A Triphosphopyridine Nucleotide-Cytochrome c Reductase from Heart Muscle*

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Extensive studies by numerous workers have served to characterize the reduced diphosphopyridine nucleotide and succinic oxidase systems as important enzymatic pathways in the terminal respiratory chain of mammalian tissue [see reviews by Chance (1) and Slater (2)]. These systems have been shown to consist of an integrated complex of components including flavins, various cytochromes, metal ions, and lipidic constituents. Recent experiments in this laboratory have implicated vitamin E as a co-factor of the cytochrome c reductases derived from the mitochondria of rat skeletal and beef heart muscle tissues (3-6). Tocopherol was indicated to act at a level between cytochromes and to reverse competitively the inhibition by antimycin A (4).

Less attention has been devoted to the properties and significance of TPN-cytochrome c reductase first purified and characterized from animal tissues (pig liver) by Horecker as a solubilized FAD-enzyme highly specific for TPNH (7). More recently Phillips (8) investigated a TPN-cytochrome c reductase occurring principally in the mitochondrial fraction of rat liver. Neither of these preparations was studied with regard to a possible vitamin E requirement nor to an effect of antimycin A or somewhat similar inhibitors on this system. Kaplan et al. (9) suggested an indirect pathway of electron transport from TPNH in liver mitochondria via transhydrogenase and DPN-linked systems, and Talalay and Williams-Ashman (10) implicated steroid hormones as electron carriers in the transhydrogenase system.

The present work describes the purification and characterization of a specific TPN-cytochrome c reductase from bovine heart muscle tissue which is insensitive to antimycin A. In addition to the apparent lack of a tocopherol requirement other evidence is presented to show that the pathway of electron transport between TPNH and cytochrome c is different from the DPNH-cytochrome c reductase isolated from the same source.

EXPERIMENTAL

Cofactors and Other Substances—TPNH of 80 per cent purity, DPNH of 90 to 95 per cent purity, horse heart cytochrome c, 90 to 100 per cent, FAD, and riboflavin phosphate were obtained from the Sigma Chemical Company. Solutions of the reduced pyridine nucleotides were assayed spectrophotometrically with the value, 6.24 × 10⁴ cm⁻² per mole as the extinction coefficient at 340 μM (11). Antimycin A and sodium Amytal were supplied by the Wisconsin Alumni Research Foundation and Eli Lilly Company, respectively; the compound, 4-hydroxy-n-heptyl quinoline N-oxide, was kindly provided by Dr. Britton Chance.

Assay Procedures—All assay procedures were carried out at 22-23° with a Beckman DU spectrophotometer and cuvettes of 1 cm. light path.

Cytochrome c Reductase—The reaction mixture of 1.0 ml. final volume was routinely composed of the following: 0.05 ml. of enzyme, 0.1 ml. of 0.01 M KCN, 0.1 ml. of 2 per cent aqueous cytochrome c, 0.50 ml. of 0.05 M Tris buffer, pH 7.6, and 0.25 ml. of TPNH containing 3.85 μmoles per ml. In the fractionation and preliminary experiments, nonsaturating amounts of TPNH varying from about 0.25 to 0.35 μmole we used in the assay. The TPNH was added at zero time, and the increase in optical density at 550 μM was measured at 30-second intervals for 3 minutes. A unit of enzyme activity was defined as that amount of enzyme which caused an increase in log I/I₀ of 0.001 in the interval from 30 to 90 seconds, all values being corrected for the endogenous rate without added TPNH. Under these conditions the rate of cytochrome c reduction was directly proportional to the enzyme concentration (Fig. 1). With the use of the above reaction mixture and assay method, the DPN and succinate cytochrome c reductases were also determined by substituting in place of TPNH, 0.1 ml. of DPNH (1.15 μmoles per ml) or 0.1 ml. of 0.1 M sodium succinate and an appropriate increase in buffer volume. Specific activity was defined as units of enzymatic activity per mg. of protein. Protein concentration was determined by the procedure of Lowry et al. (12) with crystalline human serum albumin as the standard.

TPNH Oxidase—The reaction mixture for this assay consisted of the following: 0.05 ml. of enzyme, 0.05 ml. of 2 per cent aqueous cytochrome c, and 0.70 ml. of 0.1 M phosphate buffer at pH 7.5. Upon the addition of 0.20 ml. of TPNH (5.5 μmoles per ml.) changes in optical density at 340 μM were recorded at 1-minute intervals for 5 minutes. A concenmit control reaction without added cytochrome c was also measured.

TPN-Glutathione Reductase—To 0.1 ml. of 0.1 M oxidized glutathione were added 0.05 ml. of enzyme, 0.60 ml. of 0.1 M phosphate buffer, pH 7.5, and 0.20 ml. of TPNH (5.5 μmoles per ml.). The decrease in optical density at 340 μM was determined at 30-second intervals for 5 minutes.

* The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.
RESULTS

Purification of Enzyme—All steps of the purification procedure, summarized in Table I, were carried out at 0–4°C.

A sample of beef heart obtained from a freshly killed animal and frozen until use was homogenized in a Waring Blender with 5 times its weight of 0.1 M phosphate buffer, pH 7.5, followed by homogenization in a TenBroeck tissue grinder. The homogenate was centrifuged at 15,000 × g for 30 minutes, with the resulting supernatant solution (Fraction 1) containing 20 to 60 per cent of the total TPN-cytochrome c reductase activity. Recentrifugation of Fraction 1 in a Spinco ultracentrifuge at 105,000 × g for 60 minutes yielded a supernatant solution (Fraction 2) with slightly more activity than that of the previous fraction. The addition to Fraction 2 of ammonium sulfate to 60 per cent saturation and dilute ammonium hydroxide to maintain the pH near 7 gave a precipitate which was then dissolved in a volume of 0.1 M phosphate buffer, pH 7.5, one-third that of Fraction 2. This was dialyzed for 3 to 4 hours against distilled water to yield Fraction 3 with a 2-fold greater specific activity compared to the previous fraction. In a typical experiment, 10 ml. of Fraction 3 containing 89 mg. protein were stirred intermittently for 20 minutes with an equivalent dry weight of calcium phosphate gel (aged 9 months or more) previously concentrated by centrifugation in order to minimize dilution of the enzyme. The supernatant solution (Fraction 4) after centrifugation at 1,000 × g for 10 minutes possessed 70 per cent of the enzyme units with a 2-fold increase in specific activity. The TPN-cytochrome c reductase of Fraction 4 was completely adsorbed to another aliquot of concentrated gel equivalent to 300 mg. dry weight and quantitatively recovered by successive elution for 15-minute periods with 3- to 4-ml. aliquots of water (1st eluate), 0.05 M Tris buffer (0.05 M), whereas phosphate (0.1 M) gave a 50 per cent lower but sharper maximum around pH 7.3. The activity in pyrophosphate buffer was of the same order as in phosphate buffer but with a maximum at pH 7.5.

Pyridine Nucleotide Affinity—TPNH appears to be quite specific as the hydrogen donor for this enzyme (Fig. 3). Although DPNI has some effect, the activity was very low and was not increased with 5 times greater amounts of enzyme. The dissociation constant of the TPNH-enzyme complex as estimated from the curve is 1.9 × 10⁻⁴ M.

Other Enzymes Present—In the most highly purified fractions no detectable DPN-cytochrome c reductase could be found with usual amounts of enzyme or with 5 times greater quantities. These fractions were also devoid of succinate-cytochrome c reductase and TPN-glutathione reductase activity. The TPNH oxidase activity without added cytochrome c was negligible, but was detectable with added cytochrome c.

pH Optimum—As shown in Fig. 2, the enzymatic activity exhibited a broad maximum in the region of pH 7.2 to 8.4 with Tris buffer (0.05 M), whereas phosphate (0.1 M) gave a 50 per cent lower but sharper maximum around pH 7.3. The activity in pyrophosphate buffer was of the same order as in phosphate solution but with a maximum at pH 7.5.

Stability of Enzyme—No loss of activity was detectable in any of the enzyme fractions of varying degrees of purity when stored for several weeks at −15°C. At 4°C all fractions were completely stable for at least 5 days. Exposure of the enzyme for 5 minutes to 50, 70, and 100°C resulted in a decrease in activity of 40, 62, and 100 per cent, respectively. The enzyme was stable to dialysis against water, 0.01 M phosphate buffer, pH 7.5, or 0.005 M ethylenediaminetetraacetic acid in phosphate buffer for 4 hours at 4°C.

The most highly purified enzyme preparation had a turnover number of 3 assuming a molecular weight of 100,000 for the TPN-cytochrome c reductase and with the extinction coefficient of reduced cytochrome c of 2.8 × 10⁻³ cm² per mole (13). This is about 1/10 the value, 1140, reported for the liver TPN-cytochrome c reductase (14). How-
Effect of pH on activity of TPN-cytochrome c reductase. The standard assay system was used with purified enzyme (95 μg of protein).

Effect of reduced pyridine nucleotide concentration on activity of TPN-cytochrome c reductase. The assay described was used with purified enzyme (95 μg of protein) and DPNH or TPNH at the final concentrations indicated.

Effect of cytochrome c concentration on activity of TPN-cytochrome c reductase. The assay described was used with purified enzyme (95 μg of protein) and cytochrome c at the final concentrations indicated.

Flavin Effects—FAD and riboflavin phosphate at final concentrations of 10⁻⁴ M failed to stimulate enzymatic activity when preincubated with purified fractions previously dialysed for prolonged periods under various conditions or precipitated with ammonium sulfate at acid pH.

Effect of Metals, Metal-binding Agents, and Other Inhibitors—Fe⁺⁺ and Cu⁺⁺ at a final concentration of 10⁻⁴ M and Zn⁺⁺ and Hg⁺⁺ at 10⁻³ M inhibited 50 per cent of the TPN-cytochrome c reductase activity under standard assay conditions, while Co⁺⁺ at 10⁻⁴ M inhibited 20 per cent. The following ions were found to have no effect at 10⁻⁴ M: Fe⁺⁺⁺, Mn⁺⁺⁺, Ca⁺⁺, Mg⁺⁺, Ba⁺⁺, borate, oxalate, citrate, and molybdate. The enzyme was insensitive to ethylenediaminetetraacetate, α,α'­dipyridyl, and azide at final concentrations of 10⁻³ M, whereas it was inhibited by 8-hydroxyquinoline at 10⁻⁴ M. p-Chloromercuribenzoate at 10⁻³ M was also without effect.

Effect of electron transport inhibitors. The addition of 0.3 μg of antimycin A, sodium Amytal, or 4-hydroxy-n-heptyl quinoline N-oxime to the standard reaction mixture did not alter the enzymatic rate. This amount of the various compounds was 10 to 100 times the quantities required to inhibit significantly DPN-cytochrome c reductase. Although 5 or more extractions with isooctane (4) finally decreased TPN-cytochrome c reductase by approximately 50 per cent, the addition of α-tocopherol failed to restore activity. The inactivation by isooctane extraction is probably due to a denaturation of the enzyme and is similar to that observed with the TPN-cytochrome c reductase of liver (4).

Cytochrome c Affinity—The effect of cytochrome c concentration on the rate of cytochrome c reduction is shown in Fig. 4. The dissociation constant of the complex as estimated from the graph is 4.0 × 10⁻⁴ M indicating a relatively high affinity of the enzyme for cytochrome c. Because of the rapid reduction of low concentrations of cytochrome c, the initial reaction rates at those levels were difficult to assess accurately.

Flavin Effects—FAD and riboflavin phosphate at final concentrations of 10⁻⁴ M failed to stimulate enzymatic activity when preincubated with purified fractions previously dialysed for prolonged periods under various conditions or precipitated with ammonium sulfate at acid pH.

Effect of Metals, Metal-binding Agents, and Other Inhibitors—Fe⁺⁺ and Cu⁺⁺ at a final concentration of 10⁻⁴ M and Zn⁺⁺ and Hg⁺⁺ at 10⁻³ M inhibited 50 per cent of the TPN-cytochrome c reductase activity under standard assay conditions, while Co⁺⁺ at 10⁻⁴ M inhibited 20 per cent. The following ions were found to have no effect at 10⁻⁴ M: Fe⁺⁺⁺, Mn⁺⁺⁺, Ca⁺⁺, Mg⁺⁺, Ba⁺⁺, borate, oxalate, citrate, and molybdate. The enzyme was insensitive to ethylenediaminetetraacetate, α,α'-dipyridyl, and azide at final concentrations of 10⁻³ M, whereas it was inhibited by 8-hydroxyquinoline at 10⁻⁴ M. p-Chloromercuribenzoate at 10⁻³ M was also without effect.

Several known inhibitors of electron transport were examined. The addition of 0.3 μg of antimycin A, sodium Amytal, or 4-hydroxy-n-heptyl quinoline N-oxime to the standard reaction mixture did not alter the enzymatic rate. This amount of the various compounds was 10 to 100 times the quantities required to inhibit significantly DPN-cytochrome c reductase. Although 5 or more extractions with isooctane (4) finally decreased TPN-cytochrome c reductase by approximately 50 per cent, the addition of α-tocopherol failed to restore activity. The inactivation by isooctane extraction is probably due to a denaturation of the enzyme and is similar to that observed with the TPN-cytochrome c reductase of liver (4).

DISCUSSION
Heart muscle TPN-cytochrome c reductase described in the present paper differs in several respects from the liver enzyme studied by Horecker (7) and Phillips (8). First, the former system is obtained in soluble form from fresh heart homogenates fractionated according to the procedure of Hogeboom (14). However, the possibility has not been eliminated that this enzyme preparation is a solubilized modified form of an originally particulate enzyme. It also appears in the supernatant solution of frozen heart preparations after centrifugation at 105,000 × g for 1 hour. The same enzyme in liver, however, is associated with the mitochondria or microsomes. Secondly, the heart muscle TPN-cytochrome c reductase is in contrast to that in liver described by Phillips was stable to dialysis against a variety of buffer solutions. Thirdly, the addition of p-chloromercuribenzoate, which increased the activity of the latter enzyme by 30 per cent, neither inhibited nor stimulated the activity of the heart enzyme.

A present conception of terminal respiration is the transport of electrons from DPNH → flavoprotein → cytochrome b → cytochrome c → cytochromes a + a₃ → oxygen. Kaplan et al. (9) have presented evidence in support of the idea that the oxidation of TPNH is mediated via the above pathway through the intervention of transhydrogenase enzymes. The separation of TPN-cytochrome c reductase from associated respiratory en...
zymes, such as DPN- and succinate-cytochrome c reductases, suggests that this enzyme may possibly function independently of the above schemes. Furthermore, a different role is suggested, since a decreased oxidative phosphorylation (P:O less than 1) has been reported to be associated with the TPNH pathway when transhydrogenase was suppressed by use of DPN-depleted mitochondria (15, 16).

The treatment of particulate heart muscle preparations with antimony A has been shown to inhibit the reduction of cytochrome c by DPNH or by succinate through interference with the transfer of electrons from cytochrome b to cytochrome c (17). DPN-cytochrome c reductase prepared in this laboratory by relatively mild fractionation procedures such as digitonization and gel adsorption retain this sensitivity, which can be reversed by the addition of vitamin E (4). On the other hand, preparations solubilized by more extreme procedures involving alcohol are no longer inhibited by this compound (18, 19). These findings have served in part as a basis for interposing cytochrome b between flavoprotein and cytochrome c in the sequence of electron transport. They also suggest that alcohol-solubilized DPN-cytochrome c reductase is now radically modified and no longer reflects the true electron transport system in cells. In the present study TPN-cytochrome c reductase purified by relatively gentle procedures was found to be uninhibited by antimony A and by other inhibitors of steps preceding cytochrome c, and to be unresponsive to vitamin E, thereby suggesting a different pathway of terminal respiration for this enzyme that does not involve cytochrome b or vitamin E.

Further distinguishing characteristics of the TPN enzyme in contrast to its DPN counterpart from heart muscle are its insensitivity to pyrophosphate and p-chloromercuribenzoate and its 25-fold greater affinity for cytochrome c. A difference in affinity for calcium phosphate gel was also noted in that TPN-cytochrome c reductase required 8 to 10 times more gel for complete adsorption.

**SUMMARY**

The partial purification and characterization of a triphosphopyridine (TPN)-cytochrome c reductase in bovine heart muscle is described. Unlike similar enzymes isolated from particulate fractions of liver, this enzyme was purified from the 105,000 X g supernatant fraction of homogenized frozen heart tissue. Fractionation by ammonium sulfate and calcium phosphate gel procedures resulted in a 30-fold purified fraction that was essentially devoid of diphosphopyridine nucleotide- and succinate-cytochrome c reductase activities. Furthermore, TPNH oxidase in the absence of cytochrome c and TPNH-glutathione reductase were not detectable. All fractions in the purification procedure were stable to dialysis and to storage at -15° for several weeks. The enzymatic activity in tris(hydroxymethyl)aminomethane buffer showed a broad pH optimum from 7.2 to 8.6 and was twice as high as in phosphate or pyrophosphate buffers, which showed sharper optima around pH 7.4. The enzyme was inhibited by Fe++, Cu++, Zn++, and Hg++ ions and by 8-hydroxyquinoline. However, ethylenediaminetetraacetate, α,α'-dipyridyl, azide, and p-chloromercuribenzoate as well as flavin adenine dinucleotide and riboflavin phosphate had no effect. In addition, high concentrations of known electron transport inhibitors, antimony A, Amytal, and 4 hydroxy α-bethyl N-oxide did not inhibit, nor did α-toopherol stimulate the activity of aged or isooctane extracted preparations. The characteristics of TPN-cytochrome c reductase are discussed in regard to other similar respiratory enzymes in heart muscle.

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