THE INVOLVEMENT OF PYRIDINE NUCLEOTIDE TRANSHYDROGENASE IN ATP-LINKED TPN REDUCTION BY DPNH

Takashi Kawasaki, Kenshi Satoh, and Nathan O. Kaplan

Graduate Department of Biochemistry
Brandeis University
Waltham, Massachusetts

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Animal tissue pyridine nucleotide transhydrogenase has been found to catalyze the following reversible reaction (Kaplan et al., 1953). It has been previously reported that

$$TPNH + DPN \rightleftharpoons DPNH + TPN \qquad (1)$$

the rate of reaction from left to right is somewhat faster than the rate observed in the reverse direction (Stein et al., 1959). Klingenberg and Schollmeyer (1961) and Estabrook et al. (1961) have demonstrated energy-controlled reduction of endogeneous TPN by DPNH in intact mitochondria. Danielson and Ernster (1963a,b) have reported on ATP-stimulated reduction of TPN by DPNH which is catalyzed by submitochondrial particles derived from rat liver and beef heart. These authors suggested that the energy-linked TPN reduction by DPNH was due to a different enzyme than the transhydrogenase reaction of Kaplan et al. (1953) which did not involve ATP. Hommes and Estabrook (1963) and Estabrook, Hommes and Gonze (1963) have demonstrated the ATP-dependent exogenous TPN reduction by DPNH in beef heart submitochondrial particles, although addition of magnesium inhibited the reduction of TPN. They suggested that the reaction was similar to the transhydrogenase of Kaplan et al. (1953) in sensitivity to triiodothyronine and having a similar high temperature coefficient.

Recently, a purified transhydrogenase preparation that carries out reaction (1) has been obtained from beef heart in our laboratory. This preparation contains a trace of DPNH-DPN transhydrogenase activity (Kawasaki and Satoh, 1964).* Although catalyzing the reduction of TPN by DPNH, it is not affected by the addition of ATP. It was, therefore, of interest to determine whether the purified transhydrogenase is or is not involved in the ATP-dependent TPN reduction by DPNH in the submitochondrial particles discussed by Slater (1963).

Submitochondrial particles were used as the source of the ATP-stimulated DPNH-TPN-linked reaction and were obtained by sonication of beef heart mitochondria. Substrate amounts of DPNH were used as donors in the reaction and TPN analogues as acceptors rather than the DPNH-generating system used by others to study the ATP-linked transhydrogenase reaction. The methods for following the transhydrogenase reaction with the coenzyme analogues have been described elsewhere (Stein et al., 1959; Kaufman and Kaplan, 1961).

We have found that the acetylpyridine analogue of TPN (AcPyTPN) can readily replace TPN as an acceptor in the DPNH-TPN reaction. The time course of the AcPyTPN reduction in the presence and absence of ATP is shown in Fig. 1; it can be seen that ATP stimulates the reduction of the TPN analogue severalfold. The enhancement induced by ATP is completely abolished by oligomycin, which agrees with the results reported by Danielson and Ernster (1963b) using a DPNH generating system. Addition of oligomycin to the reaction in which ATP was added produces a rate that is identical to the reaction without ATP (see Fig. 1). Oligomycin also has no effect

* A detailed description of the method of purification is now in preparation for publication by T. Kawasaki, K. Satoh and N. O. Kaplan.

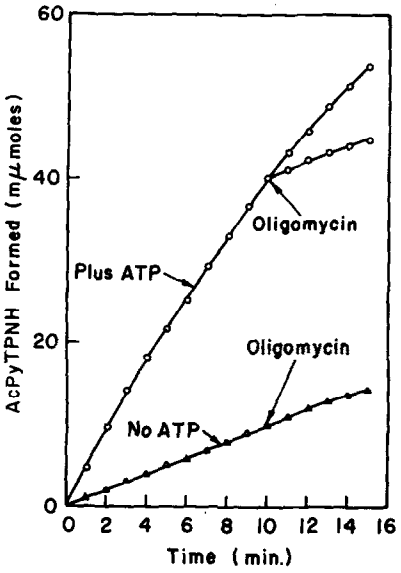

Fig. 1. Time course of and effect of oligomycin on the ATP-linked AcPyTPN reduction by DPNH. Reaction mixture of DPNH-AcPyTPN transhydrogenase assay was composed of 50 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of KCN, 5 μmoles of $MgCl_2$, 0.3 μmole of DPNH, 0.6 μmole of AcPyTPN, and 0.35 mg of beef heart submitochondrial particles in a volume of 1 ml. The reaction was started by the addition of AcPyTPN and the reduction of AcPyTPN was followed at 375 mμ. The blank cuvette was omitted by AcPyTPN. Reaction mixture for ATP-linked AcPyTPN reduction by DPNH was composed of the same component as that for the DPNH-AcPyTPN reaction except for the addition of 2 μmoles of ATP to start the reaction. The reaction was also followed at 375 mμ. At the indicated time, 2 μg of oligomycin were added. The millimolar extinction coefficient of AcPyTPNH at 375 mμ was taken as 5.1.

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on the TPNH-DPN reaction catalyzed by either the purified transhydrogenase preparation or the submitochondrial particle.

Table I shows that the ratio of the rates of reduction of thionicotinamide-TPN (TNTPN) to that of AcPyTPN is similar in the energy-linked reaction in the submitochondrial particle and the purified enzyme which is not influenced by ATP. This agreement was obtained for three different preparations of purified enzyme and submitochondrial particles. In each experiment the reactions with the purified enzyme were carried out at the same time as those with the submitochondrial particles.

TABLE I

Analogue Ratios of Rates of Reaction of DPNH-TPN Transhydrogenase and ATP-linked TPN Reduction by DPNH

The reaction mixtures were the same as described in Fig. 1, except that 0.6 μmole of TNTPN, instead of AcPyTPN, for the assay of DPNH-TNTPN reaction was used. 0.05 mg of the purified enzyme was used and 0.35 mg of the submitochondrial particles.

	$\frac{\text{DPNH-TNTPN}}{\text{DPNH-AcPyTPN}}$[a]		
	Experiment		
	1	2	3
DPNH-TPN with purified transhydrogenase	2.4	1.9	1.8
ATP-linked TPN reduction by DPNH with submitochondrial particles	2.2	2.3	1.7

[a] The TNTPN was prepared by the method of Walter and Rubin (1963) and the AcPyTPN by the method of Kaplan and Stolzenbach (1957). The millimolar extinction coefficient of TNTPNH at 400 mμ was taken as 11.0.

In order to obtain more direct evidence for the involvement of the purified transhydrogenase in the ATP-dependent TPN reduction in mitochondria, an antibody against the purified enzyme was prepared in rabbits. The antibody was obtained after an intramuscular injection of the transhydrogenase, which was followed by two intravenous injections. The serum was collected 3 weeks after the initial injection of the enzyme. Ouchtolony plates showed only one band and suggested a homogeneity of the enzyme preparation. γ-globulin prepared by ammonium sulfate fractionation was used for the studies of inhibition.

The antibody was found to inhibit to the same extent the TPNH-AcPyDPN and the DPNH-AcPyTPN reaction catalyzed by the purified enzyme. As illustrated in Table II, the submitochondrial TPNH-AcPyDPN, DPNH-AcPyTPN, and the ATP-

TABLE II

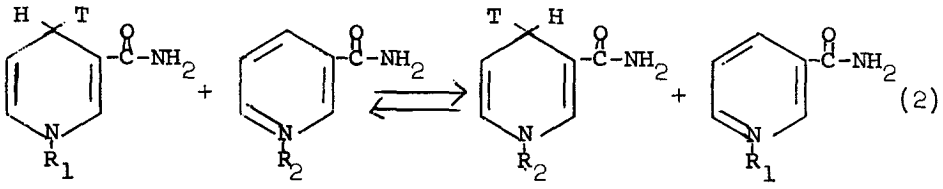
Inhibition of ATP-linked AcPyTPN Reduction by Anti-transhydrogenase γ-globulin

TPNH-AcPyDPN, DPNH-AcPyDPN transhydrogenases and DPNH-diaphorase, were determined according to the method described by Kaufman and Kaplan (1961). DPNH-AcPyTPN transhydrogenase and ATP-linked DPNH-AcPyTPN reactions were the same as those given in Fig. 1. Reaction mixture for ATP-linked DPN reduction by succinate was composed of the following: 50 µmoles of Tris-HCl, pH 7.5; 1 µmole of KCN; 5 µmoles of $MgCl_2$; 5 µmoles of succinate; 5 µmoles of DPN; 2 µmoles of ATP; and 0.32 mg of submitochondrial particles in a final volume of 1 ml. The reaction was started by the addition of ATP and followed at 340 mµ.

Reactions	Submitochondrial particles (mg)	Normal γ-globulin (mg)	Anti-transhydrogenase γ-globulin (mg)	mµmoles reduced /min.	Inhibition (%)
TPNH-AcPyDPN	0.08	0.21	-	8.0	37.5
	0.08	-	0.21	5.0	
DPNH-AcPyTPN	0.32	0.84	-	0.78	38.5
	0.32	-	0.84	0.48	
DPNH-AcPyDPN	0.08	0.21	-	23.5	1.7
	0.08	-	0.21	23.2	
DPNH-diaphorase	0.08	0.21	-	14.7	1.4
	0.08	-	0.21	14.5	
ATP-linked DPNH-AcPyTPN	0.32	0.84	-	3.8	44.7
	0.32	-	0.84	2.1	
ATP-linked DPN reduction by succinate	0.32	0.84	-	3.5	8.5
	0.32	-	0.84	3.2	

dependent DPNH-AcPyTPN reactions are all inhibited to the same degree by the anti-transhydrogenase γ-globulin. The other reactions listed in the table are not significantly inhibited by the antibody.

Recently, we have found by the use of tritiated coenzymes that the purified transhydrogenase catalyzed a stereospecific transfer between the B side of TPNH and the A side of DPNH. This is a reversible transfer, as illustrated in equation (2). Recent reports by Griffiths and by Ernster and Lee† indicate a like stereospecificity in submitochondrial particles for the ATP-stimulated reduction of TPN by DPNH.



(2)

B-TPNH DPN A-DPNH TPN

The results reported in this paper strongly support the view that the purified transhydrogenase originally described by Kaplan et al. (1953) is involved in the energy-coupled reduction of TPN by DPNH. The data also indicate that more than one enzyme is involved in the energy-coupled reaction. We have been unable to find any evidence that would indicate that the transhydrogenase is not involved in the ATP-linked reduction of TPN by DPNH. Studies are now under way to determine whether a pyridine nucleotide derivative can act as a more effective substrate with the purified transhydrogenase preparation.

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† Papers presented by D. E. Griffiths and by L. Ernster and and C. Lee, Sixth International Congress of Biochemistry, New York, 1964, Symposium 3, Intermediates in oxidative phosphorylation in animal tissues.

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