THE HYDROXYLATION OF NICOTINE: THE ORIGIN OF THE HYDROXYL OXYGEN

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The bacterial degradation of nicotine by Arthrobacter oxydans, strain P34, has been shown to proceed via 6-hydroxynicotine (6-HN). Indirect evidence suggested that the process was initiated by a hydration of the pyridine moiety of nicotine to yield a pseudo-base which in turn was oxidized to 6-HN (Hochstein and Rittenberg, 1959b). Direct verification of this pathway had to await purification of the enzyme in order to separate the reaction sequence responsible for the formation of 6-HN from an enzyme responsible for the oxidation of 6-HN to 6-hydroxypseudooxynicotine (Hochstein and Rittenberg, 1960). This paper reports on the purification of the enzyme responsible for the formation of 6-HN and presents direct evidence concerning the origin of the hydroxyl-oxygen of 6-HN. Recently, Decker and Bleeg (1965) have reported the purification of an enzyme, isolated from A. oxydans, which oxidized nicotine to 6-HN and have designated this enzyme nicotine oxidase.

Cells were grown as previously described, except that they were harvested after 12 hours of growth (Hochstein and Rittenberg, 1959a). A 0.15% cell suspension in 0.001 M sodium pyrophosphate (pH 7) - 0.001 M EDTA buffer (PPE buffer) was disrupted in a Branson Sonifier for 30 minutes while maintaining the cell suspension at a temperature of from 2 to 5°C. The suspension was centrifuged for 60 minutes at 20,000 x g at 4°C and the clear yellow supernatent (the crude extract) was fractionated with ammonium sulfate in order to obtain that fraction precipitating between 21 and 50 per cent saturation with respect to ammonium sulfate. The precipitate was recovered by centrifugation at 20,000 x g for 15 minutes at 4°C, dissolved in PPE buffer (pH 7),

and designated as fraction A_1. Fraction A_1 was heated in a 70°C water bath and when the temperature of the extract reached 48°C it was placed in a 50°C water bath and maintained at 50°C for 15 minutes. The heated fraction was sedimented by centrifugation for 10 minutes at 10,000 x g at 4°C to yield a greyish blue supernatant fraction (Fraction H). Fraction H was chromatographed on a DEAE cellulose column (1.25 x 16 cm) which had previously been equilibrated with 0.02 M potassium phosphate buffer (pH 7) - 0.001 M EDTA. Following adsorption of fraction H, the column was washed with 0.02 M potassium phosphate buffer (pH 7) - 0.2 M NaCl - 0.001 M EDTA until a major yellow band had been eluted and a blue band had moved approximately half-way down the column. The oxidase activity was then eluted by initiating a gradient consisting of 200 ml 0.02 M potassium phosphate buffer (pH 6.5) - 0.3 M NaCl - 0.001 M EDTA in the mixing chamber and 200 ml 1.0 M potassium phosphate buffer (pH 6.5) - 0.3 M NaCl - 0.001 M EDTA in the reservoir. Six ml fractions were collected, assayed for oxidase activity and protein (Lowry, et al., 1951) and those fractions exhibiting the highest specific activity were combined. The combined DEAE fractions were made 60 per cent saturated with respect to ammonium sulfate and the resulting precipitate sedimented by centrifugation for 30 minutes at 20,000 x g at 4°C. The yellow precipitate was dissolved in 0.001 M sodium pyrophosphate buffer (pH 7) - 0.001 M EDTA - 0.001 M GSH to yield the oxidase fraction (Fraction A_2).

As summarized in Table I, the A_2 fraction was approximately 50-fold purified as compared to the crude extract and represented a 14 per cent recovery of the initial units present in the crude extract. The ratio of oxidase to oxygenase was 460 and represented approximately a 370-fold enrichment of oxidase activity. The A_2 fraction was stable for at least 3 days at 4°C. It was sensitive to freezing and thawing, losing approximately 50 per cent of its initial activity following a single freeze-thawing. However, it could be stored frozen for approximately 2 weeks before losing greater amounts of

TABLE I
PURIFICATION AND RESOLUTION OF OXIDASE ACTIVITY

Fraction	Total Volume	Total Units[a] Oxidase[b]	Total Units[a] Oxygenase[c]	Oxidase / Oxygenase	% Recovery of Units	Total Protein	Specific Activity	Purification
	ml	units	units			mg/ml	units/mg	
Crude	30	4440	3540	1.25	-	462	9.6	-
A_1	30	4260	2550	1.67	96	409	10.4	1.1
H	23	2620	1660	1.58	59	65.1	40.2	4.2
A_2	13	600	1.3	460	14	1.24	485	51

a. A unit of nicotine oxidase is that amount of enzyme catalyzing the disappearance of 1 μmole of nicotine in 30 minutes. A unit of oxygenase, the enzyme catalyzing the oxidation of 6-HN to 6-hydroxypseudooxynicotine (Decker and Bleeg, 1965), is that amount of enzyme catalyzing the formation of 1 μmole of 6-hydroxypseudooxynicotine in 30 minutes.

b. Nicotine oxidase activity was determined in reaction mixtures containing approximately 2.5 units of enzyme; 10 μmoles nicotine (adjusted to pH 7.9); 50 μmoles sodium pyrophosphate buffer, pH 7.9; 100 μg catalase; and water to a final volume of 1.0 ml. The reaction was initiated by the addition of enzyme and the flasks were shaken at 30°C in an Eberbach water bath. The reactions were terminated by the addition of 9 ml of 5% TCA and appropriate aliquots were assayed by a modification of the cyanogen bromide reaction of McCormick and Smith (1946).

c. 6-hydroxypseudooxynicotine oxygenase activity was determined in reaction mixtures identical to those used in the nicotine oxidase assay except that nicotine was replaced with 10 μmoles of (l)-6-hydroxynicotine. The reactions were terminated by the addition of 9 ml of 0.1 M HCl. Appropriate aliquots were diluted with 1 M glycine buffer, pH 10, and the absorbance at 328 mμ was related to concentration by using 22×10^{-3} for the molar absorptivity of 6-hydroxypseudooxynicotine (Hochstein and Rittenberg, 1960).

activity upon initial thawing. Details concerning the properties of the purified oxidase fraction will be described in a subsequent publication.

TABLE II

ORIGIN OF THE HYDROXYL-OXYGEN OF 6-HYDROXYNICOTINE

Experiment No.	Medium	Atoms % Excess in Medium	Substrate	Atoms % Excess in 6-HN[a]
1	$H_2{}^{18}O$	10.7	Nicotine	0.560
2	$^{18}O_2$	92.5	Nicotine	0.0006
3[b]	$H_2{}^{18}O$	10.7	6-HN	0.005

a. 6-HN was isolated from reaction mixtures by chromatography on Dowex-50 using 0.8 M KCl as the eluting agent and submitted for analysis to the Analytica Corporation of New York as the silicotungstic acid salt. The data has not been corrected for dilution by silicotungstic acid.

b. No enzyme present in Experiment No. 3.

As indicated in Table II, incubation of the A_2 fraction and nicotine in water enriched with 10.7 atoms per cent excess ^{18}O resulted in the incorporation of ^{18}O into the hydroxyl oxygen of 6-HN (Expt. 1). On the other hand, incubation of nicotine and the A_2 fraction in an atmosphere enriched with 92.5 atoms per cent excess ^{18}O resulted in no significant incorporation of ^{18}O (Expt. 2). Nor was any incorporation observed when 6-HN was incubated in water containing 10.7 atoms per cent excess ^{18}O (Expt. 3). The data, as presented in Table II, are not corrected for dilution of the oxygen in 6-HN by silicotungstic acid. These data are consistent with the proposed hydration-oxidation mechanism for the formation of 6-HN thus indicating that the so-called hydroxylation of nicotine, like that of nicotinic acid (Hunt, et al., 1957) does not involve an oxygenase-like enzyme. Furthermore, the data

implies either the existence of at least 2 enzymes, one responsible for the hydration of the pyridine moiety of nicotine and the other the oxidation of the resulting pseudobase, or that a single enzyme possesses both of these activities. To date no evidence is available permitting a choice between these possibilities.

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