MAHLER and his colleagues[14,15] and there can be little doubt that the basic enzymic unit is identical in the two cases. The most important difference is the presence of a persistent heme component which is tightly bound to the protein of the reductase extractable from ETP. While one cannot exclude the possibility of an impurity in the preparation, it should be emphasized that all attempts to eliminate this heme from the flavoprotein preparation have been unsuccessful. If this heme

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component is indeed an intrinsic part of the enzyme and represents the heme which is normally linked to the flavoprotein in ETP then it would follow that the extraction procedure leads to a mixture of about 95% flavoprotein and 5% of a flavohemoprotein (assuming one heme per flavin). It is conceivable that under different conditions of extraction a higher proportion of flavohemoprotein could be extracted from ETP.

Another difference which may be of some interest in comparing the reductase derived from ETP with that of MAHLER relates to the iron-flavin ratio which is twice as high in the MAHLER enzyme. The difference in source material and in the method of preparation may account for this variation in ratio.

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SUMMARY

1. A soluble DPNH-cytochrome-*c*-reductase has been isolated in high yield from the electron transport particle of beef heart mitochondria.

2. The enzyme shows a modified flavoprotein spectrum with maxima at 270, 360, and 450 mμ. The presence of a heme component was also found which appears to be firmly attached to the flavoprotein.

3. The enzyme has 2 iron atoms for each mole of flavin. The flavin prosthetic group appears not to be flavinadeninedinucleotide on the basis of enzymic and chromatographic tests.

4. The properties of the enzyme extracted from the electron transport particle closely resemble those of the soluble DPNH-cytochrome-*c*-reductase described by MAHLER *et al.*

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