ENZYMATIC OXIDATION OF SOME NON-PHOSPHORYLATED DERIVATIVES OF DIHYDRONICOTINAMIDE

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This paper concerns the partial purification and properties of an enzyme that catalyzes the oxidation of reduced ribosyl nicotinamide (NRH) by vitamin K_3 (menadione) and certain other quinones. The enzyme is widely distributed in mammalian tissues. It is localized in the soluble portion of the cytoplasm. In the rat, the greatest activity of the enzyme was found in kidney, liver, heart and seminal vesicle. The purified enzyme also catalyzes the oxidation of certain N^1-alkyl-dihydronicotinamides, but it is completely inert toward phosphorylated derivatives of dihydronicotinamide such as the reduced forms of nicotinamide mononucleotide (NMNH), and of di- and triphosphopyridine nucleotides (DPNH and TPNH).

Purification. Unless stated otherwise, all operations were carried out between 0° and 2°. Rat kidney (127 g) was homogenized with 9 volumes of 0.25 M sucrose-0.003 M $NaHCO_3$ in a Waring blender. Nuclei, cell debris and cytoplasmic particles were removed by centrifugation at 600 x g for 10 minutes, 10,000 x g for 20 minutes and 59,000 x g for 60 minutes respectively. The final supernatant fluid was fractionated by the addition of solid ammonium sulfate. The fraction precipitating between 50 and 60 per cent saturation was collected and dissolved in 50 ml of 0.01 M tris(hydroxymethyl) aminomethane (Tris) buffer of pH 7.4. To 50 ml of this enzyme solution

was added 25 ml of 0.5 M NaH_2PO_4, and the mixture incubated for 20 minutes in a water bath at 40°. At the end of the incubation, 25 ml of 0.5 M Na_2HPO_4 was added, and the precipitate removed by centrifugation. This heat treatment virtually abolished the activity of enzyme(s) in the preparation which catalyzed the oxidation of TPNH and DPNH by menadione. To the heat treated enzyme was added 0.5 volume of a 2 per cent aqueous solution of protamine sulfate (prepared at room temperature). After standing for 10 minutes at 25°, the mixture was centrifuged and the supernatant fluid fractionated by the addition of solid ammonium sulfate. The precipitate which formed between 40 and 60 per cent saturation was collected, dissolved in 0.01 M Tris buffer of pH 7.4, and dialyzed extensively against redistilled, deionized water. To each 1 ml of the dialyzed enzyme (1.2 mg of protein per ml) was added 5.0 ml of calcium phosphate gel (24 mg dry weight per ml, prepared by the method of Tsuboi and Hudson, 1957) and the mixture was allowed to stand for 10 minutes at 2°. All of the enzyme was adsorbed on the gel under these conditions. It was eluted by 4 ml of 0.05 M sodium phosphate buffer of pH 6.8. One mg of the enzyme prepared in this manner catalyzed the menadione-dependent oxidation of 15 μmoles of N^1-(n-propyl)dihydronicotinamide per minute at pH 8.5 and 25°. The ratio of the rate of oxidation of this dihydropyridine to that of NRH remained constant at each stage of the purification procedure.

Cofactor requirement. The oxidation of NRH by menadione at pH 8.5 was unaffected by the ethylene diamine tetra-acetic acid, and Ca^{++}, Mn^{++}, Cu^{++} and Zn^{++} ions at a final concentration of 3×10^{-4} M. If the enzyme was treated with acid in ammonium sulfate by the method of Warburg and Christian (1938), the rate of oxidation of either NRH or N^1-(n-propyl) dihydronicotinamide by menadione was diminished by more than 80 per cent.

The enzyme treated in this manner was reactivated by the addition of either FAD or FMN (3×10^{-7} M), but not by riboflavine. The activity of the untreated enzyme was not increased by FMN or FAD.

Specificity toward hydrogen or electron donors and acceptors. The purified enzyme does not catalyze the oxidation of TPNH, DPNH and NMNH by menadione or p-benzoquinone at pH 6.5 or 8.5. The following enzyme activities could not be detected in the purified enzyme: TPNH and DPNH diaphorase (methylene blue, 2,6-dichlorophenol-indophenol or ferricyanide as electron acceptors), DPNH or TPNH cytochrome c reductase, xanthine oxidase, D-amino acid oxidase and glycolic oxidase. The NRH-oxidizing enzyme can therefore be distinguished from other soluble proteins that catalyze the oxidation of DPNH and/or TPNH by quinones or dyes (Wosilait and Nason, 1954 a,b; Wosilait, 1960; Märki and Martius, 1960; Frimmer, 1960; Giuditta and Strecker, 1960; Ernster, Ljunggren and Danielson, 1960). The enzyme catalyzes the oxidation of NRH by menadione more efficiently at mildly alkaline pH (7.4-8.5), whereas when phthiocol is used as acceptor, maximal rates of oxidation occur at slight acid pH (6.1-6.5). Among the acceptors which are inert for the oxidation of NRH are: 1,4-naphthoquinone, 1,2-naphthoquinone, 9,10-phenanthroquinone, 1,4-benzoquinone, methylene blue, 2,6-dichlorophenol-indophenol, ferricyanide, oxygen, the 3-acetylpyridine analogue of DPN, lipoic acid, vitamin K_1, coenzyme Q_{10} and α-tocopherol. The latter three substances were tested as suspensions in bovine serum albumin, and corrections were made for non-enzymatic reactions when necessary. The enzyme does not catalyze the reduction of cytochrome c by NRH unless catalytic amounts of menadione are added. In the latter situation, dihydromenadione(formed by the enzymatic oxidation of NRH) reduces the cytochrome c non-enzymatically (cf. Mahler, Fairhurst

and Mackler, 1955). The NRH-oxidizing enzyme catalyzes the oxidation of a number of N^1-alkyl-dihydronicotinamides at pH 8.5, where the alkyl group is methyl, ethyl, n-propyl, n-butyl, n-pentyl or n-hexyl. The n-propyl derivative is oxidized at the greatest rate. The relative activity of various acceptors for the oxidation of this donor are summarized in Table I.

TABLE I

Acceptors for the Enzymatic Oxidation of N^1-(n-propyl)dihydronicotinamide

Acceptor	Relative rate of dihydropyridine oxidation at: pH 6.5	pH 7.4	pH 8.5
Menadione	128	100	100
Phthiocol	100	12	0
1,4-naphthoquinone	32	-	39
Vitamin $K_{2(5)}$	42	-	41
Vitamin $K_{2(10)}$	6	4	3
Coenzyme Q_2	42	-	3

The reactions were carried out in cells of 1 cm light path in a final volume of 3.0 ml containing: 250 μmoles Tris buffer pH 8.5 or 100 μmoles of sodium phosphate pH 6.5 (both buffers were tested at pH 7.4); 0.35 μmoles of N-propyl-dihydronicotinamide; 0.2 μmoles of the quinone tested in 0.02 ml ethanol and suitable amounts of purified enzyme. The oxidation of the dihydropyridine was followed at 360 mμ. Corrections were made for any non-enzymatic oxidation of the dihydropyridine by the quinones.

The following substances did not function as acceptors for the enzymatic oxidation of N-propyl-dihydronicotinamide: 1,4-benzoquinone, α-tocopherol, oxygen, the 3-acetylpyridine analogue of DPN and vitamin K_1

The Michaelis constant (K_M) for NRH is 2.2×10^{-5} M (menadione as acceptor at pH 8.5) and 3.2×10^{-5} M (phthiocol as acceptor at pH 6.1). The K_M for N^1-(n-propyl)dihydronicotinamide is 4.3×10^{-5} M (menadione

as acceptor at pH 8.5). When NRH is used as the donor, the K_M for menadione (pH 8.5) and phthiocol (pH 6.1) are 1.4×10^{-5} M and 1.6×10^{-5} M respectively.

Inhibitors. The oxidation of NRH by menadione is inhibited by atabrine (80 per cent) and chlorpromazine (40 per cent) at final concentrations of 2×10^{-5} M. In marked contrast to some other enzymes catalyzing the oxidation of DPNH by menadione (Märki and Martius, 1960; Ernster, Ljunggren and Danielson, 1960), the NRH-oxidizing enzyme is not inhibited by dicumarol in concentrations as high as 10^{-5} M. Certain phenolic estrogens (equilin, estradiol-17β, diethylstilbestrol) markedly inhibit the enzymatic oxidation of NRH at levels of 10^{-5} M. Inhibitory activity could not be correlated with estrogenic activity. By far the most potent inhibitors of the NRH-oxidizing enzyme are a group of polycyclic aromatic hydrocarbons (Liao and Williams-Ashman, 1961).

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