Isolation and Enzymatic Properties of the Mitochondrial Reduced Diphosphopyridine Nucleotide Dehydrogenase*

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SUMMARY

The DPNH dehydrogenase of the mitochondrial electron transport system has been obtained in a highly purified, soluble form after resolution of DPNH-coenzyme Q reductase (Complex I) particles by chaotropic agents. The enzyme contains 1 mole of flavin mononucleotide, 4 g atoms of iron, and 4 moles of acid-labile sulfide per 70,000 g. The soluble, but not the particle-bound, enzyme behaves as a diaphorase and catalyzes the unspecific reduction of quinones and ferric compounds such as menadione, 2,6-dichloroindophenol, coenzymes Q, ferricyanide, and cytochrome c. Mercurials inhibit all the reductase activities of the soluble dehydrogenase, and DPNH (>10^{-4} M) inhibits its menadione and coenzyme Q reductase activities. The structure and function of DPNH dehydrogenase in both the soluble and the particle-bound form are discussed.

Since 1952, several preparations of the mitochondrial DPNH dehydrogenase have been reported (1-6). On the basis of molecular weight, composition, and enzymatic activity, these preparations may be divided into two categories.

I. This category includes the dehydrogenase preparations which have a molecular weight between 70,000 and 140,000, contain flavin iron and acid-labile sulfide in a ratio of 2 to 4 iron atoms and 2 to 4 labile sulfide groups per flavin moiety, and can react with ferric complexes (ferricyanide and cytochrome c) and quinoid compounds (menadione, 2,6-dichloroindophenol, and coenzyme Q) as electron acceptors. With one exception, these dehydrogenases (1,4) have been extracted from tissue homogenates or mitochondrial particles in the presence of 9 to 11% ethanol at pH 4.8 to 5.3 and 43 to 45°C, a procedure originally devised for isolation of the Straub diaphorase (7). The exception is the preparation of King and Howard (5), which has been isolated from mitochondria by treating the particles with venom phospholipase at 37°C.

II. Ringler, Minakami, and Singer (6) and Singer (8) have isolated a DPNH dehydrogenase by a procedure similar to that of King and Howard, except that the phospholipase treatment of mitochondria was carried out at 30°C. This preparation has only about 1 nmole of flavin per mg of protein, as compared to 7 to 14 nmoles for enzymes of the first category. It has an approximate ratio of flavin to iron to labile sulfide of 1:18:28, and reacts only with ferricyanide as electron acceptor. It yields a low molecular weight dehydrogenase similar to those of the first category upon various treatments, including heat-acid-ethanol.

Systematic resolution of the component enzyme complexes of the mitochondrial electron transport system in this laboratory (9) has led to the isolation of a DPNH dehydrogenase which is the subject of this paper and the accompanying report (10). This approach has made it possible to trace the above enzyme through the various stages of mitochondrial fragmentation and to study its immediate environment and its association with other components of the electron transport system (11, 12). Furthermore, this tactic has permitted a study of DPNH dehydrogenase both as an integrated component of the electron transport system and as a soluble enzyme dissociated from the particles. Thus, it has been found that dissociation of DPNH dehydrogenase from the enzymatic assembly of the electron transport system is accompanied by significant changes in its properties. These changes are consistent with the possibility of a conformation of the active site more accessible to DPNH and electron acceptors.

METHODS AND MATERIALS

Unless otherwise specified, conditions for assay of the various activities of DPNH dehydrogenase and of Complexes I and I-III were as given in previous communications (11, 13, 14). Acid-extractable flavin was estimated spectrophotometrically (A_{450} = 10.3 liters per mmole) after extraction of flavin with 5% trichloroacetic acid followed by reduction at pH 7.0 with dithionite. Total flavin was estimated as above after a heat-denatured sample of the enzyme was digested with trypsin. Protein was estimated by the biuret method of Gornall, Barda-

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1 Acid-labile sulfide, being a relatively recent discovery, has not been reported for all the preparations in this category.

2 For a preliminary communication on the resolution of Complex I and the isolation of DPNH dehydrogenase, see Reference 11.

3 Note Added in Proof—For the assay of DPNH-DC1 reductase activity in Reference 11, DC1 concentration is shown as 0.65 mM. It should be 0.065 mM.
will, and David (15) in the presence of 1 mg of potassium deoxycholate per ml. Iron was estimated by a modification of the procedure of Doeg and Ziegler (16) as well as by the same colorimetric method after wetashing of the samples. The two procedures gave equivalent results. Acid-labile sulfide was estimated by the procedure of Fogo and Popowsky (17). Results were calculated on the basis of sodium sulfide standard curves, which gave an A₅₀₀ value of 10.7 liters per mmole. Absorption spectra and enzymatic activities were recorded with a Beckman DK-2A spectrophotometer fitted with a time drive attachment. Specific activities are expressed as micromoles of DPNH oxidized per min per mg of protein at 38°C. Complex I (13) and the binary I-III complex (18) were prepared according to the references given. All Kₘ and Vₐₚₐₚ values were calculated from appropriate Lineweaver-Burk plots.

DPN, DPNH, and acetylpyridine-DPNH were obtained from P-L Biochemicals, Milwaukee. Cytochrome c, type III, was from Sigma. Coenzymes Q were generously supplied by Dr. K. Folkers, Stanford Research Institute, and Dr. O. Isler, Hoffmann-La Roche Chemical Company.

**RESULTS**

**Isolation of DPNH Dehydrogenase**

**General Considerations**—Previous work from this laboratory has shown that the mitochondrial electron transport system can be divided into its component enzyme complexes by application of a general procedure involving low levels of bile salts, KCl, ammonium acetate, and ammonium sulfate (9). The mild conditions used for resolution of the electron transport system also afforded preservation of surface complementarity of the complexes. As a result, intermixing of adjacent complexes in the absence of high concentrations of detergents and salts resulted in characteristic recombination and reconstitution of the electron transport system (9, 14).

In studies directed toward resolution of the individual complexes, two factors became apparent: (a) the forces which hold the components of a single complex together appear to be stronger than those which hold together two adjacent complexes, and (b) considerable structural organization is involved in these complexes, which may go unrecognized if the conditions used for resolution of the individual complexes are drastic, arbitrarily chosen, and not easily controllable. For example, high concentrations of bile salts at elevated temperatures can bring about the resolution of Complex III into a cytochrome b-rich and a cytochrome c₁-rich fraction (19), or heat-acid ethanol treatment can dissociate the DPNH dehydrogenase of Complex I from mitochondrial particles. However, in our experience, these treatments are not exactly reproducible, and they do not easily permit a chemical rationalization of the resolution process. Thus, our search for more appropriate reagents led us to the use of chaotropic compounds for resolution of the complexes of the electron transport system. These reagents have been used successfully for dissociating antigen-antibody complexes without destroying the immunospecific activity of the antibody, and are known to be capable of breaking hydrophobic and hydrogen bonds and unfolding macromolecular structures (20). Although a detailed report of the effect of chaotropic agents on the membrane system of mitochondria will be presented in a separate communication, this short introduction may help convey the rationale behind our use of these substances for resolution of the mitochondrial DPNH-coenzyme Q reductase.

**Specific Details**—Preparations of DPNH-coenzyme Q reductase can be resolved by chaotropics such as urea, guanidine hydrochloride, sodium perchlorate, and thiocyanate (4). The procedure described below for isolation of DPNH dehydrogenase uses urea, the weakest of the above reagents, for resolution of the complex.

Preparations of Complex I (13) were suspended at 10 mg of protein per ml in a solution containing 0.66 M sucrose, 50 mM Tris-Cl, pH 8.0, and 1 mM histidine. Solid urea was added to a final concentration of 2.5 M (without correction for the specific volume of urea), and the crystals were dissolved at ice bath temperature. The mixture was placed in a large Erlenmeyer flask so that the solution could be warmed and cooled rapidly, and the mixture was incubated for 10 min at 38°C with occasional stirring. The mixture was then cooled in an ice bath and centrifuged for 15 min at 30,000 rpm in the Spinco model L ultracentrifuge. The supernatant layer was collected and immediately fractionated with neutral, saturated ammonium sulfate. The first fraction, at 27.5% salt saturation, and the second fraction, at 36.4% salt saturation, were removed by centrifugation for 10 min at 30,000 rpm; the third fraction, at 52.9% salt saturation, was collected and dissolved in 50 mM Tris-Cl, pH 8.0. After each addition of ammonium sulfate, the mixture was allowed to stand in the ice bath for 10 min before the precipitate was removed by centrifugation. The first ammonium sulfate fraction contained the iron-sulfur protein of Complex I, the third fraction was DPNH dehydrogenase, and the middle fraction was removed in order to minimize cross-contamination between the dehydrogenase and the iron-sulfur protein fractions.

Electrophoresis of DPNH dehydrogenase so prepared on starch block at pH 8.6 with a buffer composition of 45 mM Tris, 25 mM boric acid, and 1 mM EDTA gave a single protein spot, and chromatography on hydroxylapatite resulted in a confluent double peak with virtually identical specific activities and spectral properties. The latter treatment improved neither the specific activity nor the composition of the ammonium sulfate fraction. The enzyme was, therefore, used after the stage of ammonium sulfate fractionation without further attempts at purification. Similar chromatographic results with hydroxylapatite have been reported by Pharo et al. (3) for a DPNH dehydrogenase isolated by application of the heat-acid ethanol procedure. The DPNH dehydrogenase obtained by the procedure described above lost considerable activity when stored at -20°C, but could be kept as a freeze-dried powder for several months at -20°C with little loss of activity. On the basis of flavin recovery from Complex I, the yield of DPNH dehydrogenase was 55 to 70%.

At 30°C and in the presence of 2.5 M urea, the release of DPNH dehydrogenase from Complex I was linear with time and reached completion within 7 to 8 min. Presence of DPNH in the incubation mixture impeded the appearance of dehydrogenase activity. Also, when no attempt was made to exclude oxygen, the urea resolution process was accompanied by a very rapid lipid oxidation, which appeared to be a consequence of the breakdown of Complex I. The effect of chaotropic agents in inducing lipid

Recent studies in this laboratory have suggested that the action of SCN⁻, ClO₄⁻, and other chaotropic ions is due mainly to their effect on the structure and lipophilicity of the surrounding water.
and cytochromes. For comparison, the most recent preparation determined, the preparation is free of acid-nonextractable flavin and 58 to 60 nmoles of acid-labile sulfide. As far as can be discussed in a separate communication.5

aud iron-sulfur protein oxidation in mitochondrial particles is 4, oxidized enzyme treated with solid sodium mersalyl; S, difference spectrum of 4; 5, oxidized enzyme treated with dithionite; 6, the mersalyl-treated enzyme of 4 after reduction with dithionite.6, the mersalyl-treated enzyme of 4 after reduction with dithionite.

The enzyme, at a protein concentration of 1.6 mg per ml, was dissolved in 50 mm Tris-Cl, pH 9.1, containing 2 a urea. The presence of urea at slightly alkaline pH prevented the development of turbidity during various treatments. The experiments were carried out in a closed cuvette which was evacuated several times and flushed with repurified nitrogen. Trace 1, oxidized enzyme; 2, after the addition of 1.5 mm DPNH from a side arm; 3, oxidized enzyme treated with solid sodium mersalyl; 4, difference spectrum of 1 – 2; 5, oxidized enzyme treated with dithionite; 6, the mersalyl-treated enzyme of 4 after reduction with dithionite.

The spectral characteristics of DPNH dehydrogenase are shown in Fig. 1. The oxidized form of the enzyme (Trace 1) has a major peak at about 445 nm, a shoulder at about 420 nm, and one at 550 nm. When the enzyme is reduced with DPNH (Trace 2) in a nitrogen atmosphere, the 445 nm peak is reduced by about 50%, but the 550 nm absorption shows only a small change. Treatment of the enzyme with sodium mersalyl leads to destruction of the iron-sulfur system. The result (Trace 4) is the spectrum of the oxidized flavoprotein. It is seen that this spectrum is devoid of both the 550 nm and the 420 nm peaks, and that the 445 nm absorption is equivalent to the change at this wave length when DPNH was added to the oxidized enzyme. Subtraction of the spectrum of the mersalyl treated enzyme from that of the oxidized enzyme yields the contribution of the iron-sulfur system (Trace 3) to the oxidized spectrum. It is seen that almost all of the 550 nm and the 420 nm and half of the 445 nm absorptions of the oxidized enzyme are due to the iron-sulfur components. Similar results for the iron-sulfur spectrum were obtained when flavin was extracted from the dehydrogenase according to the procedure of Rajagopalan and Hanfeler (24). The iron-sulfur spectrum of DPNH dehydrogenase is basically very similar to those of xanthine oxidase (24), aldehyde oxidase (24), and a highly purified succinic dehydrogenase prepared in this labora-

TABLE I

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activitya</th>
<th>aDPNH max</th>
<th>acceptor max</th>
<th>kDPNH</th>
<th>kacceptor</th>
<th>n</th>
<th>% inhibitionb</th>
<th>DPNH inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPNH → Fe(CN)6</td>
<td>210</td>
<td>330</td>
<td>400</td>
<td>0.5</td>
<td>100</td>
<td>None</td>
<td>&gt;1.3 mm, slight</td>
<td></td>
</tr>
<tr>
<td>DPNH → cytochrome c</td>
<td>43</td>
<td>76</td>
<td>220</td>
<td>6.4</td>
<td>60</td>
<td>&gt;1.2 mm, slight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPNH → coenzyme Q</td>
<td>150–160</td>
<td>280</td>
<td>175</td>
<td>13.3</td>
<td>16.7</td>
<td>10–20</td>
<td>&gt;0.25 mm, sharp</td>
<td></td>
</tr>
<tr>
<td>DPNH → menadione</td>
<td>160–170</td>
<td>330</td>
<td>190</td>
<td>13.3</td>
<td>16</td>
<td>10</td>
<td>&gt;0.25 mm, sharp</td>
<td></td>
</tr>
<tr>
<td>DPNH → 2,6-dichloroindophenol</td>
<td>100</td>
<td>125</td>
<td>13.3</td>
<td>6.2</td>
<td>&gt;0.3 mm, sharp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylpyridine-DPNH → coenzyme Qa</td>
<td>0.3</td>
<td></td>
<td></td>
<td>71.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Expressed as micromoles of DPNH oxidized per min per mg of protein at 38º.

b Superscripts on Vmax and Kmax refer respectively to infinite and half-saturating concentrations of the component indicated.

c p-Chloromercuriphenylsulfonate (0.1 mM) gave 100% inhibition; no inhibition was seen with 1 mm antimycin A.

d At 0.75 mm DPNH.

and iron-sulfur protein oxidation in mitochondrial particles is discussed in a separate communication.5

Composition of DPNH Dehydrogenase

DPNH dehydrogenase contains, per mg of protein, 13.5 to 14.5 moles of acid-extractable flavin, 60 to 65 ng atoms of iron, and 58 to 60 moles of acid-labile sulfide. As far as can be determined, the preparation is free of acid-nonextractable flavin and cytochromes. For comparison, the most recent preparation of Kumar, Rao, Felton, Huennekens, and Mackler (21) has a similar flavin content, only half as much iron, and one-fourth as much sulfide, and that of Pharo et al. (3) contains about 10 moles of flavin, 42 ng atoms of iron, and 50 to 53 moles of flavin. Pharo et al. (3) contains more labile sulfide than iron. In fact, an earlier preparation from this laboratory was reported to contain twice as much labile sulfide as iron (22). If these findings were not due to a specific retention of sulfide and loss of iron, then the preparations of Pharo et al. (3) and of Sanadi, Pharo, and Sordahl (22) are the only iron-sulfur proteins yet reported to contain a greater than 1:1 ratio of sulfide to iron. Kumar et al. (21) have reported the amino acid composition of a heat-acid-ethanol-extracted dehydrogenase. It has already been shown that flavin in Complex I, the parent particle of DPNH dehydrogenase, is exclusively FMN (23).
tory (25) & see also Reference 26). In detail, however, there are distinct differences among these spectra, especially between the iron-sulfur spectra of DPNH dehydrogenase and succinic dehydrogenase. It may be added that the contribution of the iron-sulfur system of DPNH dehydrogenase at 450 nm (Trace S in Fig. 1) amounts to a molar extinction coefficient of approximately 2500 per atom of iron.

Whereas DPNH treatment showed little effect, under the conditions used, on the iron-sulfur spectrum of the oxidized enzyme (compare Traces 2 and 3), dithionite treatment had a substantial effect (compare Traces 3 and 5). This observation differs from the spectral studies of Pharo et al. (3) and of Kumar et al. (21) on their heat-acid-ethanol-extracted DPNH dehydrogenases. The spectra reported by these workers show little change upon dithionite treatment of DPNH-reduced enzyme. Addition of dithionite to the mersalyl-treated enzyme (Trace 4) resulted in Trace 6, indicating that after destruction of the iron-sulfur spectrum by mersalyl and reduction of the flavin by dithionite the enzyme has no characteristic absorption between 400 and 700 nm.

**Enzymatic Properties of DPNH Dehydrogenase**

The dehydrogenase activity of the enzyme with various electron donors and acceptors and some of the pertinent kinetic constants are shown in Table I. The enzyme is specific for DPNH as substrate. It has no TPNH dehydrogenase or transhydrogenase activity as tested with TPNH plus DPN as substrate. It reacts slowly with acetylpyridine-DPNH, the $V_{\text{max}}$ with coenzyme $Q_1$ as electron acceptor being about 15% that of DPNH. At high concentrations, both DPN and DPNH inhibit the enzyme, and the extent of their inhibition is related to the nature of the electron acceptor used. Thus, with menadione and $Q_1$ as electron acceptors, DPNH is a much more potent inhibitor than DPN, whereas with ferricyanide, cytochrome $c$, and DCI, only DPNH is an effective inhibitor at high concentrations (Fig. 2).

The dehydrogenase reacts with ferricyanide, cytochrome $c$, coenzyme $Q_1$, menadione, and DCI as electron acceptors (Table I). A consideration of the values for both $V_{\text{max}}$ and $V_{\text{m}}$ shows that the enzymatic activity of DPNH dehydrogenase is extremely high and of the same order of magnitude with each of these electron acceptors. Coenzymes $Q_2$, $Q_6$, and $Q_{10}$ are also reduced by this enzyme (11). However, because of their insolubility in water and their tendency to coacervate in aqueous media, their rates of reduction are about one order of magnitude smaller than the reduction rates of the water-soluble electron acceptors given in Table I. Also, rate measurements with water-insoluble coenzymes $Q$ are reproducible only with difficulty and are apt to vary if the source of the quinone or components of the assay mixture are changed. At the optimal DPNH concentrations given for each assay in "Methods and Materials," DCI inhibits the enzyme at concentrations above 0.1 mM, menadione above 0.4 mM, and ferricyanide above 2 mM. Cytochrome $c$ up to 0.17 mM and $Q_1$ up to 0.9 mM showed no inhibitory effect. In the cases of DCI, menadione, and ferricyanide, it is seen from the corresponding values of $K_m$ for the acceptor concentration necessary for saturating the enzyme, the earlier is the onset of inhibition as the acceptor concentration is increased.

The $K_m$ values determined with various electron acceptors (Table I) are rather interesting. The $K_m^{\text{DPNH}}$ for the reactions $\text{DPNH} \rightarrow Q_1$, $\text{DPNH} \rightarrow$ menadione, and $\text{DPNH} \rightarrow$ DCI is in each case about 133 $\mu$M, whereas for $\text{DPNH} \rightarrow$ ferricyanide and...
In general, the enzymatic properties of DPNH dehydrogenase, i.e. the ability to catalyze electron transfer from a reduced pyridine nucleotide to oxidized compounds and ferric complexes, are best classified as diaphorase type activities, which in principle are shared by many other pyridine nucleotide-linked flavoprotein dehydrogenases (for a list of such enzymes see the earlier work of Hatefi (9) and References 27 to 33). However, as will be seen in the following section, the catalytic properties of the soluble dehydrogenase shown in Table I are not representative of the activities of the enzyme as an integrated component of the electron transport system.

**Dissociated and Integrated Forms of DPNH Dehydrogenase**

An interesting phenomenon, which has been observed in recent years by researchers in several laboratories working with membrane-bound enzymes, is that often the properties of such enzymes undergo vast changes when the protein is detached from the membrane assembly. Among examples of such dislocation (or allotopic) effects are the $K_m$ of Neurospora malate dehydrogenase (34, 35), the oxidation-reduction potential of mitochondrial cytochrome b (37), and the inhibitor sensitivity and catalytic properties of mitochondrial ATPase (38). In addition, recent studies in this laboratory (25) and by Bruni and Racker (39) suggest to us that the coenzyme Q reductase activity of succinic dehydrogenase might also be a property which is manifest only when the dehydrogenase is particle-bound.

The case of the mitochondrial DPNH dehydrogenase also, our studies show that the catalytic and the inhibitor response properties of the enzyme are altered when it is detached from particles and rendered soluble. As seen in Tables I and II, the specific activity for menadione reduction by the soluble DPNH dehydrogenase is about 160 to 170, whereas in Complex I the specific activity for menadione reduction is only 50 to 60. Thus, per mole of flavin the soluble dehydrogenase has 8 to 9 times the menadione reductase activity of the integrated enzyme. In other words, in reactions with two-electron acceptors the value of $K_m$ for DPNH is almost exactly twice that obtained for the reactions with one-electron acceptors. Assuming that DPNH interaction with the enzyme involves a hydride ion transfer regardless of the nature of the electron acceptor, it is then possible that the above phenomenon is a reflection of the difference in the mechanism of reoxidation of the enzyme by one-electron and two-electron acceptors. The acceptor-dependent DPNH inhibitions discussed earlier might be related to differences in the reoxidation mechanism of the enzyme when two-electron acceptors such as Q$_1$ and Q$_0$, or one-electron acceptors such as cytochrome c and ferricyanide are the oxidants.9

9) The behavior of the system with DCI as electron acceptor does not fall into the above pattern.

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**Table II**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme complex</th>
<th>Specific activity</th>
<th>$K_m$ DPNH $\times 10^4$</th>
<th>$K_m$ acceptor $\times 10^4$</th>
<th>Inhibition by</th>
<th>DPNH inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPNH $\rightarrow$ Fe(CN)$_6$ &amp;</td>
<td>I 100$^a$</td>
<td></td>
<td>0.7</td>
<td>400$^a$</td>
<td>None</td>
<td>&gt;0.1 mM, sharp</td>
</tr>
<tr>
<td>DPNH $\rightarrow$ cytochrome c &amp;</td>
<td>I 20$^b$</td>
<td></td>
<td>1.4</td>
<td>1.2</td>
<td>None</td>
<td>&gt;0.3 mM, slight</td>
</tr>
<tr>
<td>DPNH $\rightarrow$ coenzyme Q &amp;</td>
<td>I-III 25-30</td>
<td></td>
<td>1.4</td>
<td>1.2</td>
<td>None</td>
<td>&gt;0.25 mM, sharp</td>
</tr>
<tr>
<td>DPNH $\rightarrow$ menadione &amp;</td>
<td>I 14</td>
<td></td>
<td>1.5</td>
<td>4.4</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>DPNH $\rightarrow$ DCI &amp;</td>
<td>I 1.9</td>
<td></td>
<td>1.5</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

$^a$ Per mole of flavin, this activity is actually higher in Complex I than in the isolated DPNH dehydrogenase.

$^b$ At 0.15 mM DPNH; $V_{\text{max}}$ $= 685$.

$^c$ This activity is due to the presence in Complex I of 0.5 to 1% Complex III contamination.

$^d$ Higher concentrations of antimycin A inhibit, and the inhibition is progressive with time (40).
the urea-induced detachment of DPNH dehydrogenase from Complex I is accompanied by a rise in menadione reductase activity up to about 8.5 times the activity of Complex I in 2.5 mM urea before incubation at 30°C. Similar results are obtained for antimony-insensitive cytochrome c reductase, DCI reductase, and the rotenone- and p-mercuribenzoate-insensitive coenzyme Q$_1$ reductase activities of Complex I in 2.5 mM urea before and after incubation at 30°C. Also, as shown in Tables I and II, the ferriyanide reductase activity of Complex I is insensitive to mercurials, whereas the same activity in the soluble dehydrogenase is inhibited by these reagents. Thus, as shown in Fig. 3, the mersalyl-insensitive ferriyanide reductase activity of Complex I was converted to a mersalyl-sensitive activity as incubation of Complex I in 2.5 mM urea proceeded at 30°C. It may also be noted that, like the rise in menadione reductase activity, the appearance of mersalyl-sensitive ferriyanide reductase activity was complete by about 8 min of incubation at 30°C. That the urea-liberated DPNH dehydrogenase had considerable ferriyanide reductase activity in the absence of mersalyl is shown in Fig. 3 (c).

As seen in Tables I and II, the values for $K_{DPNH}$ and $K_{acceptor}$ (cytochrome c and coenzyme Q) are several times lower with Complex I (or the binary I-III complex), than with the soluble dehydrogenase, but for the artificial acceptor, ferriyanide, $K_{acceptor}$ is higher with Complex I than with the free dehydrogenase. Although we only wish to emphasize the difference in these constants between the two systems, it is interesting, nevertheless, that the integrated systems appear to be more "efficient" than the isolated enzyme in reacting with the natural substrates (coenzyme Q and cytochrome c) and less so with ferriyanide. Another point of interest is the difference between the $K_{DPNH}$ values in Table II with ferriyanide, cytochrome c, and coenzyme Q as electron acceptors. It may be recalled from Table I that the value varied by a factor of 2 depending on whether the oxidant was a one-electron or a two-electron acceptor. A similar picture exists in Complex I (or the binary I-III complex), provided it is taken into account that reduction of cytochrome c in these systems is perforce preceded by the reduction of two-electron-accepting intermediates such as coenzyme Q.

In addition to the above differences between the bound and the free DPNH dehydrogenases, the meager response of the latter to rotenone and antimycin A, the classical inhibitors of the mitochondrial DPNH oxidase system (Table II), is indicated in Table I. Moreover, as seen in Fig. 4, ferriyanide reduction by the bound dehydrogenase (in Complex I or the binary I-III complex) is sharply inhibited above 0.1 mM DPNH, but the same reaction catalyzed by the soluble dehydrogenase does not show such an effect. It is also interesting that acetylpyridine-DPNH, which has a high $K_m$ (16 × 10^-8 M for acetylpyridine-DPNH versus 0.7 × 10^-6 M for DPNH) in the ferriyanide reductase reaction catalyzed by the particle-bound dehydrogenase, does not inhibit ferriyanide reduction at concentrations at which DPNH inhibits (Fig. 5). A more detailed study of the effect of inhibitors on Complex I and DPNH dehydrogenase is presented in the accompanying communication (10).

**DISCUSSION**

The studies reported in this and other communications on Complex I and its component proteins (10-12) bring to light several interesting aspects of the structure and function of these systems.

**Structure of Complex I**

In Complex I both DPNH dehydrogenase and the iron-sulfur protein appear to be protected by a hydrophobic sheath of lipids and structural proteins. Such a structure is consistent with (a) the unique ability of chaotropic agents to effect the release of DPNH dehydrogenase and iron-sulfur protein from Complex I, (b) the fact that dithionite is unable to reduce DPNH dehydrogenase and iron-sulfur protein in the intact complex, but does so readily when these components are released into the aqueous phase, (c) the fact that ionic chelators such as Tiron and bathophenanthroline sulfonate do not readily react with the iron moiety of Complex I, but do so after treatment of the complex with chaotropic agents, and (d) the observation that the inhibitor potency of barbiturates, Demerol, rotenone, and piericidin A is in keeping with their hydrophobic character and lipid solubility.

**Active Site of DPNH Dehydrogenase**

The soluble DPNH dehydrogenase appears to have a more accessible conformation at the active site, and the receptor site for the electron donor appears to be located near the site of interaction of the electron acceptor with the enzyme. This postulate is consistent with the following observations.

In the ferriyanide reductase reaction catalyzed by the soluble DPNH dehydrogenase, the value of $K_{DPNH}$ is nearly 10 times that of the Complex I-catalyzed reaction. Moreover, in the Complex I-catalyzed reactions high concentrations of DPNH (>0.1 mM) sharply inhibit ferriyanide reduction, whereas the dehydrogenase-catalyzed reduction of ferriyanide is unaffected up to 1 mM DPNH (see also the effect of acetylpyridine-DPNH under "Dissociated and Integrated Forms of DPNH Dehydrogenase").

In Complex I, ferriyanide reduction is not inhibited by p-chloromercuri phenyl sulfonate or mersalyl, whereas in the soluble dehydrogenase this reaction is completely inhibited by either mercurial at a concentration of about 50 μM. This difference is consistent with the possibility that in Complex I the sulfhydryl group (or groups) involved in ferriyanide reduction is inaccessible to mercurial attack, while the less hindered conformation of the soluble dehydrogenase allows such access.

In contrast to the soluble dehydrogenase, Complex I has a very low menadione and DCI reductase activity and no anti-
mycin-insensitive cytochrome c reductase activity. This differ-
ence is also consistent with the possibility that in Complex I the
interaction of DPNH dehydrogenase and the above compounds
is hindered, but that the more accessible active site of the soluble
dehydrogenase permits such an interaction with considerable
efficiency.

In the reduction of menadione catalyzed by the soluble dehy-
drogenase, the onset of DPNH inhibition is inversely related to
the concentration of menadione in the assay mixture. Thus at 0.9
mm menadione DPNH inhibition begins at about 0.15 mm
DPNH, whereas at 0.3 mm menadione the reaction rate increases
with increasing DPNH concentration up to about 0.25 mm
DPNH, and DPNH inhibition begins only above this concen-
tration. These results also suggest that the site of quinone
reduction in the soluble dehydrogenase might lie near the site of
DPNH interaction with the enzyme.

**Diaphorase Activity of Soluble DPNH Dehydrogenase**

Many flavoproteins which contain either FMN or FAD as
prosthetic group (or in which the apoprotein can use either FMN
or FAD) and which catalyze electron transfer from either DPNH
or TPNH or both to various quinones and ferric compounds are
known (27–29, 32, 33, 41). Among the electron acceptors used
are ferric chloride, ferri cyanide, cytochrome c, methylene blue,
DCI, tetrachloramine dyes, unsubstituted and substituted p-benzo-
quinones, including coenzymes Q, and various naphthoquinones,
including menadione. Many of these enzymes are inhibited by
mercurials, especially those that can use DPNH as substrate.
These common features, which are also shared by the purified
DPNH dehydrogenase of the electron transport system, suggest,
therefore, that in these enzymes abstraction of two electrons and
one proton from pyridine nucleotides involves the same basic
mechanism and such moiety of these flavoproteins as can inter-
act with quinones and ferric compounds. Also, the fact that,
unlike mitochondrial DPNH dehydrogenase, no labile sulfide-iron
system has been reported for the above enzymes suggests that
these components of DPNH dehydrogenase may not be required
for its diaphorase activity. This possibility is supported by the
following findings.

In some preparations of DPNH dehydrogenase, treatment
with DPNH does not give rise to a g = 1.94 electron paramag-
netic resonance signal, whereas dithionite treatment does.
However, the enzyme is highly active as a diaphorase (see also
Reference 22).

A number of iron chelators, including α,α′-bipyridyl, batho-
phanthrazone, S-hydroxyquinoline, EDTA, and Tiron, can
react with the iron moiety of DPNH dehydrogenase without
either inhibiting or activating substantially its diaphorase
activity (10).

The dehydrogenase preparations of others (4, 21, 22), which
have lost 50% or more of their iron and labile sulfide content, are
still capable of catalyzing (although at a lowered rate) electron
transfer from DPNH to quinones and ferric compounds. The
possible noninvolvement of iron and labile sulfide in the diaph-
orase activity of DPNH dehydrogenase is not necessarily in
agreement with Hatfield's previous demonstration (12) that the
loss of labile sulfide induced by incubation of the enzyme at pH
4.8 and 38° parallels the loss of dehydrogenase activity, as labile
sulfide loss could simply result in structural alterations which
would be detrimental to the diaphorase activity of the dehydro-
genase.

The above situation is somewhat similar to the results of recent
studies in this laboratory on succinic dehydrogenase (20).* Highly
purified preparations of succinic dehydrogenase, containing,
per mg of protein, 6 to 7 mmoles of flavin and about 50 mmoles
each of iron and labile sulfide and capable of catalyzing phen-
azine methosulphate reduction at a rate of 20 to 32 μmoles of
succinate oxidized per min × mg of protein, were found to be
completely inactive for reconstitution of succin oxidase activity
when added back to alkali-inactivated electron transport par-
ticles. Treatment of the dehydrogenase with ferrous ions,
sodium sulfide, and mercaptoethanol restored reconstitution
activity. These studies show that even when the labile sulfide-
iron system of succinic dehydrogenase was damaged enough to
be incapable of transmitting electrons from succinate to the
respiratory chain, the enzyme was nevertheless highly active as
a succinic dehydrogenase in the presence of an appropriate,
although artificial, electron acceptor.

With regard to the cytochrome c reductase activity of DPNH
dehydrogenase, the following points might be of interest.

1. Massey (42) has shown that cytochrome c can be reduced
by dihydroliopoloside produced in the lipoyl dehydrogenase
reaction.

\[
\text{lipoyl dehydrogenase} \\
\text{DPNH} + \text{H}^+ + \text{Lip}_2 \rightarrow \text{DPN}^+ + \text{Lip(SH)}_2
\]

where Lip₂ and Lip(SH)₂ represent oxidized and reduced lipoic
acid.

2. The existence of an active disulfide in the mitochondrial
DPNH dehydrogenase has been postulated by Tyler et al. (43)
and by Mersmann, Luthy, and Singer (44).

3. The inaccessibility for mercurial attack of the active sulphy-
dryl groups of the integrated DPNH dehydrogenase and the
availability of these groups for mercurial inhibition in the soluble
dehydrogenase are consistent with the appearance of cytochrome
\( c \) reductase activity as the dehydrogenase is detached from
the particles.

4. Ziegler, Green, and Doeg (41) have shown that a diaphorase
enzyme (later shown to be indistinguishable from lipoyl dehy-
drogenase) acquired cytochrome c reductase activity after aging
or repeated freezing and thawing.

Therefore, it is possible that cytochrome c reduction by DPNH
dehydrogenase results from interaction of cytochrome c with the
enzymatically reduced sulfhydryl groups of the dehydrogenase
and that the appearance of this reaction is a consequence of a
more accessible conformation of the isolated dehydrogenase (*i.e.*
as regards the sulfhydryl groups) as compared with the integrated
form of the enzyme.

**Apparent Michaelis Constants and Pyridine Nucleotide Inhibitions**

As pointed out earlier, there appears to be a pattern in the
magnitude of \( K_m \) for both the soluble and the particle-bound
DPNH dehydrogenases depending on whether the oxidant (or
intermediate oxidants in the case of particle-bound dehy-
drogenase) is a one-electron or a two-electron acceptor. Thus,
with one-electron acceptors the value of \( K_m \) was the same
and exactly one-half of that obtained for two-electron acceptors
(Tables I and II). Also, with the exception of DCI, the reduc-
tion of two-electron acceptors by DPNH dehydrogenase was
inhibited at high concentrations of DPNH whereas, in contrast,
the reduction of one-electron acceptors did not exhibit such
tsensitivity. These phenomena might be a consequence of the
dissimilar affinity of DPNH for the various oxidation-reduction
states of the enzyme (i.e. oxidized, half-reduced, and fully
reduced) produced during electron transfer to one-electron versus
two-electron acceptors.

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