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.

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_{244} = 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.
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 chaotropic agents such as urea, guanidine hydrochloride, sodium perchlorate, and thiocyanate.* 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 flask 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 (21). 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⁻, CI⁻, and other chaotropic ions is due mainly to their effect on the structure and lipophilicity of the surrounding water.
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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

and iron-sulfur protein oxidation in mitochondrial particles is

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were carried out in a closed cuvette which was evacuated several
times and flushed with repurified nitrogen.

ence spectrum of

The enzyme, at a protein concentration of 1.6 mg per ml, was
dissolved in 50 mM Tris-Cl, pH 9.1, containing 2 mM urea. The
presence of urea at slightly alkaline pH prevented the develop-
ment of turbidity during various treatments. The experiments
were carried out in a closed cuvette which was evacuated several
times and flushed with repurified nitrogen.

DPNH + 2,6-dichloroindophenol. 100

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dissolved in 50 mM Tris-Cl, pH 9.1, containing 2

Acetylpyridine-DPNH + coenzyme Q1.. 125

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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 nmoles 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
nmoles of flavin, 42 ng atoms of iron, and 50 to 53 nmoles of

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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 destruc-
tion of the iron-sulfur system. The result (Trace 4) is the spec-

trum 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
dependence of the latter two enzymes (heat-acid-ethanol) is not

surprising, and Hatefi has shown (12) that incubation of the
present dehydrogenase at 38° and pH 4.8 can result in a complete
loss of labile sulfide and a parallel loss of enzymatic activity.

It is surprising, however, that the preparation of Pharo et al. (3)
contains more labile sulfide than iron. In fact, an earlier prepa-
ration 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

T able I

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activitya</th>
<th>μDPNHb max</th>
<th>μacceptorb max</th>
<th>KDPNHc</th>
<th>Kacceptorb</th>
<th>Inhibitiond by 1 μm rotenone</th>
<th>DPNH inhibition</th>
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<tr>
<td>DPNH → Fe(CN)4</td>
<td>210</td>
<td>330</td>
<td>400</td>
<td>0.5</td>
<td>165</td>
<td>None</td>
<td>&gt;1.3 mM, slight</td>
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<tr>
<td>DPNH → cytochrome c</td>
<td>43</td>
<td>76</td>
<td>230</td>
<td>6.4</td>
<td>60</td>
<td>&gt;1.2 mM, slight</td>
<td>&gt;0.25 mM, sharp</td>
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<tr>
<td>DPNH → coenzyme Q1</td>
<td>130–140</td>
<td>600</td>
<td>175</td>
<td>13.3</td>
<td>16</td>
<td>10–20</td>
<td>&gt;0.25 mM, sharp</td>
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<tr>
<td>DPNH → menadione</td>
<td>100</td>
<td>150</td>
<td>13.3</td>
<td>16</td>
<td>6.2</td>
<td>&gt;0.3 mM, sharp</td>
<td></td>
</tr>
<tr>
<td>Acetylpyridine-DPNH → coenzyme Q1</td>
<td>100</td>
<td>125</td>
<td>13.3</td>
<td>6.2</td>
<td>71.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 μm antimycin A.

d At 0.75 mM DPNH.

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10.75 mM DPNH.
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 8), 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% of 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 $K_m$ 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 $Q_i$ 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 $K_m$ in Table I that the lower 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 $K_m$ for the reactions DPNH $\rightarrow Q_1$, DPNH $\rightarrow$ menadione, and DPNH $\rightarrow$ DCI is in each case about 133 $\mu$M, whereas for 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 quinoid 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 Hafei (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 oxygenation-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.

In 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 by the soluble DPNH dehydrogenase is about 160 to 170, whereas in Complex I the menadione reductase activity of the integrated enzyme is only 9 times the menadione reductase activity of Complex I. The menadione reductase activity of the integrated enzyme is only 9 times the menadione reductase activity of Complex I. The enzyme concentration in all assay mixtures was 10 μg per ml.

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An earlier demonstration of this phenomenon is the work of Huennekens on the malate dehydrogenase of mammalian mitochondria (36). He showed that the properties of the conjugated and the dissociated malate dehydrogenases differed, among other things, in pH optimum and in $K_m$ for substrate.
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 antimycin-insensitive cytochrome c reductase, DCI reductase, and the rotenone- and piericidin-insensitive coenzyme Q 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 ferriyamine 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 ferriyamine 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 ferriyamine reductase activity was complete by about 8 min of incubation at 30°C. That the urea-liberated DPNH dehydrogenase had considerable ferriyamine reductase activity in the absence of mersalyl is shown in Fig. 3 (c).

As seen in Tables I and II, the values for $K_{m}^{DPNH}$ and $K_{m}^{acceptor}$ (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, ferriyamine, $K_{m}^{acceptor}$ 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 ferriyamine. Another point of interest is the difference between the $K_{m}^{DPNH}$ values in Table II with ferriyamine, cytochrome c, and coenzyme Q as electron acceptors. It may be recalled from Table I that this 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, ferriyamine 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 $K_{m}$ ($16 \times 10^{-4}$ mM for acetylpyridine-DPNH versus $0.7 \times 10^{-4}$ mM for DPNH) in the ferriyamine reductase reaction catalyzed by the particle-bound dehydrogenase, does not inhibit ferriyamine 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 dithiothreite 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 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 ferriyamine reductase reaction catalyzed by the soluble DPNH dehydrogenase, the value of $K_{m}^{DPNH}$ 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 ferriyamine reduction, whereas the dehydrogenase-catalyzed reduction of ferriyamine 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, ferriyamine reduction is not inhibited by p-chloromercuriphenylsulfonate 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 ferriyamine 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 difference 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 dehydrogenase, 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 concentration. 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, ferricyanide, cytochrome c, methylene blue, DCI, tetrazolium dyes, unsubstituted and substituted \( p \)-benzoquinones, 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 moieties of these flavoproteins as can interact 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 paramagnetic 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 \( \alpha, \alpha ' \)-bipyridyl, bathophenanthroline, 8-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 diaphorase activity of DPNH dehydrogenase is not necessary in disagreement with Hatefi's previous demonstration (12) that the loss of labile sulfide induced by incubation of the enzyme at pH 4.8 and 38\°C 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 dehydrogenase.

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 phenazine methosulfate reduction at a rate of 20 to 32 mmoles of succinate oxidized per min \( \times \) mg of protein, were found to be completely inactive for reconstitution of succinimidase activity when added back to alkali-inactivated electron transport particles. 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 dihydrolipoate produced in the lipoyl dehydrogenase reaction.

\[
\text{Lipoyl dehydrogenase} \\
\text{DPNH} + \text{H}^+ + \text{Lip}_2 \rightarrow \text{DPNH}^+ + \text{Lip(SH)}_2 + 2 \text{fold cytochrome c} \\
\text{Lip}_2 + 2 \text{fold cytochrome c} + 2\text{H}^+ \\
\]

where Lip\(_2\) 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 sulfhydryl groups of the integrated DPNH dehydrogenase and the availability of these groups for mercurial inhibition in the soluble dehydrogenase is 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 lipoym dehydrogenase) which 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 dehydrogenase) 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 reduction 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 sensitivity. 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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