PYRIDINE NUCLEOTIDE TRANSHYDROGENASE ACTIVITY OF SOLUBLE CARDIAC NADH DEHYDROGENASE AND PARTICULATE NADH-UBIQUINONE REDUCTASE

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SUMMARY--Pyridine nucleotide transhydrogenase activities of a highly purified soluble NADH dehydrogenase and particulate NADH-ubiquinone reductase (Complex I) differ in their pH optima (5.0 and 6.0, respectively) and in their sensitivity to inhibition by Mg^{2+} and ATP. The oxidation of NADPH with ferricyanide as acceptor is very similar in both preparations with a pH optimum around 5.0. It is concluded that Complex I possesses two types of transhydrogenase activity, whereas only one has been found in the soluble dehydrogenase.

Recently, the subject of NADPH oxidation and pyridine nucleotide transhydrogenase in mitochondrial preparations has come under vigorous investigation, after nearly 20 years moderate progress but with little controversy. Hatefi and Hanstein (1, 2) have reported that "Complex I" (NADH-ubiquinone reductase) catalyzes the oxidation of NADPH by ferricyanide and that it also shows considerable pyridine nucleotide transhydrogenase activity. From visible and EPR spectroscopy they concluded that NADPH interacted with EPR Center 2 of NADH dehydrogenase (3) and proposed that this site was involved in the transhydrogenase reaction. Their conclusions are at variance with those previously reported by Ernster *et al.* (4, 5) and have been challenged by other investigators (*e.g.* 6, 7). In this report we compare the interactions of NADPH with Complex I and with a soluble NADH dehydrogenase isolated in this laboratory (8).

MATERIALS AND METHODS--Preparations of Complex I (1, 9) and soluble NADH dehydrogenase (8) were obtained and specific activities of NADH-ferricyanide reductase at Vmax for ferricyanide (1, 8) were assayed as previously

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described. Pyridine nucleotides were purchased from Sigma. Protein was estimated by the method of Lowry et al. (10). Pyridine nucleotide transhydrogenase activity with APAD[1] as acceptor was measured spectrophotometrically at 363 nm according to Kaplan (11) as adapted by Hatefi and Hanstein (2). Variations in assay procedures are indicated in the respective figure and table legends.

RESULTS AND DISCUSSION--Figure 1a shows the pH dependence of NADH and NADPH oxidation with ferricyanide as acceptor catalyzed by the soluble NADH dehydrogenase. In agreement with previous findings, NADH oxidation showed a broad pH optimum centered around pH 8 (8), while the optimum for NADPH oxidation was about pH 5 (12). At the respective pH optima, the rate of NADPH oxidation was still only 2.8% of the rate of NADH oxidation; this fact may account for previous failures to observe the oxidation of NADPH by ferricyanide (4), especially at pH 7.5. Figure 1b shows the pH dependence with APAD as acceptor. Both activities were considerably lower than the corresponding activities with ferricyanide as acceptor, but the pH profiles were the same regardless of the nature of the electron acceptor, i.e. optimum at pH 5 for NADPH oxidation and about pH 8 for NADH oxidation.

The NADH- and NADPH-ferricyanide activities of Complex I are shown in Fig. 1c. The NADH-ferricyanide activity was approximately one-third of that of the soluble dehydrogenase, while the NADPH dehydrogenase activity was approximately the same. Figure 1d shows the activities of Complex I with APAD as acceptor. The NADH-APAD activity is lower than that of the soluble dehydrogenase. The pH optimum is at pH 8, the same optimum found for NADH-APAD activity of the soluble enzyme or NADH-ferricyanide activity of either preparation. However, the pH profile of NADPH-APAD activity is entirely different. The optimum was between pH 6.0 and 6.5, at which pH the soluble enzyme has an activity only one-tenth of the maximal activity. Unlike the

[1] Abbreviations: APAD, acetylpyridineadeninedinucleotide; DCIP, 2,6-dichlorophenolindophenol.

soluble enzyme, the pH profile of NADPH oxidation with APAD as acceptor by Complex I was distinctly different from that with ferricyanide as acceptor.

These facts suggest that Complex I may have two types of transhydrogenase activity. One of these resembles that of the soluble dehydrogenase, has a pH optimum at 5.0 and appears to be associated with an NADPH-ferricyanide activity of similar pH dependence. The other transhydrogenase activity, found only in Complex I, has a pH optimum at 6.0 to 6.5 and does not appear to catalyze NADPH oxidation by ferricyanide. These properties

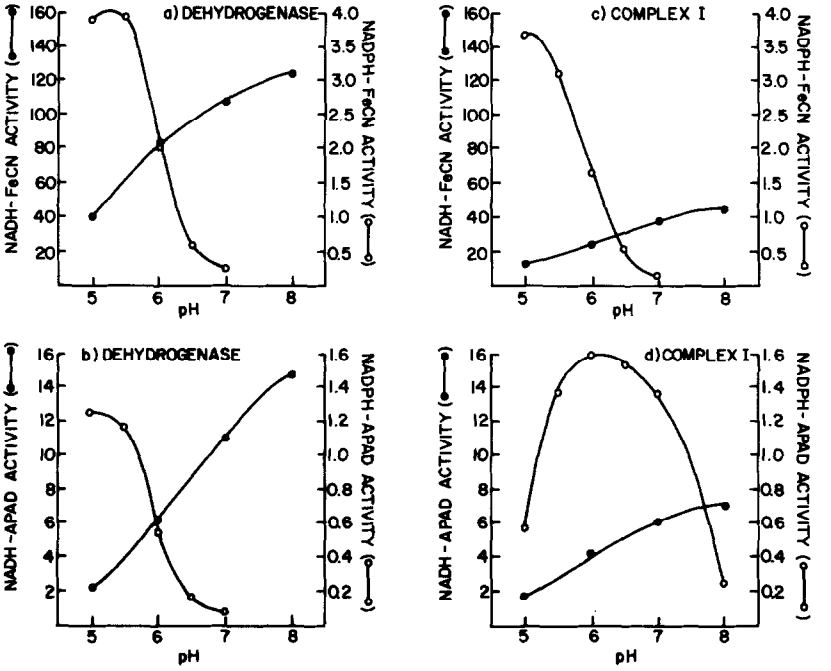

Fig. 1. Effect of pH on NADH- and NADPH-acceptor activities of Complex I and soluble NADH dehydrogenase. NADH-ferricyanide (NADH-FeCN) activity was measured at 420 nm with 0.125 mM NADH, 1 mM $K_3Fe(CN)_6$ and 5 µg of enzyme protein per ml. NADPH-ferricyanide (NADPH-FeCN) activity was measured at 420 nm with 0.5 mM NADPH, 1 mM $K_3Fe(CN)_6$ and 25 µg of enzyme protein per ml. NADH-APAD transhydrogenase activity was measured at 363 nm with 0.5 mM NADH, 1.0 mM APAD and 5 µg of enzyme protein per ml. NADPH-APAD transhydrogenase activity was measured at 363 nm with 0.5 mM NADPH, 1.0 mM APAD and 25 µg of enzyme protein per ml. The media were 0.1 M K-acetate (pH 5.0 and 5.5) or 0.1 M potassium phosphate (pH 6.0, 6.5, 7.0 and 8.0). Temperature was 38°. Appropriate corrections were made for small non-enzymic activities. Specific activities of the soluble NADH dehydrogenase and Complex I in the NADH-ferricyanide assay were 833 and 340 µmoles NADH/min/mg of protein, respectively, in terms of V_{max} for ferricyanide at 30°.

are reminiscent of the mitochondrial pyridine nucleotide transhydrogenase isolated by Kaplan (11), which has been partially purified by means of digitonin. This transhydrogenase has a pH optimum of 6.0. It may be significant to note that different detergents were used for the purification of the transhydrogenase (digitonin), the soluble NADH dehydrogenase (Triton X-100 and cholate), and Complex I (bile salts alone). It may be mentioned that the transhydrogenase activity of Complex I, shown in Fig. 1d, is considerably greater than that reported by Hatefi and Hanstein (2). In part, this is probably due to removal of inhibitory bile salts during the preparation; however, we also find considerable variation in the activity from preparation to preparation of Complex I, occasionally to as low as 0.7 μmoles/min/mg of protein.

In view of the similarity between the transhydrogenase activity of Complex I and that found in submitochondrial particles, some inhibitors of the mitochondrial transhydrogenase were tested on both Complex I and the soluble NADH dehydrogenase. The results are summarized in Table I. NADPH-ferricyanide activity was assayed at pH 5.5 with both enzymes and was found to be relatively little affected by the presence of 10 mM $MgSO_4$ or 1 mM ATP. Transhydrogenase activity with APAD as acceptor was assayed at pH 5.5 with the soluble enzyme, and at pH 7.5 with Complex I to give good separation of the two types of reaction. In the soluble dehydrogenase, little inhibition by Mg^{2+} or ATP was observed, while the transhydrogenase activity of Complex I was inhibited 52% by either Mg^{2+} or ATP. Similarly, NADPH-DCIP activity of the soluble dehydrogenase, assayed at pH 6.0, was substantially unaffected by Mg^{2+} or ATP, while in Complex I, assayed at pH 7.5, the activity was about 60% inhibited. Previous reports that NADPH-DCIP activity is a partial reaction of the mitochondrial transhydrogenase (4, 6) would thus appear to be correct only at high pH, since considerable activity was found with the soluble enzyme at low pH. The data of Table I, therefore, support the view that there may be two pathways for NADPH dehydrogenation in Complex I, only one of which appears in the purified soluble dehydrogenase.

TABLE I. NADPH Oxidation Catalyzed by Soluble NADH Dehydrogenase and by Particulate NADH-Ubiquinone Reductase ("Complex I").

		NADPH oxidation with indicated acceptor					
		$K_3Fe(CN)_6$		APAD		DCIP	
	Addition	Rate	Inhibition	Rate	Inhibition	Rate	Inhibition
		μmoles/min/mg	%	μmoles/min/mg	%	μmoles/min/mg	%
Soluble NADH dehydrogenase	None	2.70	-	1.00	-	1.05	-
	$MgSO_4$, 10 mM	2.52	7	0.98	2	0.99	6
	ATP, 1 mM	2.45	9	0.82	18	0.95	10
Complex I	None	3.04	-	0.45	-	0.59	-
	$MgSO_4$, 10 mM	2.53	16	0.21	52	0.21	64
	ATP, 1 mM	2.48	18	0.21	52	0.24	59

Activities of the soluble dehydrogenase were assayed as follows: NADPH-$K_3Fe(CN)_6$ and NADPH-APAD activities of NADH dehydrogenase were assayed under the same conditions as described in the Legend of Fig. 1 at pH 5.5; NADPH-DCIP activity was determined spectrophotometrically at 600 nm in 1 ml final volume containing 0.1 μmole of K-acetate pH 6.0, 0.5 μmole of NADPH, 50 nmoles of DCIP and 25 μg of enzyme protein at 38°. NADPH-$K_3Fe(CN)_6$ and NADPH-APAD activities of Complex I were assayed as in Fig. 1 at pH 5.5 and pH 7.5, respectively. NADPH-DCIP activity of Complex I was determined as that of the dehydrogenase but at pH 7.5.

The results presented here clarify the situation regarding NADPH oxidation by Complex I and submitochondrial particles. Further evidence of two pathways of NADPH dehydrogenation might be obtained through consideration of the stereospecificity of the reactions involved, since the mitochondrial transhydrogenase involves the 4A-hydrogen atom of NADH (13, 14) while the respiratory chain NADH dehydrogenase for both Type I and Type II (see Ref. 15 for the terminology) is 4B specific for all preparations so far examined (14). Likewise, the oxidation of NADH by oxygen or ferricyanide in the presence of submitochondrial particles is also 4B specific (14). It is true that no direct experiment has yet been made on the stereospecificity of the soluble NADH dehydrognease described. Definitive results (see for example 16, 17) have demonstrated beyond a reasonable doubt that the various NADH dehydrogenase preparations are due to cleavage of the soluble enzymes at different sites in the NADH dehydrogenase-containing segment of the mitochondrial inner membrane by different solubilizing agents under varying conditions (as depicted in Fig. 1 of Ref. 17).

In view of all these considerations, such as the reactivity, inhibition behavior, pH optima and stereospecificity (by deduction), we think that the transhydrogenase activity, and the activities of NADH and NADPH oxidation are from one soluble enzyme--the respiratory chain-linked NADH dehydrogenase.

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