Studies on the Electron Transfer System

XL. PREPARATION AND PROPERTIES OF MITOCHONDRIAL DPNH-COENZYME Q REDUCTASE*

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Previous studies from this laboratory have shown that the bound coenzyme Q of mitochondrial particles could be reduced by pyridinoprotein-linked substrates as well as by reduced di-

phosphopyridine nucleotide itself (1). It was also shown that externally added coenzyme Q10 was reduced by pyruvate-

malinate as well as by reduced diphosphopyridine nucleotide in the presence of beef heart mitochondria. These studies indicated that coenzyme Q reduction by reduced diphosphopyridine nucleotide.

A simple spectrophotometric assay for the DPNH-coenzyme Q reductase activity of mitochondria and submitochondrial preparations was developed in which the short chain homologue of coenzyme Q (coenzyme Q1) was used as the electron acceptor (2). Long chain homologues are less reactive than coenzyme Q1, presumably because of their insolubility in aqueous media. By means of this assay, it was shown that DPNH-coenzyme Q reductase activity is highly concentrated in the DPNH-cyto-

chrome c preparations purified from beef heart mitochondria (2, 3). DPNH-cytochrome c reductase preparations have now been resolved into a DPNH-coenzyme Q reductase fraction, which is essentially free of cytochromes, and a cytochrome-rich fraction, which catalyzes the oxidation of reduced coenzyme Q by cytochrome c.

The present communication deals with the preparation of DPNH-coenzyme Q reductase from mitochondrial DPNH-cyto-

chrome c reductase and the general properties of this enzyme system. Preliminary reports of this work have already been presented (4, 5).

EXPERIMENTAL PROCEDURE

Materials

The sources of materials used in these studies were the same as described previously (2). Coenzyme Q homologues, varying in the number of isoprenoid units in the side chain, were synthe-

sized at the Merck, Sharp and Dohme Laboratories and were kindly supplied by Dr. K. Folkers. 2,3-Dimethoxy-5,6-di-
methyl benzoquinone was a gift from Dr. F. L. Crane. Cholic acid was obtained from Eastman Kodak Company. It was recrystallized from 50% ethanol in the presence of EDTA-
washed Norit A.

Methods

Assay of DPNH-Coenzyme Q Reductase Activity—Coenzyme Q1 (100 μmoles) was dissolved in 1.5 ml of absolute ethanol, and the volume was made up to 100 ml with water. At this concen-

tration, coenzyme Q1 appears to be completely soluble. An addition of 0.1 ml of this solution (100 μmoles of coenzyme Q1) was made to each assay mixture. Other conditions of the assay procedure were the same as described earlier (2).

The assay procedure for measurement of DPNH-ferricyanide reductase activity and the methods for determination of the flavin of DPNH-flavoprotein, coenzyme Q, and cytochrome content have already been described (3, 4). Protein was deter-

mined by the biuret method of Gornall, Bardawill, and David (6).

EPR spectroscopy was performed with a standard Varian V4500-10A spectrometer equipped with a 100-kc. field modulation unit and an accessory for work at variable temperatures. Samples were contained in quartz tubes (with a wall thickness of 0.5 mm and an inner diameter of 3 mm) which had been matched for quantitative work. The samples were frozen in liquid nitrogen and examined at −171°. The records are pres-

ented in the form of the first derivative of the absorption curve.

Preparation of DPNH-Coenzyme Q Reductase—DPNH-coen-

zyme Q reductase was isolated from purified preparations of DPNH-cytochrome c reductase. The latter enzyme complex, recently isolated in this laboratory from beef heart mitochondria, contains DPNH dehydrogenase flavoprotein, nonheme iron, co-

enzyme Q, cytochrome b, and cytochrome c. The procedure for its isolation and the properties of this enzyme complex have been described elsewhere (3, 4).

All operations involved in the preparation of DPNH-coenzyme Q reductase were performed at 0-2°C. Particles of DPNH-cyto-

chrome c reductase are suspended in a buffered sucrose solution of 0.5 mm and an inner diameter of 3 mm) which had been matched for quantitative work. The samples were frozen in liquid nitrogen and examined at −171°. The records are presented in the form of the first derivative of the absorption curve.

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All operations involved in the preparation of DPNH-coenzyme Q reductase were performed at 0-2°C. Particles of DPNH-cyto-

chrome c reductase are suspended in a buffered sucrose solution of the following composition: 0.05 M Tris-chloride, pH 8.0, 0.66 M succrose, and 0.001 M histidine. This solution will be referred to subsequently as buffered sucrose. The protein concentration is adjusted to 10 mg per ml with buffered sucrose, and sufficient cold neutral potassium-cholate (20% weight per volume) added to a final concentration of 0.4 mg of cholate per mg of protein. Then cold saturated ammonium sulfate (neutralized) is added to a

1 The abbreviations used are: Q1, Q2, and Q10, coenzymes Q with 1, 2, and 10 isoprenoid units in the side chain, respectively; EPR, electron paramagnetic resonance.
Table I

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (μmole/mg protein)</th>
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<tr>
<td>DPNH dehydrogenase flavoprotein</td>
<td>1.4-1.5</td>
</tr>
<tr>
<td>Coenzyme Q</td>
<td>4.2-4.5</td>
</tr>
<tr>
<td>Nonheme iron</td>
<td>26</td>
</tr>
<tr>
<td>Cytochromes b + c</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

*DPNH-coenzyme Q reductase preparations contain about 0.22 mg of lipid per mg of protein. Data of S. Fleischer.*

† Acid-extractable flavin (3).

RESULTS

Composition of DPNH-Coenzyme Q Reductase—DPNH-cytochrome c reductase, from which DPNH-coenzyme Q reductase is obtained, contains DPNH dehydrogenase flavoprotein, nonheme iron, coenzyme Q, and cytochromes b and c (3). The flavoprotein and the cytochromes are present in almost equimolar amounts. However, DPNH-coenzyme Q reductase contains only trace amounts of cytochromes, but is enriched in DPNH dehydrogenase flavoprotein, coenzyme Q, and nonheme iron (cf. Table I). The concentrations of DPNH dehydrogenase flavoprotein and of nonheme iron are about 70% higher than the corresponding values in the parent particle, the DPNH-cytochrome c reductase. The fact that both DPNH-dehydrogenase flavoprotein and nonheme iron have been concentrated to the same extent in DPNH-coenzyme Q reductase suggests a strong association between these two components.

Spectrum of DPNH-Coenzyme Q Reductase—The difference spectrum of the enzyme is shown in Fig. 1. The dashed line represents the difference spectrum of oxidized enzyme (5 mg of protein per ml) in both the experimental and reference cuvettes. The solid line is the difference spectrum after addition of DPNH to the experimental cuvette. The fact that in the region of the spectrum between 570 and 540 μm very little change occurs on reduction of the enzyme with DPNH indicates only that a residual amount of cytochromes b and c (less than 0.1 μmole per mg of protein) remains in the preparation. However, the absorbance of cytochromes b and c and their combined Soret bands (between 425 and 430 μm) are clearly discernible. The trough between 445 and 450 μm is in part due to reduction of the flavoprotein moiety of the enzyme. Calculations based on the concentration of this component (on the assumption that Δabs (oxidized-reduced) = 10.3 mm (cm)²) indicate that only about 50% of the change at 450 μm can be accounted for by reduction of the flavoprotein. The extra absorbance change in this region suggests the possibility that another component may be present in the enzyme, which is reduced by DPNH and oxidized by coenzyme Q. Earlier studies on the parent particle, the DPNH-cytochrome c reductase, also indicated that a new component may be involved in the enzyme (cf. Figs. 1 and 4 in (7) and the pertinent discussions in the text). These studies also suggested that the rapid reduction of this component is inhibited by Amytal (7).

Examination of DPNH-coenzyme Q reductase by EPR spectroscopy indicates the presence of a paramagnetic metal ion and two other components in the enzyme preparation (see below for further details). It is possible that those spectral changes of the enzyme unrelated to the flavoprotein content and the residual amount of cytochromes are caused by the new components seen in the EPR spectra.

EPR Studies on DPNH-Coenzyme Q Reductase—A set of typical results is shown in Fig. 2. Trace A shows an EPR spectrum of the enzyme preparation in the oxidized state. On addition of DPNH to the enzyme preparation, the spectrum, B, was recorded. Signal a of this spectrum has been interpreted by Beinert and Lee (9) to be due to paramagnetic metal ions, tentatively identified as reduced iron in some as yet unknown specific linkage, which does not involve porphyrin. According to these authors, the iron compound represents only a small portion of the total nonheme iron fraction.† Signal b, Trace B, appears to be due to a free radical of flavin. Traces C, D, E, F, and G show successive stages during reoxidation of the reduced enzyme preparation. In the state of oxidation-reduction of the enzyme preparation marked by Trace E, two other signals, c and d, are observed. These signals are due to thus far unidentified components in the enzyme preparation. Trace G was recorded after nearly complete reoxidation of the enzyme preparation. In this

† Also see the studies of Ziegler (10) on the chemical determination of nonheme iron.
traces, Signals a, c, and d have almost completely disappeared while a residual amount of Signal b (flavin radical) remains. Although the new components detected by EPR spectroscopy are capable of undergoing cyclic oxidation-reduction, their involvement as electron carriers in the DPNH-coenzyme Q reductase system still remains to be established.

Signal a, which has tentatively been identified as being due to a nonheme iron component, has also been found in DPNH-cytochrome c reductase and in submitochondrial particles (11). The oxidation-reduction component represented by this EPR signal appears to be specifically reducible by DPNH. It is of interest that on fractionation of DPNH-cytochrome c reductase, this component is concentrated exclusively in the flavoprotein containing DPNH-coenzyme Q reductase fraction and is completely absent from the cytochrome containing reduced coenzyme Q (QH) cytochrome c reductase fraction.

Activities of DPNH-Coenzyme Q Reductase—Oxidation-reduction dyes, such as 2, 6-dichlorophenol indophenol and K$_2$Fe(CN)$_6$ have been used successfully in the assay of succinic-coenzyme Q reductase activity. These compounds react with the succinoxidase system at the site of coenzyme Q and their reduction requires the presence of catalytic amounts of coenzyme Q in the assay medium (12). For the assay of mitochondrial DPNH-coenzyme Q reductase activity, however, such compounds cannot be employed as electron acceptors, since they interact with the DPNH oxidase system at a site before the locus of coenzyme Q.

In mitochondria and derivative particles, the reduction of coenzyme Q by DPNH is specifically inhibited by Amytal whereas the reduction of potassium ferricyanide, 2, 6-dichlorophenol indophenol, or methylene blue is not. Among the homologues of coenzyme Q, the homologue with only one isoprene unit (coenzyme Q$_2$) was found to be a suitable electron acceptor. This compound is slightly soluble in water and its reduction by DPNH, as catalyzed by mitochondrial particles, is sensitive to the inhibitory effect of Amytal (2).

The enzymic properties of DPNH-coenzyme Q reductase are shown in Table II. It is seen that among the homologues of coenzyme Q tested, only coenzyme Q$_1$ is rapidly reduced. The rate of reduction of coenzyme Q$_2$ is usually about one-fourth of that of coenzyme Q$_1$. This compound (coenzyme Q$_2$), although water-insoluble, forms a very fine suspension when added in small volumes of ethanol to the aqueous assay medium. It has already been pointed out (2) that the DPNH-coenzyme Q reductase enzyme system of mitochondria is highly specific for homologues of coenzyme Q. The DPNH oxidation observed in the presence of quinones such as 2,3-dimethoxy-6-methylbenzoquinone and 2,3-dimethoxy-5,6-dimethylbenzoquinone was Amytal-insensitive as was the reaction in the presence of menadione. Vitamin K$_1$ and α-tocopherylquinone were completely inactive in the assay.

The enzyme preparation is specific for DPNH as substrate. Since TPNH in the presence of DPNH cannot replace DPNH, the presence of transhydrogenase activity is ruled out. The enzyme is free of lipoic dehydrogenase, succinic-coenzyme Q reductase as well as succinic-cytochrome c reductase activity. As seen in Fig. 1 and Table I, a residual amount of cytochromes b and c$_1$ is usually present in the preparations of DPNH-coenzyme Q reductase. Consequently, a residual amount of antimycin-sensitive DPNH-cytochrome c reductase activity remains. The enzyme is capable of catalyzing the rapid reduction of ferricyanide by an Amytal-insensitive reaction. This property seems to be

![Fig. 2. EPR spectra (first derivative) of DPNH-coenzyme Q reductase, (3.5 mg of protein in 0.10 ml of Tris-sucrose-histidine, pH 8.0). A, untreated; B, reduced with 0.26 μmole of DPNH (2 μl of 0.13 m DPNH), frozen after 30 seconds at 0°; C, D, E, F, G, successive stages of reoxidation on shaking in air at 0°. All spectra were recorded at a sample temperature of -171°. For further experimental details, see (9) and (11).]

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Electron acceptor</th>
<th>Specific activity*</th>
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<tbody>
<tr>
<td>DPNH</td>
<td>Coenzyme Q$_1$</td>
<td>27.6</td>
</tr>
<tr>
<td>DPNH</td>
<td>Coenzyme Q$_2$</td>
<td>7.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>Coenzyme Q$_3$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DPNH</td>
<td>2,3-Dimethoxy-6-methylbenzoquinone</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>2,3-Dimethoxy-5,6-dimethylbenzoquinone</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>DPNH</td>
<td>Menadione$^+$</td>
<td>3.1</td>
</tr>
<tr>
<td>DPNH</td>
<td>Vitamin K$_1$</td>
<td>0.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>α-Tocopherylquinone</td>
<td>0.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>K$_2$Fe(CN)$_6$</td>
<td>200-210</td>
</tr>
<tr>
<td>TPNH (± DPN)</td>
<td>Coenzyme Q$_1$</td>
<td>0.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>Lipoic acid</td>
<td>0.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>Coenzyme Q$_1$ or cytochrome c</td>
<td>0.0</td>
</tr>
<tr>
<td>DPNH</td>
<td>Cytochrome c</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Specific activity is expressed as micromoles of electron acceptor (one electron equivalent) reduced per mg of protein per minute at 38°.

$^+$ In contrast to the reaction with coenzyme Q$_1$ and coenzyme Q$_2$, these reactions are not inhibited by Amytal.
characteristic of the mitochondrial DPNH dehydrogenases isolated by various means (Ziegler et al. (15); Ringer et al. (14)).

Inhibitors of DPNH-Coenzyme Q Reductase System—Previous studies from this laboratory have shown that the reduction of mitochondrion-bound coenzyme Q by pyridinoprotein-linked substrates is inhibited by Amytal (1). The DPNH-coenzyme Q reductase activity of the purified enzyme is also strongly inhibited (90%) by Amytal (3 mM) as well as by Demerol (0.5 mM) and p-chloromercuriphenyl sulfonate (6 mM). It has already been shown that antimycin A and 2-nonyl-4-hydroxyquinoline-N-oxide do not inhibit DPNH-coenzyme Q reductase activity while Dicumarol at concentrations of about 0.25 mM is an effective inhibitor (2). Treatment of the enzyme with organic solvents, such as acetone, ethanol, butanol, and t-amyl alcohol, as well as high concentrations of detergents, such as bile salts and Triton, results in a destruction of enzymic activity. Incubation of the enzyme with Naja naja venom (boiled) or Crotalus venom (unboiled) is also destructive, whereas these treatments have little or no effect on DPNH-ferrixyanide activity.

**DISCUSSION**

The studies reported in this communication indicate that there exists in heart mitochondria a distinct enzyme system capable of catalyzing the Amytal-sensitive reduction of coenzyme Q by DPNH. Purified preparations of the enzyme contain the DPNH dehydrogenase flavoprotein, nonheme iron, and coenzyme Q, and are essentially free of succinic dehydrogenase flavoprotein, cytochromes b, c1, and c, and cytochrome oxidase. The absence of significant amounts of cytochromes in the DPNH-coenzyme Q reductase preparations provides the first evidence that the reduction of mitochondrial coenzyme Q by DPNH is not mediated by any one of the homoproteins.

It has been shown that preparations of DPNH-coenzyme Q reductase are capable of combining with preparations of reduced coenzyme Q-cytochrome c reductase to reconstitute a DPNH-cytochrome c reductase unit (5). These reconstituted systems are capable of catalyzing electron flow between donor and acceptor at a rate twice as fast as the rate of reduction of coenzyme Q, given in Table II. It seems, therefore, that the assay system described in this communication does not fully indicate the capacity of these preparations of DPNH-coenzyme Q reductase for electron transfer. Reduced coenzyme Q-cytochrome c reductase is composed of cytochrome b and cytochrome c1. It may be concluded, therefore, that DPNH coenzyme Q reductase is the enzyme system that links the pyridinoprotein dehydrogenases of the citric acid cycle to the cytochrome system of the respiratory chain.

Studies of the effect of inhibitors on succinic-coenzyme Q and DPNH-cytochrome c reductase, coupled with spectroscopic studies of the changes in the visible spectrum between 400 and 600 m/ have suggested that oxidation-reduction components other than flavoproteins and cytochromes are involved in these reactions (7, 10). Similar conclusions have been reached in the case of the present preparation of DPNH-coenzyme Q reductase as a result of the following observations.

1. The spectroscopic changes between 400 and 600 m/ on oxidation-reduction of the enzyme preparation cannot be accounted for by the flavoprotein content of the preparation.
2. Electron paramagnetic resonance spectroscopy indicates the presence of a paramagnetic metal (probably Fe) and unidentified components which undergo oxidation-reduction.

3. As a result of fractionation of DPNH-cytochrome c reductase, the oxidation-reduction components detected by electron paramagnetic resonance spectroscopy have been concentrated exclusively in the flavoprotein-containing DPNH-coenzyme Q reductase fraction. This suggests a functional role for these components in the DPNH-coenzyme Q reductase system.

4. The high levels of nonheme iron and the evidence from electron paramagnetic resonance spectroscopic studies suggest that nonheme iron plays an oxidation-reduction role in the DPNH-coenzyme Q reductase system. Such evidence, although not conclusive, points to the conclusion that the Amytal-sensitive reduction of coenzyme Q by DPNH requires a series of reactions involving at least one oxidation-reduction component other than the DPNH dehydrogenase flavoprotein.

Ernst, Ljundggren, and Danielson (15) have described a preparation from liver of a soluble DPNH-TPNH quinone reductase that is capable of catalyzing the reduction of coenzyme Q. This enzyme appears to be completely different from our preparation of DPNH-coenzyme Q reductase. The former enzyme can oxidize TPNH, is insensitive to Amytal, and is inhibited by very low concentrations of Dicumarol (50% at 1 x 10^-4 M). By contrast, the mitochondrial DPNH-coenzyme Q reductase preparations do not oxidize TPNH, are inhibited by Amytal, and are far less sensitive to the inhibitory effect of Dicumarol (60% inhibition of DPNH-coenzyme Q, reductase activity at 1 x 10^-4 M). Sensitivity to Amytal seems to be peculiar to the mitochondrial DPNH-coenzyme Q reductase system in that it has not been observed for any other preparation of quinone reductase reported by others (15-17). This characteristic property may be related to nonheme iron and the other components detected in preparations of DPNH-coenzyme Q reductase by EPR spectroscopy.

**SUMMARY**

The procedure for the preparation of a highly active reduced diphosphopyridine nucleotide (DPNH)-coenzyme Q reductase and the properties of this enzyme complex are described. The enzyme complex is isolated from preparations of DPNH-cytochrome c reductase derived from beef heart mitochondria. Preparations of DPNH-coenzyme Q reductase contain DPNH dehydrogenase flavoprotein, nonheme iron, coenzyme Q, and lipid. The enzyme is highly specific for DPNH as substrate and coenzyme Q homologues as electron acceptor. The enzymic activity is inhibited, as in mitochondria, by Amytal, Demerol, and p-chloromercuriphenyl sulfonate. In addition to a signal for a paramagnetic metal ion, electron spin resonance spectra show three other signals (characteristic of free radicals) in the enzyme preparation. These signals appear and disappear as the enzyme preparation is reduced and oxidized. DPNH-coenzyme Q reductase is the Amytal-sensitive system in heart mitochondria that links the pyridinoprotein dehydrogenases of the citric acid cycle to the cytochrome system of the respiratory chain.

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