A New Anthranilic Acid Hydroxylase from Aspergillus niger

PURIFICATION AND PROPERTIES

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SUMMARY

A new anthranilic acid hydroxylase which catalyzes the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid, has been partially purified from crude extracts of the mycelial felts of Aspergillus niger, grown in the presence of anthranilic acid. Neither 3-hydroxyanthranilic acid nor salicylic acid is an intermediate in this conversion.

The enzyme is stabilized by glutathione and requires substrate amounts of NADPH. The optimum pH for anthranilic acid hydroxylase activity is around 8.2. The $K_m$ values for anthranilic acid and NADPH at pH 8.2 and 30° are 0.15 and 0.16 mm, respectively. The partially purified enzyme is highly specific for anthranilic acid and does not act on related substances such as 3-hydroxyanthranilic acid, salicylic acid, m-hydroxybenzoic acid, methylanthranilic acid, and ethylanthranilic acid. Cupric, mercuric, and cadmium ions have a pronounced inhibitory effect on enzyme activity. Metal-chelating agents like $o$-phenanthroline, $o$, $o'$-dipyrindyl, salicyldioxime, and diethyl dithiocarbamate also inhibit the reaction. Sulfhydryl reagents such as p-hydroxymercuribenzoate and N-ethylmaleimide irreversibly inhibit anthranilic acid hydroxylase activity.

Crude extracts of the mycelium have the ability to hydroxylate anthranilic acid to 3-hydroxyanthranilic acid in addition to 2,3-dihydroxybenzoic acid. This activity, however, is lost on purification.

EXPERIMENTAL PROCEDURE

Materials

Anthranilic acid purchased from Eastman Kodak Company, Rochester, was recrystallized from water. 2,3-Dihydroxybenzoic acid was obtained from K and K Laboratories, Inc., California, and recrystallized from hot water. Protamine sulfate was obtained from Mann. Hydroxylapatite (Bio-gel, HTP) and alumina Cy gel were purchased from Calbiochem. NAD, NADP, NADH, NADPH, FMN, FAD, folic acid, glucose 6-phosphate dehydrogenase, and DEAE-cellulose were from Sigma. Glucose 6-phosphate (barium salt) was purchased from Reanal Chemical Company, Budapest, Hungary. Sephadex G-25 was from Pharmacia, Uppsala. Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid with platinum oxide as catalyst (8). Other chemicals were of analytical reagent grade available commercially.

Organism—Aspergillus niger UBC 814 was obtained from Dr. R. J. Bandoni, Department of Botany, University of British Columbia.

Media and Conditions of Cultivation—A. niger was grown for 48 to 60 hours at 30° in 1-liter flasks on a synthetic medium (9) supplemented with 0.1% anthranilic acid. Stock cultures were maintained on the same medium supplemented with 1.5% agar. The mycelia were harvested before the advent of sporulation and washed thoroughly with distilled water. The mycelium was used fresh or stored at −20° until required.

Methods

Assay of Anthranilic Acid Hydroxylase—Anthranilic acid hydroxylase activity was measured by incubating for 20 min at 30° a reaction mixture (1 ml) containing Tris-HCl buffer, pH 8.2
(30 μmoles), anthranilic acid (0.2 μmole), NADP (0.2 μmole),
glucose 6-phosphate (0.25 μmole), glucose 6-phosphate dehydro-
genase (10 milliunits), and 0.5 ml of anthranilic acid hydroxylase
(4 to 8 milliunits). The components of the NADPH-regenerating
system (NADP, glucose 6-phosphate, and glucose 6-phosphate
dehydrogenase) were incubated with the buffer for 5 min
prior to the addition of anthranilic acid hydroxylase and
substrate. The reaction was stopped by the addition of 0.2 ml
of 0.5 N HCl and the mixture was extracted with 2.5 ml of per-
oxide free ether. One milliliter aliquots were added to
a dryness

Oxygen Consumption—Oxygen consumption was measured by
using Gilson Oxygraph, model K.

Ammonia Formation—Ammonia liberated during the course
of the reaction was determined by nesslerization by using the
Conway microdiffusion method (12).

Determination of Protein Concentration—The protein con-
centrations of various fractions obtained in the course of purification
were determined with Folin-Phenol reagent (13) with bovine
serum albumin as the standard.

| RESULTS |

Stabilization of Anthranilic Acid Hydroxylase

The mycelium can be stored at −20° for 1 month without
appreciable loss in activity. The enzyme activity is rapidly lost
during purification. Thiol compounds have a profound protec-
tive action, GSH being the most effective.

Purification of Anthranilic Acid Hydroxylase

All operations were performed at 0–4°.

Preparation of Crude Extract—The mycelium (30 g) was
macerated in a chilled mortar for 15 min with an equal weight of
glass powder and extracted with 90 ml of 0.025 M sodium phos-
phate buffer (pH 7) containing 0.001 M GSH. The extract was
passed through a cheesecloth and centrifuged at 12,000 × g for
20 min. The supernatant solution containing about 5 to 6 mg
of protein per ml was worked up for purification.

Treatment with Protamine Sulfate—A 2% solution of protamine
sulfate in 0.025 M sodium phosphate buffer, pH 7 (9 ml) was
added to the crude extract (81 ml) with gentle stirring. After
15 min, the mixture was centrifuged for 10 min at 12,000 × g
and the precipitate was discarded.

DEAE-cellulose Treatment DEAE-cellulose was washed ac-
cording to the procedure described by Peterson and Sober (14)
and suspended in 0.025 M sodium phosphate buffer (pH 7). An
aliquot of the suspension was filtered through a Buchner funnel.
To the protamine sulfate extract (90 ml) were added 6 g of the
washed DEAE-cellulose cake and the mixture was stirred
mechanically for 30 min. The suspension was filtered through a
Buchner funnel and the filtrate was collected.

Alumina Cy Treatment—The enzyme solution obtained after
DEAE-cellulose treatment (70 ml) was treated with alumina
Cy gel (solids obtained from 70 ml of a preparation containing
10 mg per ml) and stirred for 15 min. The gel was separated by
centrifugation and washed with 70 ml of 0.025 M sodium phos-
phate buffer, pH 7, containing 0.001 M GSH. The enzyme was
finally eluted from the gel twice with 70 ml (35 ml each time) of
a solution containing sodium phosphate buffer (0.06 M, pH 8)
and GSH (0.001 M).

Hydroxylapatite Treatment—The enzyme preparation from the
previous step (50 ml) was treated with hydroxylapatite (500 mg).
After stirring for 15 min, the suspension was centrifuged and the
precipitate was discarded. The clear supernatant was used as
anthranilic acid hydroxylase.

The balance sheet for the purification of anthranilic acid
hydroxylase is shown in Table I. Crude extracts of the
mycelium exhibited very low anthranilic acid hydroxylase
activity. These preparations were found to contain a potent
NADPH oxidase. Even in the presence of a sufficient excess of
the NADPH-regenerating system (NADP, 1 μmole; glucose
6-phosphate, 1.25 μmoles; and glucose 6-phosphate dehydro-
genase, 50 milliunits), there was no further increase in the enzyme
activity in crude extracts. These results seem to suggest that
there are endogenous inhibitors other than NADPH oxidase in
crude preparations. The enzyme activity, however, increases
rapidly after treatment with DEAE-cellulose, giving over-all
recoveries as high as 850%. Preparations obtained after DEAE-
treatment were completely free from NADPH oxidase activity.

Attempts to purify the enzyme extensively have been limited
by its extreme instability. The enzyme was inactivated by
treating with ammonium sulfate or acetone, or by positive
adsorption on DEAE-cellulose or carboxymethyl cellulose.
Filtration through Sephadex G-25 or dialysis against 0.025
M sodium phosphate dehydrogenase (10 units), and partially purified
anthranilic acid hydroxylase (2 units) was incubated at 30°.
After 2 hours, the pH of the reaction mixture was adjusted to
2 with 1 N HCl and it was converted twice with equal volumes of
peroxide-free ether. The organic layer was taken to dryness
at room temperature after shaking with anhydrous sodium sulfate.
The residue was dissolved in ethyl acetate and chromatographed
on Whatman No. 3 filter paper with 2% formic acid as solvent.
The fluorescent band corresponding to 2,3-dihydroxybenzoic
The enzymic product was identified as 2,3-dihydroxybenzoic acid by comparing its properties with those of an authentic sample. The $R_f$ values on paper chromatograms in benzene-acetic acid-water (10:7:3) and benzene-ethyl methyl ketone-formic acid-water (900:100:2:98) were 0.52 and 0.38, respectively. Color reactions with phenolic reagents like p-nitroaniline, diazotized sulfanilic acid, and ferric chloride (15, 16) were identical with those of synthetic, 2,3-dihydroxybenzoic acid. The ultraviolet spectrum of the isolated compound which showed absorption maxima at 248 and 316 m$\mu$ in ethanol was indistinguishable from that of 2,3-dihydroxybenzoic acid.

Decarboxylation of Anthranilic Acid Hydroxylase

Reaction Product to Catechol

Incubation of the enzymatically isolated product from anthranilic acid with purified 2,3-dihydroxybenzoic acid carboxylase from A. niger, resulted in its decarboxylation to catechol (17). The identity of the decarboxylation product was established by comparing its properties with those of authentic catechol. The decarboxylation product, like authentic catechol, gave a melting point of 105°. It was indistinguishable from catechol when subjected to paper chromatography in water-ethyl methyl ketone-diethylamine (77:921:2) and formic acid-water (2:98) as solvents, and the $R_f$ values were 0.88 and 0.73, respectively. The infrared and ultraviolet spectra of the decarboxylated product and authentic catechol were identical. In ethanol each showed a peak at 278 m$\mu$.

Properties of Partially Purified Anthranilic Acid Hydroxylase

Effect of pH on Enzyme Activity—Fig. 1 shows the effect of pH on anthranilic acid hydroxylase activity in the range 7.2 to 10. The maximum activity was observed around pH 8.2. For the pH range between 5 and 8, activity determinations were made in buffer consisting of sodium citrate (30 mmol) and disodium phosphate (60 mmol) and it was found that there was a steady increase in activity when the pH was raised from 5 to 8. It was also observed that the activities were invariably higher when citrate-phosphate buffer was used. For example, at pH 7.5 the activity in citrate-phosphate buffer was about 25% higher than the corresponding value in Tris-HCl buffer. The enzyme was only about 26% active at pH 5 when compared to the activity at the optimum pH.

Stoichiometry—the stoichiometry of the reaction catalyzed by the purified anthranilic acid hydroxylase from A. niger is shown in Table II. For the formation of 1 mole of 2,3 dihydroxybenzoic acid from anthranilic acid there was a consumption of 2 atoms of oxygen and oxidation of 1 mole of NADPH. From the data it is evident that the over-all reaction catalyzed by the enzyme is

$$\text{COOH} \quad \text{NH}_2 \quad \text{NADPH} \quad \text{H}^+ \quad \text{O}_2 \rightarrow \quad \text{COOH} \quad \text{OH} \quad \text{OH} \quad \text{NADP}^+ \quad \text{NH}_3$$

FIG. 1. pH activity curve for the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid. A reaction mixture (1 ml, total volume) consisting of enzyme, 8 milliunits; anthranilic acid, 0.2 mmol; NADP, 0.2 mmol; glucose 6-phosphate, 0.25 mmol, and glucose 6-phosphate dehydrogenase, 10 milliunits, was incubated with 30 wales of Tris-HCl (pH 7.2 to 9) or sodium carbonate-sodium bicarbonate buffer (pH 9 to 10). After incubation for 26 min at 30°, the enzyme activity was assayed as described under "Experimental Procedure."

Substrate Specificity for Pyridine Nucleotides—The enzyme showed an absolute requirement for NADPH. There was approximately 2% activity when NADPH was replaced by NADP, FMN, FAD, or tetrahydrofolic acid had no effect on enzyme activity. The reaction rate increased with an increase in the concentration of NADPH, the optimum concentration being 0.2 mm (Table III). The absorption spectrum of the purified enzyme in 0.06 M sodium phosphate buffer (pH 7) was that of a simple protein and did not show any indication for the presence of a flavin prosthetic group.

Substrate Specificity—Anthranilic acid is the only substrate known to be hydroxylated by the A. niger enzyme. No activity was detected with 3-hydroxyanthranilic acid, benzoic acid, salicylic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, p-aminobenzoic acid, m-aminobenzoic acid, methylanthranilate, and ethylanthranilate.

Substrate Affinities—A study of the effect of different concentrations of NADPH and of anthranilic acid on the rate of 2,3-dihydroxybenzoic acid formation in the presence of partially purified anthranilic acid hydroxylase gave typical Lineweaver-Burk plots. The $K_m$ values for anthranilic acid and NADPH at pH 8.2 were 0.15 and 0.16 mm, respectively.

Effect of Metal Ions—Anthranilic acid hydroxylase is not activated by any divalent cation. Mercu
cupric ions inhibited the enzyme activity to the extent of 100, 75, and 50%, respectively, at a final concentration of 0.5 mm.

Effect of Inhibitors—Table IV shows the effect of various inhibitors on the reaction. Even though the enzyme activity was not enhanced by any metal ion, partial inhibition by agents forming complexes with metals like o-phenanthroline, $\alpha,\alpha'$-dipyridyl, salicylaldoxime, diethyl dithiocarbamate, and 8-hydroxyquinoline seems to suggest that a tightly bound metal ion,

### Table II

**Stoichiometry of reaction catalyzed by anthranilic acid hydroxylase from A. niger**

In Experiment 1, each reaction mixture (2 ml) contained Tris-HCl buffer, pH 8.2, 0.5 mmole; anthranilic acid, 0.5 mmole; NADP, 0.4 mmole; glucose 6-phosphate, 0.5 mmole; glucose 6-phosphate dehydrogenase, 20 milliunits, and anthranilic acid hydroxylase from Step V, 9 milliunits. The reaction mixtures were incubated at 30° for various time intervals. The consumption of oxygen, anthranilic acid disappearance, and formation of 2,3-dihydroxybenzoic acid and ammonia were estimated as described under "Experimental Procedure," and expressed as millimicromoles per ml of standard reaction mixture. To determine the amount of NADPH oxidized, the reaction was allowed to proceed until all the anthranilic acid was utilized. The 2,3-dihydroxybenzoic acid formed was converted quantitatively to catechol by the addition of anthranilic acid. 2,3-Dihydroxybenzoic acid was determined colorimetrically as described under "Experimental Procedure."

<table>
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<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
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<td>Salicylaldoxime</td>
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<td>Aminopterin</td>
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### Table III

**Effect of different concentrations of NADPH on reaction**

The standard assay conditions were used except for the replacement of regenerating system (NADP, 0.2 mmole, glucose 6-phosphate, 0.25 mmole; and glucose 6-phosphate dehydrogenase, 10 milliunits) with the indicated concentration of NADPH. The amount of anthranilic acid hydroxylase added to the reaction mixtures was 8 milliunits.

<table>
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<tr>
<th>NADPH concentration</th>
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<tr>
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<td>160</td>
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<tr>
<td>200</td>
<td>130</td>
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<tr>
<td>Regenerating system</td>
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</tbody>
</table>

### Table IV

**Effect of different inhibitors on anthranilic acid hydroxylase activity**

The substrate-free standard reaction system was first incubated for 10 min with the test compound and the reaction was started by the addition of anthranilic acid. 2,3-Dihydroxybenzoic acid was determined colorimetrically as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>EDTA</td>
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<td>8-Hydroxyquinoline</td>
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<td>Aminopterin</td>
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<td>0</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

probably Fe++, is required for enzyme activity. The inhibition, however, was found to be irreversible as neither Fe++ nor any other metal ion could restore the activity.

As might be expected from the protective effect of GSH, the enzyme is sensitive to inhibition of thiol groups (Table IV), even though an excess of GSH is present.

Inhibitors like Atabrine and aminopterin had no effect on anthranilic acid hydroxylase activity.

**Effect of Structural Analogues**—Structurally related compounds like salicylic acid, methylantranilate, and ethylantranilate had no effect on the enzyme activity. 3-Hydroxyanthranilic acid and $m$-hydroxybenzoic acid inhibited the formation of 2,3-dihydroxybenzoic acid to the extent of 30%.

**Formation of 3-Hydroxyanthranilic acid in Crude Extracts**

A reaction mixture consisting of Tris-HCl buffer, pH 8.2 (300 mmole), anthranilic acid (2 mmoles), NADP (2 mmoles), glucose...
6-phosphate (2.5 µmoles), glucose 6-phosphate dehydrogenase (2 units), and 5 ml of crude cell-free extract was incubated for 30 min at 30°. The reaction was arrested by acidifying to pH 2 with 1 n HCl and the precipitate was removed by centrifugation. The supernatant solution was extracted thrice with equal volume of peroxide-free ether. The ether extract was taken to dryness in a vacuum and the residue was dissolved in 0.5 ml of ethyl acetate. Suitable aliquots were spotted on Whatman No. 1 filter paper and subjected to two-dimensional chromatography (16). In addition to 2,3-dihydroxybenzoic acid and anthranilic acid, the chromatograms contained an additional spot corresponding to 3-hydroxyanthranilic acid. The identity of this compound was established by its characteristic fluorescence and color reactions (15). Partially purified preparations, however, did not convert anthranilic acid to 3-hydroxyanthranilic acid, and 2,3-dihydroxybenzoic acid was invariably detected as the only product of anthranilic acid hydroxylation in such preparations. The second hydroxylase activity, i.e., hydroxylation of anthranilic acid to 3-hydroxyanthranilic acid, which is confined to crude preparations, was lost during purification and attempts to purify this enzyme were unsuccessful.

**DISCUSSION**

Results presented in this paper show that partially purified preparations from A. niger catalyze the stoichiometric conversion of anthranilic acid to 2,3-dihydroxybenzoic acid. Terui, Enata, and Tobata (19) identified 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid, and catechol as metabolites of anthranilic acid in the culture filtrates of A. niger. Radioactive studies by Subba Rao et al. (20) with tryptophan-14C also showed that the label from tryptophan is incorporated into anthranilic acid, and Tobata (19) identified 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid, and catechol as metabolites of anthranilic acid. Subsequently, anthranilic acid was isolated from preparations of A. niger. The enzyme hydroxylates anthranilic acid with tryptophan and converts anthranilic acid to 3-hydroxyanthranilic acid, which is confined to crude preparations, was lost during purification and attempts to purify this enzyme were unsuccessful.

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**REFERENCES**


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