Oxidation of Nicotinic Acid by a Bacillus Species: Purification and Properties of Nicotinic Acid and 6-Hydroxynicotinic Acid Hydroxylases

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The enzymes of a Bacillus species that hydroxylate nicotinic acid to 6-hydroxynicotinic acid and 6-hydroxynicotinic acid to 2,6-dihydroxynicotinic acid were purified and characterized. The purified enzymes contained approximately two molecules of flavine and eight molecules of iron per molecule of enzyme. The enzymes were large (molecular weight, 400,000 to 450,000) and appeared to consist of sub-units.

Ensign and Rittenberg (6) reported the isolation of a Bacillus species that grows on nicotinic acid (NA) as its sole carbon and energy source and produces a soluble blue pigment as a by-product. They identified 6-hydroxynicotinic acid (6-HNA) and 2,6-dihydroxynicotinic acid (2,6-DHNA) as the first two pathway intermediates. The inducible enzymes, which hydroxylate NA to 6-HNA and 6-HNA to 2,6-DHNA, were found in the soluble fraction of crude cell-free extracts and required an external electron acceptor for activity.

Hughes (15) and Behrman and Stanier (1) studied the degradation of NA by Pseudomonas species. The pathway involved is similar to the Bacillus pathway in that 6-HNA is the first metabolite intermediate, but different in that the 6-HNA is oxidatively decarboxylated, yielding 2,5-dihydroxypyridine (1). This latter pathway is also used by an Arthrobacter species (S. L. Kington, Ph.D. thesis, Univ. of Wisconsin, Madison, 1969).

The products of NA fermentation by a Clostridium species were described by Harary (8, 9). The intermediate products of the fermentation and the enzymes involved in the conversion of NA to 6-HNA and 1,4,5,6-tetrahydroxynicotinic acid have been studied (9, 14, 19, 22). This paper describes the purification of the NA and 6-HNA hydroxylases from the Bacillus species and their characterization as large, iron-containing flavoproteins.

MATERIALS AND METHODS

Growth conditions. The Bacillus species of Ens

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and Rittenberg (6) was grown on their medium with the trace salts solution modified to contain (grams per liter of 0.1 N HCl): CaCl₂·2H₂O, 2.0; MnSO₄, 1.0; FeSO₄·7H₂O, 0.5; CoCl₂·6H₂O, 0.01; CuSO₄·5H₂O, 0.01; ZnSO₄·7H₂O, 0.01; and Na₂MoO₄·2H₂O, 0.01. Large batches of cells were grown in a 30-liter stainless-steel fermentor with vigorous forced aeration at 30 C. Cells were harvested in a Sharples Super Centrifuge and either used immediately or stored as a cell paste at −20 C.

Enzyme assays. The enzymatic conversion of NA to 6-HNA by NA hydroxylase was assayed by following the increase in absorbance at 295 nm, a peak in the absorption spectrum of 6-HNA, in a recording spectrophotometer (Cary 15). Reaction mixtures contained 30 μmoles of potassium phosphate buffer (pH 7.4), 0.1 μmole of methylene blue, 0.5 μmole of NA, and enzyme, in a 3.0-ml total volume. The conversion of 6-HNA to 2,6-DHNA by 6-HNA hydroxylase was assayed at 322 nm, a peak in the absorption spectrum of 2,6-DHNA, in a similar reaction mixture which contained 6-HNA instead of NA. A unit of activity is defined as the conversion of 1 μmole of substrate to product in 1 min at room temperature (about 25 C). The further metabolism of 6-HNA formed in the NA assay did not interfere with the assay.

Urease activity was assayed by following the increase in pH which accompanied the hydrolysis of urea to CO₂ and ammonia. The assay was performed by adding 2.0 ml of 3% urea in 0.03 M phosphate buffer (pH 7.0) to the enzyme sample (1.0 ml) and then measuring the pH at 2-min intervals with a pH meter (Corning model 12) employing the expanded scale.

Analytical methods. Protein was determined by the method of Lowry et al. (18) with a Spectronic 20 colorimeter. Protein in column effluents was monitored with an LKB Ulvacord at 280 nm. Other spectrophotometric measurements were performed with a Beckman DB-G spectrophotometer.

The iron content of the enzyme was determined on the residue after wet combustion, with H₂SO₄/HNO₃ (1:1), of an enzyme sample containing approximately
20 nmoles of iron. The iron was then reduced with thioglycollic acid and assayed by complexing with bathophenanthroline (5).

Flavine was assayed by using the difference in absorption between oxidized and reduced forms at 450 nm, 9.8 × 10⁻⁴ M⁻¹ cm⁻¹ (21).

Discontinuous gel electrophoresis was performed with the Davis system (2). Samples were applied above the spacer gel in 20% sucrose. Enzyme activity was located in the gels by incubating them in 0.02 M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.4 (buffer A), containing 1 mm substrate and 1% neotetrazolium chloride. Dark purple bands of formazan formed at the site of hydroxylase activity. Protein was stained with 0.09% Ponceau-S.

Chemicals and reagents. Diethylenetriamine (DEAE)-cellulose was obtained from Sigma Chemical Co. and washed by the method of Peterson and Sober (20). NA and 6-HNA were obtained from the Aldrich Chemical Co. Beef pancreas deoxyribonuclease, jack bean urease, and α-phosphatase were also obtained from Sigma. Agarose, Sagarose, and Bio-gel P-10 were purchased from Bio-Rad Laboratories. Neotetrazolium and 6-HNA were obtained from Nutritional Biochemicals Corp., and α, α-dipryridyl from the Eastman Chemical Co.

RESULTS

Purification of the NA and 6-HNA hydroxylating activities. Step 1 was the preparation of crude extracts. Fresh or frozen cell paste (25 to 30 g) was suspended in about twice its volume of 0.02 M potassium phosphate, pH 7.4 (buffer B). The thick suspension was forced twice through a chilled French pressure cell. This and all subsequent procedures were performed at 4 °C. Approximately 1 mg of deoxyribonuclease was added to reduce the viscosity, and the preparation was centrifuged at 20,000 × g for 1 hr.

The second step was high-speed centrifugation. The 20,000 × g supernatant fluid was centrifuged at 105,000 × g for 2.5 hr. Both of the hydroxylase activities were found in the supernatant fluid. About 30% of the starting protein was sedimented as a reddish pellet. An increase in the total units of both the NA and 6-HNA hydroxylating activities was obtained, resulting in a 1.5- to 2-fold purification of each.

The third step was ammonium sulfate fractionation. Dry, crystalline ammonium sulfate was added to the 105,000 × g supernatant fluid. The protein which precipitated between 35 and 55% saturation contained the hydroxylating activities. This precipitate was dissolved in 10 to 15 ml of 0.05 M potassium phosphate, pH 7.4 (buffer C), and desalted by passage through a 1.2 by 20 cm column of Biogel P-10, equilibrated with the same buffer. This step resulted in a two- to threefold purification.

Step 4 was DEAE-cellulose chromatography. The desalted material from step 3 was applied to a column (1.5 by 25 cm) of DEAE-cellulose equilibrated with buffer C. Unbound protein was removed by washing with 400 ml of the buffer. The bound protein was eluted with a 500-ml linear gradient of 0 to 0.5 M NaCl in buffer C. Both hydroxylase activities were eluted by about 0.3 M NaCl (Fig. 1). The active fractions were pooled, precipitated by adjusting to 60% saturation with ammonium sulfate, and suspended in 5 to 10 ml of buffer B.

In some purifications, the hydroxylases were eluted from the column by a stepwise increase in NaCl concentration. In either case, a three- to fivefold purification resulted. Sufficient NaCl was carried over into the assay reactions to inhibit the activities significantly. No correction for this inhibition was made in the calculations of activity and purification (Table 1). When the salt was removed during gel filtration (step 5), the activity was recovered.

Gel filtration (step 5) was conducted as follows. The active material from step 4 was applied to a column (2.5 by 50 cm) of Agarose A-1.5m equilibrated with buffer B, and the column was developed with the same buffer. The hydroxylase activities were both eluted in the major protein peak (Fig. 2). The peak fraction of NA hydroxylase activity emerged from the column slightly before the peak of the 6-HNA hydroxylase activity, but most of the active fractions contained both. The position of elution indicates that these enzymes are large proteins. The purification at this step was two- to threefold. Essentially the same results were obtained when a column of Sagarose 9F was employed. The ac-

![Fig. 1. Purification of the hydroxylating activities by diethylenetriamine (DEAE)-cellulose chromatography.](http://jb.asm.org/)

Desalted 35 to 55% ammonium sulfate fraction (13 ml of protein solution containing 530 mg of protein) was applied to a DEAE-cellulose column (1.5 by 25 cm). Unbound protein was washed through with buffer B. Bound protein was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions (3.0 ml) were collected, and every other one was assayed for NA and 6-HNA hydroxylating activity and for protein content.)
Table 1. Purification of the nicotinic acid and 6-hydroxynicotinic acid hydroxylase activities

<table>
<thead>
<tr>
<th>Step</th>
<th>Substrate</th>
<th>Vol (ml)</th>
<th>Total protein (mg)</th>
<th>Total units</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>NA</td>
<td>81</td>
<td>1,815</td>
<td>66.2</td>
<td>0.036</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>6-HNA</td>
<td></td>
<td></td>
<td>56.0</td>
<td>0.031</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>2. High-speed centrifugation</td>
<td>NA</td>
<td>77</td>
<td>1,055</td>
<td>72.8</td>
<td>0.069</td>
<td>110</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>6-HNA</td>
<td></td>
<td></td>
<td>67.5</td>
<td>0.064</td>
<td>120</td>
<td>2.1</td>
</tr>
<tr>
<td>3. Ammonium sulfate fractionation</td>
<td>NA</td>
<td>13</td>
<td>530</td>
<td>75.7</td>
<td>0.143</td>
<td>114</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>6-HNA</td>
<td></td>
<td></td>
<td>82.0</td>
<td>0.155</td>
<td>145</td>
<td>5.0</td>
</tr>
<tr>
<td>4. DEAE-cellulose</td>
<td>NA</td>
<td>82</td>
<td>105</td>
<td>32.7</td>
<td>0.313</td>
<td>50</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>6-HNA</td>
<td></td>
<td></td>
<td>30.8</td>
<td>0.295</td>
<td>55</td>
<td>9.5</td>
</tr>
<tr>
<td>5. Gel filtration</td>
<td>NA</td>
<td>6.0</td>
<td>52</td>
<td>42.9</td>
<td>0.83</td>
<td>65</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>6-HNA</td>
<td></td>
<td></td>
<td>49.8</td>
<td>0.93</td>
<td>89</td>
<td>30.0</td>
</tr>
</tbody>
</table>

A summary of the purification data is shown in Table 1. Many repetitions of the purification procedure yielded final purifications varying from 15- to 25-fold for NA hydroxylase and from 25- to 40-fold for 6-HNA hydroxylase. Overall recoveries were 65% for the NA hydroxylase and 89% for the 6-HNA hydroxylase. The increase in total units of both activities after the high-speed centrifugation and ammonium sulfate fractionation steps may be the result of removal of some inhibitory substance.

These purification procedures did not separate the NA and 6-HNA hydroxylating activities. Several other methods were utilized in an attempt to accomplish this. Neither of the hydroxylating activities was eluted from a DEAE-cellulose column by its substrate. They were not separated by passage through a Sephadex G-200 column or by elution from a hydroxylapatite column with a phosphate-buffer concentration gradient. The purified enzyme protein was denatured when an attempt was made to separate the hydroxylases on a pH 7 to 10 electrofocusing gradient.

**Characterization of the purified hydroxylases.** To determine electrophoretic mobility, a sample of the purified hydroxylase preparation (step 5) was tested for homogeneity by using the disc gel electrophoresis technique. Thirteen protein bands were resolved (Fig. 3C). All of the protein bands except bands 10 and 12 reduced tetrazolium dye with NA as substrate (Fig. 3A). With 6-HNA as substrate, dye-reduction activity occurred with bands 2, 4, 6, 8, 11, 12, and 13 (Fig. 3B). Protein band 10 showed no activity with either substrate. Thus, bands 1, 3, 5, 7, and 9 contained only NA hydroxylase activity; band 12 contained only 6-HNA hydroxylase activity; and bands 2, 4, 6, 8, 11, and 13 contained both enzymes. No dye reduction occurred in the absence of the substrates. Virtually identical patterns of protein bands and activity were obtained when several independent enzyme preparations were used and when the buffer concentration and current applied to the gels were changed.

For molecular weight determination, a mixture of 5 mg of purified enzyme and 5 mg of urease, in 1 ml of buffer B, was applied to a column (2.5 by 50 cm) of Agarose A-5m, and the column was eluted with the same buffer. The hydroxylase
activities were eluted very shortly after urease (molecular weight, 480,000 (4)). The peak of the NA hydroxylase activity emerged slightly before the 6-HNA peak (Fig. 4). The molecular weights of the hydroxylases were estimated to be 400,000 to 450,000 by comparing their elution positions with that of urease (3).

The apparent $K_m$ values for NA and 6-HNA are $0.7 \times 10^{-4}$ M and $0.5 \times 10^{-4}$ M, respectively, as determined by Lineweaver-Burk plots.

The hydroxylations of both NA and 6-HNA were inhibited 30 to 50% by $10^{-4}$ M a-atabrine, $10^{-4}$ M $\alpha$, $\alpha$-dipyridyl, and $10^{-3}$ M o-phenanthroline. This suggests that a flavine and a metal, probably iron, are involved in the enzymatic reactions. The flavine and iron remained with the enzyme complex through the purification procedures, indicating that they are tightly bound.

Difference spectra of the enzymes showed a reduction of flavine absorbance at 450 nm with both NA and 6-HNA as substrates (Fig. 5). This strongly implicates a flavine as a primary electron acceptor for both hydroxylations.

Acidification of a sample of purified enzyme to pH 4.5 with HCl resulted in a turbid solution.

**FIG. 4.** Determination of molecular weights for hydroxylases by gel filtration through Agerose A-5m. A solution containing 5.0 mg of urease and approximately 5 mg of the purified enzymes in a 1.0-ml volume was applied to a column (2.5 by 50 cm). Fractions (5.0 ml) were collected, and each was assayed for NA and 6-HNA hydroxylating activity and for urease activity.

**FIG. 5.** Reduction of the purified enzymes by NA, 6-HNA, and dithionite. Purified enzyme preparation (8.3 mg of protein per ml) was flushed three times with $N_2$ in an anaerobic cuvette. Reactions were blanked against a cuvette containing the same amount of enzyme incubated aerobically. A, Control, no additions; B, 10 min after adding 1 $\mu$ mole of NA; C, 10 min after adding 1 $\mu$ mole of 6-HNA; D, reduction by excess dithionite.
After centrifugation, a yellow supernatant fluid and a light grey pellet were obtained. The pellet was dissolved in buffer B and assayed for hydroxylating activity; 5 to 10% of the starting activity remained. In another experiment, the precipitated protein and yellow supernatant fluid were recombined and then neutralized. This resulted in a reconstitution of over 90% of the original activity. When flavine mononucleotide (FMN) or flavine adenine dinucleotide (FAD) was substituted at concentrations from $10^{-6}$ to $10^{-7}$ M for the supernatant fluid, the activity was not restored. Spectra of the holoenzyme and the apoenzyme are shown in Fig. 6. The shoulder at 450 nm was removed by the acid treatment, indicating that the loss of activity was due to the removal of a flavine. The amount of flavine removed by acid treatment was calculated, assuming a molecular weight for the enzymes of 430,000, to be 1.5 molecules of flavine per molecule of enzyme. This represents a minimum value, because there was some residual activity in the acid-precipitated apoenzyme. A reasonable estimate of the flavine content would be two molecules per molecule of enzyme.

Iron determinations were performed on duplicate samples of three different purified enzyme preparations. An overall average of approximately eight molecules of iron per molecule of enzyme (assuming a molecular weight of 430,000) was found. (Individual values were 8.7, 9.1, and 7.1 for experiments 1, 2, and 3, respectively.) The iron was not removed from the holoenzyme by acid precipitation at pH 4.5.

**DISCUSSION**

The NA and 6-HNA hydroxylating activities were not separated by the purification procedures employed. This indicates either that two distinct enzymes with very similar properties exist or that both activities are components of a multifunctional enzyme complex. Our experiments did not distinguish between these alternatives. The high molecular weight of the hydroxylases and the resolution of 12 bands of enzymatic activity during disc electrophoresis suggest that the native enzyme unit consists of subunits. One reasonable explanation for the observation that some bands of activity contained both NA and 6-HNA hydroxylating activity, whereas others were active with only one or the other substrate, is that these bands represent various associations of subunits. Presumably, disaggregation occurs during electrophoresis, and the subunits reaggregate randomly in the gel to form various multimers with the ability to hydroxylate NA, 6-HNA, or both. Further study of the molecular weights of the enzymes and possible subunits is necessary to clarify this.

The hydroxylases are iron-containing flavoproteins. Neither FMN nor FAD activated the apoenzyme. The flavine removed by acid treatment did activate the apoenzyme, however. Possibly, the acid treatment removed a third cofactor (other than flavine or iron), or an unknown flavine is the natural cofactor. Attempts to identify the flavine by thin-layer chromatography procedures were not successful.

In several studies, hydroxylation of the pyridine ring in the alpha position has been reported. In each instance, water is the source of the hydroxyl groups. This was true for the hydroxylation of the number 6 carbon atom of nicotinic acid by *Pseudomonas fluorescens* (16) and a *Clostridium* species (13), of nicotine by *Arthrobacter oxydans* (11), and of picolinic acid by an *Arthrobacter* species (R. L. Tate, unpublished data). The *Bacillus* species employed in this study incorporates water into both alpha positions of nicotinic acid (10). The hydroxylation enzymes of *A. oxydans* (12), the *Clostridium* species (13), the *Arthrobacter* sp. (R. L. Tate, unpublished data), and the *Bacillus* sp. used in this investigation have been characterized well enough to note some interesting similarities. All are large proteins containing both iron and flavine as prosthetic groups.

In the *Bacillus* sp. used in this study, NA is hydroxylated via two sequential and identical reactions. Both the NA and 6-HNA hydroxylases are coordinately induced by either substrate in a 1:1 ratio of activity (R. Hirschberg, unpublished data). It seems reasonable for two en-
zymes with very similar properties, which catalyze an identical type of reaction and which are synthesized coordinately, to be combined into a multifunctional complex. Multienzyme complexes have been reported in the pyruvate dehydrogenase system of Escherichia coli (17), the tryptophan pathway of Neurospora crassa (7), and in the fatty acid synthetase of yeast (F. Lyen, Fed. Proc., p. 941, 1961).

ACKNOWLEDGMENT

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LITERATURE CITED