ISOLATION AND PROPERTIES OF THE DPNH DEHYDROGENASE OF THE RESPIRATORY CHAIN FROM HEART MITOCHONDRIA *

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Although a number of soluble preparations of mitochondrial origin capable of oxidizing DPNH have been reported in the literature, the properties of none of them suggest that it represents, in a native form, the primary dehydrogenase component of the DPNH oxidase chain. From heart mitochondria, wherein DPNH oxidation has been most extensively studied, two soluble DPNH dehydrogenases were isolated some time ago: Mahler's cytochrome c reductase (Mahler et al., 1952), and Straub's diaphorase (Straub, 1939). The function of the former remains obscure, but speculations that it might be an artifact have not been abandoned (Ziegler et al., 1959), while the latter has now been shown to be the lipoyl dehydrogenase component of the α-ketoglutarate oxidase complex and not a part of the respiratory chain (Massey, 1960a, b). More recently a lipoflavoprotein was described by Ziegler et al. (1959) which may be considered a fragment of the respiratory chain, but, in view of its high particle weight and lipid content, it can not be considered a soluble preparation of the primary dehydrogenase. It was felt desirable, therefore, to elaborate a mild method for the isolation of the DPNH dehydrogenase of the respiratory chain, free from lipids and without the use of bile salts or organic solvents, which might modify the properties of the protein.

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It was found some time ago in this laboratory that the digestion of brain mitochondria with phospholipase A, besides solubilizing α-glycerophosphate dehydrogenase (Ringler and Singer, 1958) liberates DPNH dehydrogenase in a soluble form. That treatment with phospholipase is suitable for the solubilization of the latter enzyme is further indicated by the report of King and Howard (1960), who have obtained a soluble preparation capable of DPNH oxidation from a Keilin-Hartree preparation of heart muscle by this means. However, since the starting material used by these workers is known to contain a number of enzymes which oxidize DPNH, the identification of the respiratory chain-linked DPNH dehydrogenase in the resulting extract is inherently difficult.

Past experience in the isolation of the various respiratory chain-linked dehydrogenases in this laboratory has shown that the ultimate success hinges primarily on two factors: a reliable assay method and the choice of the starting material. The details of the assay method are presented in the next paper in this issue (Minakami et al., 1960). The DPNH oxidase of Crane et al. (1956) seemed to offer an ideal starting material since it is reported to represent a purified particulate form of the DPNH oxidase system free from many contaminating mitochondrial enzymes, in particular, diaphorases, and in which the oxidation of DPNH is completely antimycin A — and amytal — sensitive (Crane et al., 1956; unpublished observations, this laboratory). Thus, if the flavoprotein component of this preparation could be solubilized and separated from the other components of the electron transport system, one might justifiably conclude that it represents the DPNH dehydrogenase of the respiratory chain.

Treatment of the DPNH oxidase preparation (Crane et al., 1956) with Naja naja venom, an excellent source of phospholipase A, resulted in extensive solubilization of the DPNH dehydrogenase activity (Table I). Under the conditions of Table I, the solubilization varied from about 62 to 78%, and most of the activity remaining in the residue (Table I) could be solubilized by longer incubation or by a second treatment with the venom. Little activity is lost on digestion with the phospholipase under these conditions and essentially all of the activity is accounted for by analysis of the supernatant solution and of the

residue, despite the difficulties of the assay of this enzyme in particulate preparations (Minakami et al., 1960).

The fact that the enzyme is in true solution is indicated by the observations that it fails to sediment at 144,000 x g for one hour even after extended dialysis against buffer of low ionic strength or repeated freezing and thawing, and that it behaves as a typical soluble enzyme on fractionation by classical methods.

TABLE I

DISTRIBUTION OF ENZYMATIC ACTIVITY FOLLOWING DIGESTION WITH NAJA NAJA VENOM

Step	Activity (μmoles DPNH oxid./min.)	Per cent
DPNH oxidase	3,330	
Same after venom treatment	3,030	(100)
Soluble enzyme	1,870	62
Residue	940	32

The DPNH oxidase preparation was incubated for 80 minutes at 30°, pH 7.4, with Naja naja venom, 0.04 mg. venom per mg. mitochondrial protein and then centrifuged for 20 min. at 144,000 x g. The activity determinations are V_{max} values, ferricyanide assay. For assay conditions see Minakami et al. (1960).

Although the starting material is completely amytal— and antimycin A—sensitive in the DPNH oxidase assay and partially sensitive in the ferricyanide assay (the fraction of the activity inhibited represents the reaction of ferricyanide with cytochrome c in respiratory chain preparations), the soluble enzyme is insensitive to these two inhibitors and also to cyanide. The circumstance that amytal does not inhibit the activity of the soluble enzyme nor that part of the activity in particulate preparations which represents the reaction of ferricyanide with the dehydrogenase itself (Minakami et al., 1960) suggests that amytal interrupts the flux of electrons between the dehydrogenase and the respiratory chain and not between DPNH and the flavoprotein, as is also the case in the choline oxidase system of rat liver mitochondria (Packer et al., 1960).

The soluble enzyme is completely precipitated by 0.4 saturation of ammonium sulfate at pH 7.4. After dialysis its specific activity is about 140 μmoles of DPNH oxidized per minute per mg. protein (biuret reaction) at 30°, and it has been further purified by chromatographic procedures. The flavin present in the preparation is readily liberated by thermal denaturation and thus differs from the flavin of succinic dehydrogenase (Kearney, 1960). The minimal turnover number per mole of flavin at 30° is 97,000.

The absorption spectrum of the oxidized enzyme shows a pronounced peak at 410 mμ, pH 7.4: the position and height of this absorption band varies markedly with pH in the range studied (pH 7.1 to 9.0). DPNH and dithionite both bleach the enzyme rapidly whether measured at 410 mμ (Fig. 1) or 450 mμ. The difference spectrum with DPNH is shown in Fig. 2. The bleaching observed in the 450 and 375 mμ regions is compatible with the flavoprotein nature of the dehydrogenase.

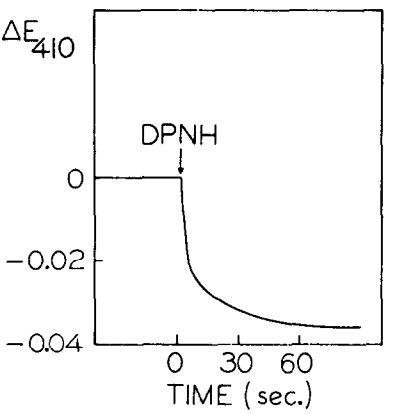

Fig. 1. The reduction of DPNH dehydrogenase at 410 mμ by 0.06 mM DPNH, pH 7.4, 30°. Protein concentration 3.62 mg./ml.

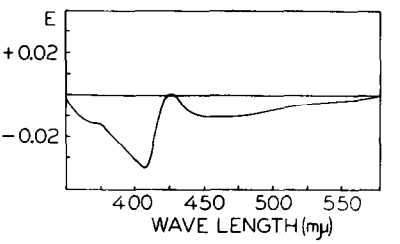

Fig. 2. The difference spectrum of DPNH dehydrogenase after reduction with DPNH. Protein concentration 3.6 mg./ml.

The 410 mμ peak resembles that seen in α-glycerophosphate dehydrogenase (Ringler, 1960) and the succinic dehydrogenase from Micrococcus lactilyticus (Warringa and Giuditta, 1958), both of which are relatively rich in non-heme iron.

Besides ferricyanide, the enzyme reacts relatively well with 2,6-dichlorophenolindophenol but not with oxygen. The solubilized enzyme, even prior to purification, shows only a trace of cytochrome c reductase activity when tested

under the conditions recommended by Mahler et al. (1952) for the assay of DPNH cytochrome c reductase. Even this slight activity might be due to a trace contamination of the preparation with the DPNH cytochrome c reductase of Mahler et al. (1952), since substances which inhibit the reduction of cytochrome c by Mahler's enzyme (0.01 M phosphate, pyrophosphate, calcium ion and magnesium ion) strongly inhibit the reduction of cytochrome c by the solubilized DPNH dehydrogenase without affecting the reaction with ferricyanide. The enzyme may be readily distinguished from diaphorase by many of its properties. Thus, under the conditions recommended by Massey (1960a) for the assay of lipoyl dehydrogenase, at pH 6.5, the ratio V_{max} ferricyanide/V_{max} lipoamide is 56 for the partially purified DPNH dehydrogenase and 0.1 for diaphorase. The complete insensitivity of DPNH dehydrogenase to dicumarol, the very slight activity with menadione, and the specificity for DPNH readily distinguish it from DT diaphorases (Ernster, 1960; Giuditta and Strecker, 1960). The DPNH dehydrogenase described here also differs markedly from the lipoflavoprotein of Ziegler et al. (1959) in regard to its absorption spectrum and in other respects.

The dehydrogenase does not oxidize TPNH. DPN is a competitive inhibitor toward the electron acceptor (ferricyanide). Unlike the preparation reported by King and Howard (1960), purified preparations of the enzyme are very stable as compared with other mitochondrial dehydrogenases studied in this laboratory.

In view of the composition of the starting material and the mild methods employed in the extraction and purification of the enzyme, it seems probable that this preparation represents a highly purified, soluble, and native form of the flavoprotein component of the respiratory chain which links DPNH oxidation to the cytochrome system.

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