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Oxidation of Nicotinic Acid by a *Bacillus* Species: Source of Oxygen Atoms for the Hydroxylation of Nicotinic Acid and 6-Hydroxynicotinic Acid

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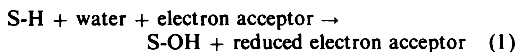
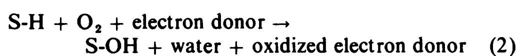
Three types of evidence are presented to show that the enzymes that hydroxylate nicotinic acid to 2,6-dihydroxynicotinic acid use water as a source of oxygen atoms. ^{18}O is incorporated into the products from $H_2{}^{18}O$. Molecular oxygen acts as a terminal electron acceptor, one-half molecule being consumed per molecule of hydroxyl groups incorporated. An external electron acceptor is required for activity in purified preparations.

The hydroxylation of aromatic ring compounds that invariably accompanies their utilization by microorganisms may occur by three kinds of reactions. Two of these reactions use molecular oxygen as the source of oxygen for the hydroxyl groups, whereas the other uses water. Hydroxylases catalyze the incorporation of oxygen from water into their substrates (reaction 1). Monoxygenases incorporate one atom of molecular oxygen (reaction 2), and dioxygenases incorporate both atoms from molecular oxygen into the substrate (reaction 3).



$$\text{S-H} + \text{water} + \text{electron acceptor} \rightarrow \text{S-OH} + \text{reduced electron acceptor} \quad (1)$$



$$\text{S-H} + O_2 + \text{electron donor} \rightarrow \text{S-OH} + \text{water} + \text{oxidized electron donor} \quad (2)$$

$$S + O_2 \rightarrow SO_2 \quad (3)$$

The three reactions are characterized by the source of oxygen for the hydroxyl groups, by the amount of oxygen consumed, and by whether there is a requirement for either an electron donor or acceptor, or neither.

Oxygenases have been studied extensively and are prominent in the oxidation of benzene ring compounds (2). Hydroxylases, on the other hand, are rarely found in nature. Those that have been reported all catalyze the initial reactions in the bacterial degradation of pyridine ring compounds. The hydroxylation of nicotinic acid (NA) by *Pseudomonas fluorescens* (6), by an *Arthrobacter* species (S. L. Kingan, Ph.D. thesis, Univ. of Wisconsin, Madison, 1969) and by a *Clostridium* species (5), of picolinic acid by an *Arthrobacter* species (R. L. Tate and J. C. Ensign, Bacteriol. Proc., p. 153, 1970), and of nicotine by *A. oxydans* (4) are all mediated by hydroxylases.

The degradation of NA by a *Bacillus* species is initiated by two hydroxylation reactions yielding 2,6-dihydroxynicotinic acid (2,6-DHNA). The enzymes have been purified and characterized as described previously (3). The purpose of this paper is to report the source of oxygen for the two hydroxylases. Three types of evidence will be presented to show that the source of oxygen atoms for both reactions is water and that, therefore, these enzymes should be classified as hydroxylases.

MATERIALS AND METHODS

Growth of the organism and preparation of the enzymes have been described (3). For ^{18}O studies, extracts were purified through the diethylaminoethyl (DEAE)-cellulose stage. The enzyme preparation contained both hydroxylases.

Mass spectra were obtained using a Cook Engineering Co. 21-103 mass spectrometer at an ionizing voltage of 15 ev. Water containing 10 atom per cent excess ^{18}O was obtained from Bio-Rad Laboratories.

Oxygen uptake was measured in a YSI model 53 polarographic oxygen electrode.

The synthesis of 2-hydroxynicotinic acid from 2-aminonicotinic acid (Aldrich Chemical Co.) was performed as described by Phillips (7).

RESULTS AND DISCUSSION

Studies of ^{18}O incorporation were conducted in 50-ml flasks containing 10.0 mg of substrate, 0.25 μmole of methylene blue, 50 μmoles of potassium phosphate buffer (pH 7.4), and approxi-

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mately 5 mg of purified enzyme protein in 5.0 ml of $H_2{}^{18}O$ or $H_2{}^{16}O$ (final atom per cent excess ^{18}O, 9.5). The reactions were carried out with either NA or 6-hydroxynicotinic acid (6-HNA) as substrate. The flasks were incubated at 30 C with gentle shaking for 4 hr, a period sufficient for nearly complete conversion of substrate to product. The reaction mixtures were acidified to *p*H 2.0 with HCl and lyophilized to dryness. The reaction products were extracted into absolute methanol, dried in vacuo, and stored under N_2 at −15 C. The ultraviolet absorption spectra of the products were compared with authentic spectra to establish their identity.

Mass spectra of the products formed are shown in Fig. 1. The molecular ions of 6-HNA and its heavy ^{18}O isotope appear at *m/e* 139 and 141 (Fig. 1A and 1B). The 2,6-DHNA which had been produced was chemically decarboxylated to 2,6-dihydroxypyridine (2,6-DHP) when the reaction mixtures were acidified (1). This was done for two reasons. The carboxyl group of 2,6-DHNA is very unstable, and some fragmentation to 2,6-DHP and CO_2 would have occurred in the mass spectrometer. Chemical conversion of all the 2,6-DHNA to 2,6-DHP before analysis eliminated this problem. Also, the decarboxylated compound has two fewer oxygen atoms, which simplifies corrections for the natural abundance of ^{18}O. The molecular ion of [^{16}O]2,6-DHP appears at *m/e* 111. The ions containing one and two heavy oxygen atoms appear at *m/e* 113 and 115.

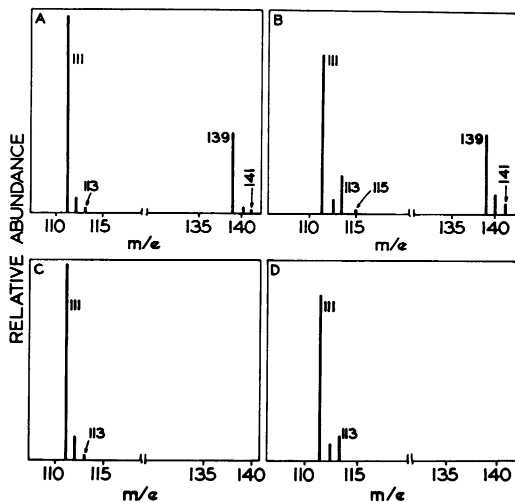

FIG. 1. *Mass spectra of the products formed enzymatically from nicotinic acid (NA) and 6-hydroxynicotinic acid (6-HNA) in the presence of $H_2{}^{16}O$ or $H_2{}^{18}O$. A, NA as substrate, $H_2{}^{16}O$; B, NA, $H_2{}^{18}O$; C, 6-HNA, $H_2{}^{16}O$; D, 6-HNA, $H_2{}^{18}O$.*

In the ^{18}O reactions with either NA or 6-HNA as the substrate (Fig. 1B and 1D), the relative abundance of the molecular ion at 113 is substantially greater than in the control reactions (Fig. 1A and 1C), indicating the enzymatic production of 2,6-DHNA with one heavy oxygen atom. With NA as substrate, an increase in the abundance of the ions at *m/e* 115 and 141 was observed (Fig. 1B). These ions represent 2,6-DHP with two heavy oxygen atoms and 6-HNA with one heavy oxygen atom.

Calculations of isotope incorporation into 6-HNA and 2,6-DHNA are shown in Table 1. The atom per cent excess in each instance agrees closely with that expected. The lack of absolute agreement probably reflects the fact that the methanol-extracted products were not completely free of salts. The di[^{18}O]2,6-DHNA peak was not large enough to measure accurately, as it would have been if a much greater atom per cent excess ^{18}O had been present in the reaction mixture. Cost considerations made the use of more highly enriched water prohibitive. From these results, we conclude that both of the hydroxyl-group atoms incorporated into 2,6-DHNA are derived from water.

When a substrate is hydroxylated by a hydroxylase, one-half molecule of molecular oxygen is consumed. This results from the transfer of the electrons liberated during the reaction. Monooxygenases and dioxygenases utilize one molecule of oxygen. It is a simple task to differentiate a hydroxylase reaction from an oxygenase reaction by measuring the oxygen consumed. Monitoring of oxygen consumption during the hydroxylation of NA, 6-HNA, and 2-hydroxynicotinic acid (an alternate substrate) showed that 0.5 μmole of oxygen was consumed per μmole of hydroxyl groups incorporated (Fig. 2). This was true whether the hydroxylation occurs at the 2 or the 6 position of the ring and confirms the ^{18}O-incorporation data.

The hydroxylation of NA and 6-HNA using enzymes in a 105,000 × *g* supernatant fraction was shown previously to require the presence of an electron acceptor (3). This requirement was observed at all stages in the purification of the enzymes, and methylene blue was the most effective electron acceptor of a large number tested. Reduced pyridine nucleotides had no effect on the activity of purified enzyme preparations whether in the presence or absence of methylene blue. These observations again indicate that water is the source of oxygen atoms for the hydroxylation reactions, since monooxygenases require the presence of an electron donor, and there is no requirement for an electron carrier with dioxygenases.

TABLE 1. *Incorporation of ^{18}O into 6-hydroxynicotinic acid (6-HNA) and 2,6-dihydroxynicotinic acid (2,6-DHNA) by nicotinic acid and 6-HNA hydroxylases*

Substrate	Product	m/e[a]	Atom % excess	
			Calculated	Observed
Nicotinic acid	mono[^{18}O]H 2,6-DHNA	113	17.3	17.2
Nicotinic acid	di[^{18}O]H 2,6-DHNA	115	0.82	Present, not calculated
Nicotinic acid	[^{18}O]H 6-HNA	141	9.5	7.6
6-Hydroxy-nicotinic acid	mono[^{18}O]H 2,6-DHNA	113	9.5	10.8

[a] Mass-to-charge ratios for the isotopes of 2,6-DHNA are those of the corresponding chemically decarboxylated 2,6-dihydroxypyridine.

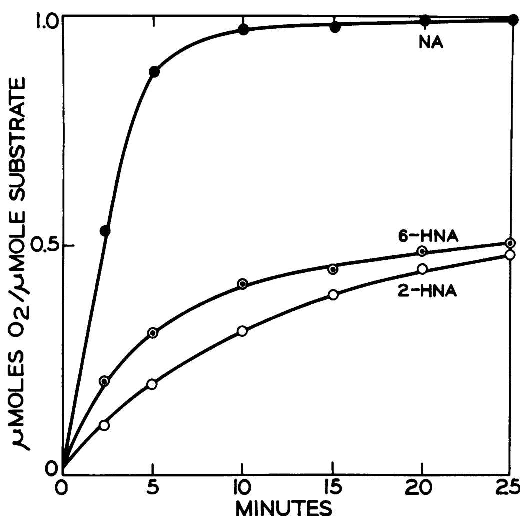

FIG. 2. *Consumption of oxygen by purified hydroxylases. Reaction mixtures contained: methylene blue, 0.1 µmole; enzyme, 25 µliters; and the specified amount of substrate, in 3.0 ml of 0.01 M potassium phosphate buffer (pH 7.4). Oxygen consumption was followed at 25 C using a polarographic oxygen analyzer. Tracings are corrected for a small amount of endogenous oxygen uptake.*

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