OXIDATION OF REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE BY SUBMITOCHONDRIAL PARTICLES FROM BEEF HEART*

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SUMMARY: Submitochondrial particles prepared from beef heart are capable of oxidizing TPNH, in the absence of added DPN, at a rate of approximately 50 nmoles/min x mg protein at 30°. TPNH oxidation by these particles occurs through the respiratory chain as evidenced from TPNH-induced reduction of the cytochromes and the inhibitory effects of rotenone, piericidin A, amytal, antimycin A and cyanide. The latter studies have indicated that the site of TPNH interaction with the respiratory chain is on the substrate side of the rotenone-piericidin block and close to that of DPNH.

It has been generally assumed that submitochondrial particles from bovine heart do not oxidize TPNH directly, but that they do so through the action of membrane-bound pyridine nucleotide transhydrogenase when DPN is also present (1). The purpose of this preliminary report is to demonstrate that bovine-heart submitochondrial particles catalyze TPNH oxidation in the absence of added DPN. The oxidation of TPNH occurs through the mitochondrial electron transport system, and is sensitive to several respiratory chain inhibitors. TPNH has been shown to reduce iron-sulfur proteins, cytochromes aa_3, $c + c_1$ and b. Results concerning phosphorylation accompanying TPNH oxidation, energy-linked TPN reduction by succinate, the site of TPNH interaction with the respiratory chain, and the relationship between mitochondrial TPNH oxidase and transhydrogenase will be presented elsewhere (2).

METHODS AND MATERIALS

Oxygen uptake was measured at 30° with a Clark-type electrode. The data shown in the figures were recorded with an Aminco-Chance double-beam spectrophotometer. Submitochondrial particles (ETP) were prepared by sonication and

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differential centrifugation of heavy beef heart mitochondria essentially according to standard procedures (3). The reaction medium was in all cases 0.25 M sucrose containing 0.05 M Tris sulfate, pH 7.5. TPNH, DPNH and DPN were obtained from PL Biochemicals, and antimycin A (Ant.A) was a gift from Kanegafuchi Chemical Industrial Co., Japan.

RESULTS

The rates of TPNH oxidation by ETP, in the absence and presence of added cytochrome *c*, are shown in Table I. For comparison, the oxidation rates of DPNH,

TABLE I

Oxidation Rates of TPNH, DPNH, and 3-Hydroxybutyrate by ETP

Substrate	Specific Activity	
	-cyt.*c*	+cyt.*c*
TPNH	49	58
DPNH	1040	1500
TPNH + DPN	310	295
3-Hydroxybutyrate	0.0	----
3-Hydroxybutyrate + DPN	280	----

Conditions: 2 mM each of TPNH and DPNH, 1 mM DPN, 20 mM 3-hydroxybutyrate, 0.05 μM cytochrome *c*, and 1.72 mg ETP protein per ml. Specific activity is defined as *nano*atoms O_2 taken up/min x mg ETP protein at 30°.

TPNH + DPN, and 3-hydroxybutyrate ± DPN are also shown. It is seen that ETP preparations oxidize TPNH at a rate of about 50 nmoles/min x mg protein at 30°, and that added cytochrome *c* has little effect on augmenting this rate. Thus, as compared to the rate of DPNH oxidation, in the presence of added cytochrome *c*, the rate of TPNH oxidation is very small, indeed. Addition of DPN increases the TPNH oxidation rate about sixfold. This increase appears to be the result of

TABLE II

Effect of Respiratory Chain Inhibitors on TPNH Oxidation by ETP

Inhibitor	% Inhibition
Rotenone , 2.5 μM	100
Piericidin A, 2.5 μM	96
Amytal , 3 mM	82
TTFA , 1 mM	0.0
NaCN , 0.5 mM	94

Conditions were the same as in Table I. TTFA: 2-thenoyltrifluoroacetone.

the action of ETP-bound pyridine nucleotide transhydrogenase and the production of DPNH, which is then rapidly oxidized. That these preparations of ETP contain TPNH → DPN transhydrogenase activity has been ascertained. Table I also shows that 3-hydroxybutyrate is not oxidized by ETP in the absence of added DPN. These results support our other findings that TPNH oxidation by ETP preparations was not due to the presence of ETP-bound DPN. The K_m for the oxidation of TPNH by ETP is approximately 55 μM. This value is of the same order of magnitude as that (20 μM) calculated by Ernster and coworkers for the submitochondrial TPNH → DPN transhydrogenase reaction (4). Similar to DPNH, the oxidation of TPNH by ETP is inhibited by appropriate concentrations of rotenone, piericidin A, and amytal, but not by 1.0 mM 2-thenoyltrifluoroacetone (Table II). These inhibitor studies suggest that the site of TPNH interaction with the respiratory chain is on the substrate side of the rotenone-piericidin A inhibition site and close to DPNH dehydrogenase. The results of EPR studies and energy-dependent reverse electron transfer from succinate to TPN agree with this conclusion (2).

The effect of TPNH on the reduction of respiratory chain components is shown in Fig. 1 to 5. Figure 1 shows the effects of TPNH, DPNH and succinate on the

bleaching at 475 minus 510 nm, where the combined light absorption of both flavoproteins and iron-sulfur proteins is measured. It is seen that TPNH affords considerable bleaching at 475 minus 510 nm in piericidin-blocked ETP. Further addition of DPNH results in a comparable amount of reduction. The total amount of reduction by TPNH and DPNH shown in Fig. 1 is equal to that afforded when DPNH alone is added to piericidin- or rotenone-blocked ETP. These results suggested that, in piericidin-blocked ETP, TPNH reduces some, but not all, of the chromophores located between DPNH and ubiquinone. Addition of succinate after TPNH and DPNH causes still further reduction. The latter reduction appears to be due to succinate dehydrogenase, since the same degree of succinate-induced bleaching occurred also in the presence of 1 mM TTFA, which inhibits electron transfer from succinate dehydrogenase to ubiquinone. In the presence of antimycin A, the succinate-induced bleaching at 475 minus 510 nm is considerably greater (see Fig. 1). A similar degree of bleaching occurs also upon addition of DPNH to antimycin-treated ETP. Other studies have indicated that the additional bleaching in the presence of antimycin A is caused mainly by ferrocytochromes *b*.

The TPNH induced reduction of cytochrome *b* is shown in Fig. 2. It is seen that under aerobic conditions, the steady-state reduction level of cytochrome *b* is very small, which is in agreement with the slow rate of TPNH oxidation by ETP. Addition of antimycin A results in reduction of nearly half of the substrate reducible *b* cytochromes. The TPNH reducible *b* of ETP under these conditions appears to be essentially b_K as judged from its absorption peak at 563 nm (Fig. 3) (see ref. 5 for absorption spectra of the three *b* cytochromes of bovine heart mitochondria). The inability of TPNH to reduce b_T under these conditions appears to be, at least in part, because the rate of b_T oxidation through the antimycin block is considerably faster than that of b_K. That in antimycin-treated ETP both b_K and b_T are reduced by DPNH, succinate or TPNH + DPN (*via* transhydrogenase) has been ascertained. The right-hand trace in Fig. 2 shows that rotenone effectively blocks *b* reduction by TPNH or DPNH in antimycin treated ETP. For control, the effect of succinate is also shown.

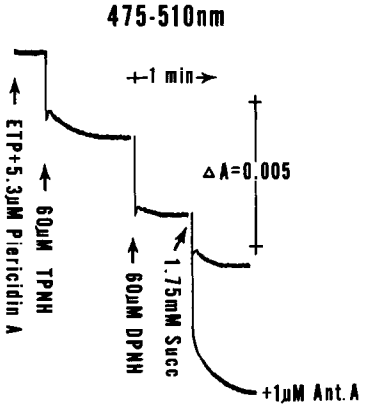

Fig. 1.

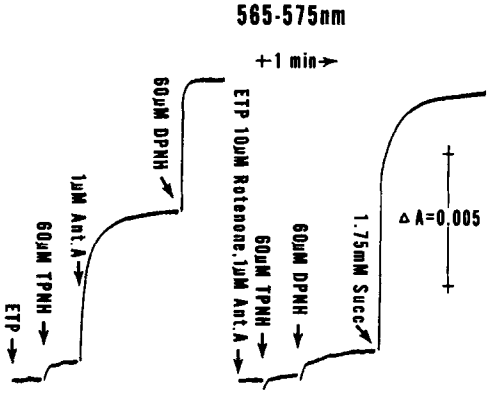

Fig. 2.

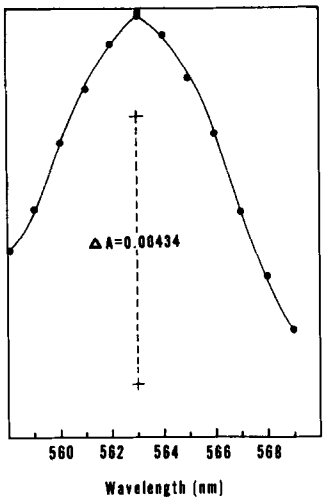

Fig. 3.

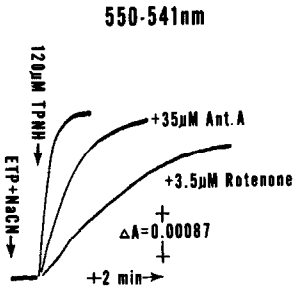

Fig. 4.

Figure 1. Effects of TPNH, DPNH, and succinate on the reduction of ETP chromophores at 475 minus 510 nm. ETP, 2.2 mg protein/ml.

Figure 2. Reduction of cytochromes *b* in ETP by TPNH, DPNH and succinate. ETP, 0.78 mg protein/ml.

Figure 3. Absorption spectrum (α region only) of the cytochrome *b* reduced by TPNH in antimycin-treated ETP. TPNH, 120 μM; ETP, 0.63 mg protein/ml; antimycin A, 1.1 μM. Reference wavelength was 541 nm, and the sample wavelength was varied as shown.

Figure 4. Reduction of cytochromes $c + c_1$ by TPNH in cyanide-treated ETP. NaCN, 1 mM; ETP, 0.82 mg protein/ml.

Figure 4 shows the TPNH reduction of cytochromes $c + c_1$ in cyanide-treated ETP. Full reduction of $c + c_1$ (and aa_3 in Fig. 5) by TPNH was contingent upon

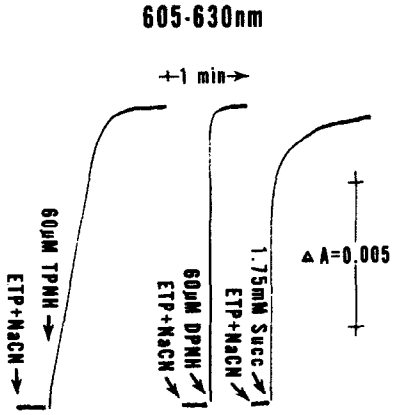

Figure 5. Reduction of cytochromes aa_3 by TPNH, DPNH and succinate in cyanide-treated ETP. NaCN, 1 mM; ETP, 1.66 mg protein/ml.

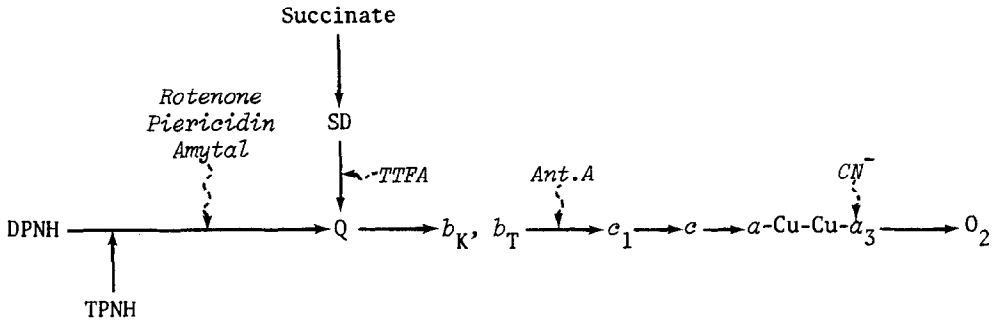

Figure 6. Site of TPNH interaction with the electron transport system relative to those of DPNH and succinate (SD: succinate dehydrogenase).

complete inhibition of cytochrome oxidase by cyanide. Figure 4 also shows the effects of antimycin A and rotenone in the above system. It is seen that even at high antimycin concentration the leak through this block is considerable and much greater than the leak through the rotenone block. Figure 5 shows the TPNH reduction of aa_3 in cyanide-treated ETP. For comparison, the reduction of these cytochromes by DPNH and succinate are also shown.

The above results are consistent with the site shown in Fig. 6 for the interaction of TPNH with the electron transport system. This conclusion also agrees with EPR data concerning the interaction of TPNH with complex I. Our studies with complex I have shown that TPNH reduces iron-sulfur centers 2 and 3 + 4, but not center 1, which appears to be associated with DPNH dehydrogenase (2).

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