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NADH oxidation by hypoxanthine dehydrogenase of avian kidney

Avian tissue, unlike mammalian tissue, catalyzes the oxidation of hypoxanthine and xanthine by an NAD specific metalloflavoprotein dehydrogenase[1-4]. In addition to NAD^+, the avian system also utilizes artificial electron acceptors such as methylene blue and hydroxylamine[1,2]. In mammalian tissue, xanthine oxidase (EC 1.2.3.2) catalyzes the oxidation of NADH as well as purine substrates[5,6]. NADH oxidation has not been characterized for the avian enzyme system.

The present study employs a highly purified enzyme preparation from the chicken kidney and examines the interaction of this enzyme with NADH. Preparation of the enzyme has been described previously[2]. Purification resulted in a several hundred fold enhancement of specific activity. The enzyme activity was assayed spectrophotometrically with a Beckman DU spectrophotometer. Oxidation of hypoxanthine was measured by the reduction of NAD^+ at 340 mμ or the formation of uric acid at 290 mμ. Oxidation of NADH was measured at 340 mμ. The reduction of acetylpyridine NAD^+ was measured at 365 and 400 mμ (ref. 7). NADH oxidation was also measured with an Aminco Bowman spectrofluorophotometer. v_{max} and K_m values were determined by plotting data according to the method of LINEWEAVER AND BURK[8]. K_i values were determined by methods described by WILSON[9].

The chicken kidney enzyme catalyzes the oxidation of NADH in the presence of methylene blue, menadione, hydroxylamine and acetylpyridine NAD^+. These acceptors are also active with hypoxanthine as substrate. NADPH oxidation does not occur. The following acceptors are not active in this preparation with either NADH or hypoxanthine: cytochrome *c*, ferricyanide and 2,6-dichlorophenolindophenol.

NADH oxidation rates with various acceptors were determined and the v_{max} for each acceptor calculated. Table I presents the relative rate of NADH oxidation with these acceptors. The rate of hypoxanthine oxidation with these acceptors is presented for comparison. Oxidation of NADH proceeds at twice the rate of hypoxanthine oxidation. The apparent K_m of these acceptors when calculated for the

TABLE I

RELATIVE v_{max} AND APPARENT K_m VALUES FOR CHICKEN KIDNEY HYPOXANTHINE DEHYDROGENASE

For comparison the value of v_{max} in the reaction between hypoxanthine and NAD^+ is designated as 1. The apparent K_m for NADH as substrate is $1.4 \cdot 10^{-5}$ M. Incubations were carried out for 5 min at 22° in 3 ml of 0.1 M potassium phosphate buffer (pH 7.4) with 1.5 units of enzyme. One unit of enzyme activity is defined as the production of 0.01 μmole of NADH per min at 22° with hypoxanthine as substrate and NAD^+ as acceptor[2].

Acceptor	*NADH substrate*		*Hypoxanthine substrate*	
	v_{max}	K_m *of acceptor* (*M*)	v_{max}	K_m *of acceptor* (*M*)
NAD^+			1.0	$5.5 \cdot 10^{-5}$
Acetylpyridine NAD^+	2.0	$5.0 \cdot 10^{-5}$	1.0	$5.5 \cdot 10^{-5}$
Menadione	3.1	$1.0 \cdot 10^{-4}$	1.5	$1.1 \cdot 10^{-4}$
Hydroxylamine	0.63	$8.7 \cdot 10^{-3}$	0.25	$8.7 \cdot 10^{-3}$

oxidation of NADH or hypoxanthine are approximately the same with both substrates. These measurements indicate that common acceptor sites are likely for the oxidation of hypoxanthine and NADH. The apparent K_m of NAD^+ as acceptor ($5.5 \cdot 10^{-5}$ M), NADH as substrate ($1.4 \cdot 10^{-5}$ M) and acetylpyridine NAD^+ as acceptor ($5 \cdot 10^{-5}$ M) are fairly close. NADH inhibits hypoxanthine oxidation when NAD^+ serves as acceptor[2]. The inhibition by NADH was examined in this study and was found competitive with NAD^+. The K_i for this NADH inhibition is $2.0 \cdot 10^{-5}$ M. NAD^+ is also a competitive inhibitor when NADH is the substrate. These data imply that NAD^+ as acceptor and NADH as substrate may share one or more enzyme sites in common.

The effect of *p*-chloromercuribenzoate, cyanide and arsenite on NADH oxidation with menadione as acceptor is shown in Table II. Cyanide is presumed to complex enzyme iron and *p*-chloromercuribenzoate and arsenite are presumed to complex enzyme sulfhydryl groups. Only 50 % of the NADH oxidation is blocked by these inhibitors. With menadione as acceptor, hypoxanthine oxidation is completely inhibited. NADH oxidation with acetylpyridine NAD^+ as acceptor is completely insensitive to these inhibitors.

TABLE II

INHIBITORS OF HYPOXANTHINE DEHYDROGENASE

Incubations were carried out for 15 min at 22° in 3 ml 0.1 M potassium phosphate buffer (pH 7.4) with $2.3 \cdot 10^{-4}$ M menadione as acceptor. 1.0 unit of enzyme was employed in each assay.

Inhibitor	*Concentration (M)*	*Substrate*	
		NADH % Inhibition	*Hypoxanthine % Inhibition*
p-Chloromercuribenzoate	$3.0 \cdot 10^{-7}$	25	25
	$4.5 \cdot 10^{-7}$	48	52
	$7.5 \cdot 10^{-7}$	48	91
Cyanide	$7.5 \cdot 10^{-4}$	26	26
	$3.0 \cdot 10^{-3}$	46	71
	$4.5 \cdot 10^{-3}$	50	85
Arsenite	$6.0 \cdot 10^{-5}$	25	45
	$1.5 \cdot 10^{-4}$	45	70
	$3.0 \cdot 10^{-4}$	48	95

In the absence of acceptor, NADH oxidation by the enzyme cannot be detected spectrophotometrically. A slow aerobic oxidation can be readily detected with a fluorimeter and is illustrated in Fig. 1. The rate of NADH oxidation is considerably less than 1 % of the rate in the presence of menadione or acetylpyridine NAD^+. The decline of fluorescence is specific for NADH and NADPH will not react. The apparent K_m of this reaction is $1.5 \cdot 10^{-5}$ M in agreement with the other data presented. The decline in fluorescence is readily reversed by hypoxanthine which regenerates the NADH. The oxidation is not sensitive to the three inhibitors tested in this study. When NADH is first added, an initial rapid quenching of NADH fluorescence is seen. Several enzymes quench NADH fluorescence on binding the coenzyme[10]. If the initial quenching of NADH fluorescence represents binding, then it appears that 3 moles of NADH are bound per mole of enzyme flavin.

In summary, the present study shows that NADH oxidation, an enzymatic activity found in mammalian xanthine oxidase, is also a property of avian kidney hypoxanthine dehydrogenase when suitable acceptors are present. Two pathways of NADH oxidation are to be found in the purified enzyme. One pathway involves iron and sulfhydryl groups common to the purine oxidation pathway. An additional pathway apparently does not involve these inhibitor-sensitive groups. This second pathway may partly account for the fact that NADH oxidation proceeds at a rate twice that of purine oxidation. The existence of two almost identical enzymes for NADH oxidation in this highly purified preparation seems a less likely interpretation of the data.

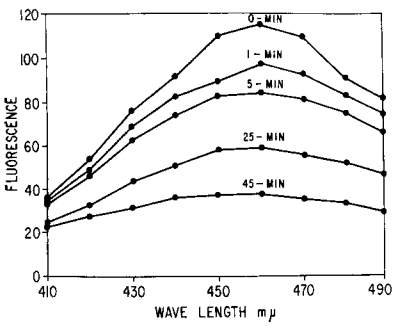

Fig. 1. The quenching of NADH fluorescence by hypoxanthine dehydrogenase. The cuvette contained 3 ml of 0.04 M potassium phosphate buffer (pH 7.4), NADH, 10^{-6} M and four units of enzyme. Activation wavelength was 360 mμ. Temperature was 22°.

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Department of Pharmacology,
Vanderbilt University, School of Medicine,
Nashville, Tenn. (U.S.A.)

ERWIN J. LANDON
MARION MYLES

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