Studies of the Mechanism of Anthranilate Synthase

EFFECT OF HYDROXYLAMINE AND N-METHYLHYDROXYLAMINE*

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SUMMARY

The mechanism of inhibition of anthranilate synthase by NH₂OH and CH₃-NHOH was investigated. The inhibition of anthranilate synthase by NH₂OH is accompanied by the formation of γ-glutamylhydroxamate. Apart from inhibiting the over-all reaction, i.e., formation of anthranilate from chorismate and glutamine, NH₂OH also inhibits the glutaminase activity exhibited by anthranilate synthase in the absence of Mg²⁺. Chorismate exerts a positive cooperative effect on the glutaminase activity.

CH₃-NHOH, the methyl derivative of NH₂OH, is a more potent inhibitor than NH₂OH but the mechanism of its inhibition is different. CH₃-NHOH depletes the enzymatic reaction of chorismate by cleaving it to form an adduct with the enolpyruvyl moiety of chorismate. The evidence obtained suggests that the structure of this adduct is α-carboxy-α,N-dimethylnitroso. The formation of the adduct is dependent on the presence of chorismate and anthranilate synthase. CH₃-NHOH does not form a derivative with glutamine, nor does it inhibit the glutaminase activity.

Anthrانيlate synthase, the first enzyme specific for the biosynthesis of tryptophan, catalyzes the formation of anthranilate from either chorismate and glutamine or chorismate and ammonia (Fig. 1). The enzyme exists as an aggregate with the next enzyme in the tryptophan pathway, anthranilate-5'-phosphoribosyltransferase (1, 2). The aggregate is capable of utilizing either glutamine or ammonia as amino donor. The anthranilate synthase protein devoid of the aggregate is capable of utilizing either glutamine or ammonia as amino donor. The anthranilate synthase protein devoid of the aggregate, termed Component I, can utilize only ammonia as amino donor (2). The formation of anthranilate from chorismate and an amino donor is a complex reaction requiring amination at carbon 2 (3), as well as elimination of a hydroxyl group and an enolpyruvyl group. Elimination of the enolpyruvyl group is accompanied by protonation to form pyruvate and this proton originates from water (4). In view of the complexity of the enzymatic reaction, efforts have been directed towards finding inhibitors which might allow the reaction to proceed in partial form only. Somervelle and Elford (5) have shown that NH₂OH, and even more so its derivative CH₃-NHOH, are inhibitors of anthranilate synthase, the inhibitors resulting in the formation of unidentified hydroxamates. The fact that hydroxamate formation is completely dependent on chorismate, glutamine, and enzyme and is inhibited by the end product, tryptophan, suggests that hydroxylamine may function by interfering with the over-all reaction. Zalkin and Kling (6), working with Component I of anthranilate synthase, observed the formation of the hydroxamate only in the presence of CH₃-NHOH. Moreover, it was dependent on chorismate and enzyme, whereas the amino donors, ammonia or glutamine, had no effect. In view of these findings it was felt that the identification of the hydroxamate may shed light on the mechanism of anthranilate synthase reaction. It was soon realized that the two inhibitors, NH₂OH and CH₃-NHOH, although very similar in structure, interfere with the enzymatic reaction in different ways. Both inhibitors formed compounds that reacted with FeCl₃. Careful separation of the products demonstrated that NH₂OH reacted with glutamine in the presence of enzyme and chorismate to form γ-glutamylhydroxamate, whereas CH₃-NHOH reacted with chorismate in the presence of enzyme to yield an adduct of pyruvate. The chorismate-dependent glutaminase activity of the enzyme was inhibited only by NH₂OH and not by CH₃-NHOH. Consequently, NH₂OH deprives the enzymatic reaction of glutamine and CH₃-NHOH deprives the reaction of its other substrate, chorismate.

EXPERIMENTAL PROCEDURE

Materials and Methods—γ-Glutamylhydroxamate was a generous gift of Dr. Amos Neidle. The following materials were obtained from commercial sources: NADH and 3-acetylpyridine-NAD, Sigma; lactate dehydrogenase, glutamate dehydrogenase, C. F. Boehringer and Soehne, Mannheim, Germany; hydroxylamine hydrochloride, Fisher Scientific Company; CH₃-NHOH, K and K Laboratories, Plainview, New York; H₂¹⁸O with 1.79 atom per cent excess from Miles Laboratories, Inc. The isolation of homogeneous anthranilate synthase from Salmonella typhimurium has been described by us (7). Chorismate was isolated from the accumulation medium of Aerobacter aerogenes 62-1 by the method described by Gibson and Gibson (8). Chorismate-U-¹³C was isolated by adding to the accumulation medium uniformly labeled glucose (6 g per liter 30 μCi per mm). Ring-labeled chorismate was prepared by enzymatically

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converting shikimate-5-phosphate-U-14C and unlabeled phos- 
phoenolpyruvate to chorismate with chorismate synthetase. 
The incubation mixture contained 20 mmoles of Tris-HCl buffer, 
ph 8.2; 1.0 mmole of MgCl₂; 0.1 mmole of dithiothreitol; 0.4 
mmole of 14C shikimate 5 phosphate (380 cpm per μmole); 0.1 
mmole of FAD; 0.6 mmole of NADH; 0.6 mmole of phosphenol-
pyruvate; 78.6 mg of crude chorismate synthetase (specific ac-
tivity, 2.5) prepared from A. aerogenes 62-1 according to the 
method of Morel et al. (9) in a final volume of 200 ml. The two 
substrates and NADH were added in 4 aliquots at 20-min inter-
vals. After 90 min at 37° the reaction mixture was cooled and 
the pH adjusted to 3.0 with 20 ml of 2 N H₂SO₄. The precipi-
tate proteins were removed by centrifugation and the chorismic 
acid was extracted with ether from the clear aqueous layer. 
The ether was removed and the crude chorismic acid was purified 
by adsorption on Dowex 1-Cl⁻ and elution with 1 N H₂SO₄. 

The inhibition by NH₃OH at the respective pH optima was 29% 
and, at the optimum pH for glutamine as amino donor (i.e. 7.4), the 
inhibition was 53% and, at the optimum pH for NH₃ as amino 
donor (i.e. 8.7), the inhibition was only 25% with CH₃-NHOH. 
The inhibition by NH₃OH was dependent only on en-
zyme concentration. With NH₃OH the absorption 
maximum was centered around 505 nm while with CH₃-NHOH 
it was around 520 nm. 

pH Optimum and Substrate Requirements for Hydroxamate Formation-The optimum pH for hydroxamate formation with either NH₃OH or with CH₃-NHOH was 7.9; at this pH, the 
inhibition of anthranilate synthase by CH₃-NHOH was 42% 
and 37% with glutamine and ammonia, respectively. However, 
at the optimum pH for glutamine as amino donor (i.e. 7.4), the 
inhibition was 53% and, at the optimum pH for NH₃ as amino 
donor (i.e. 8.7), the inhibition was only 25% with CH₃-NHOH. 
The inhibition by NH₃OH at the respective pH optima was 29% 
for glutamine and 9% for NH₃ as amino donor. 

Somerville and Elford (5) have already shown that the forma-
tion of hydroxamate with NH₃OH was dependent only on en-
zyme, chorismate, and glutamate; omission of Mg²⁺, which is 
required for the enzymatic reaction, resulted in only a 20% 
decrease. Similar results were obtained with the homogenous 
anthranilate synthase; Mg²⁺ was not necessary for the formation 
of hydroxamate with either NH₃OH or CH₃-NHOH. A linear 
relationship was observed between hydroxamate formation and 
enzyme concentration.

While studying the effect of chorismate concentration on the 
formation of hydroxamate by chorismate synthase, it was found that 
the addition of glutamine at high chorismate concentration did 
not have any influence; even at low concentrations of chorismate, 
there was only a slight stimulation of hydroxamate formation as 
measured by the FeCl₃ color reaction (Fig. 2B). However, with 

![Fig. 1. Reaction catalyzed by anthranilate synthase.](image)

![Fig. 2. A, effect of inhibitor concentration (conc.) on hydrox-
amate formation. The reaction mixture contained Tris-HCl buffer, 
ph 8.2, 50 moles; glutamine, 5.0 moles; chorismate, 0.5 
μmoles; anthranilate synthase (specific activity 45), 0.2 units; and 
neutralized NH₃OH or CH₃-NHOH at the indicated concentration 
in a final volume of 1.0 ml. The enzyme was added last. The 
inactivation was carried out for 30 min at room temperature and 
was stopped by the addition of FeCl₃ reagent (5). The color 
was allowed to develop for 10 min and the intensity was read at 500 nm. 
B, effect of chorismate concentration on hydroxamate formation 
with CH₃-NHOH. The incubation mixture contained Tris-HCl 
buffer, ph 7.9, 50 moles; EDTA, 0.1 μmole; anthranilate synthase, 
0.015 mg; neutralized CH₃-NHOH, 100 μmoles; and chorismate at 
the indicated concentration in a final volume of 0.5 ml. In the 
experiments with glutamine, it was present at a concentration of 
2.5 μmoles.](image)
TABLE I
Lack of incorporation of $^{18}O$ from $H_2^{18}O$ into pyruvate and anthranilate

The incubation mixture contained, in a total volume of 25 ml of $H_2^{18}O$, 20 μmoles of chorismic acid, 500 μmoles of MgCl₂, 1.25 mmoles of Tris-chloride buffer, pH 7.5, 300 μmoles of p-bromophenylhydrazine, 210 units of anthranilate synthase, and 500 μmoles of glutamine. The reaction was carried out at 37° for 3 hours and its course was followed by assaying the amount of glutamate formed.

The control incubation mixture contained 100 μmoles of anthranilic acid, 100 μmoles sodium pyruvate, and all other components except glutamine and chorismate.

| Experiment | Pyruvate $^a$ formed | Water medium | Pyruvate p-bromophenylhydrazone | Anthranilic acid |
|------------|----------------------|--------------|---------------------------------|-----------------|
|            | μmoles               | atom %       |                                 |                 |
| Control... | 110                  | 1.774        | 0.023                           | 0.005           |
| Control... | 1.790                | 0.000        | 0.008                           |                 |

a Since the stoichiometry of the reaction catalyzed by our enzyme preparation is 1:1 (7), the assay of glutamate gives a measure of the pyruvate formed.

$\text{NH}_2\text{OH}$ as inhibitor, the presence of glutamine was essential for hydroxamate formation. These results suggested that the mechanism of inhibition, as well as the hydroxamate formed with $\text{CH}_3\text{NHOH}$, may be different from that with $\text{NH}_2\text{OH}$. Further studies were therefore directed towards separating and identifying the hydroxamates that gave color with FeCl₃ in strong acid.

Experiments with $\text{CH}_3\text{NHOH}$—The finding that the enzymatic product of the reaction with $\text{CH}_3\text{NHOH}$ and chorismate behaved like a hydroxamate, i.e. gave color with FeCl₃ in acid; could be explained if either one or both carboxyl groups of chorismate formed an acyl enzyme intermediate in the normal enzymatic reaction. Such an acyl enzyme would yield a hydroxamate with $\text{CH}_3\text{NHOH}$. The occurrence of an acyl enzyme complex with chorismate could be studied by running the complete enzymatic reaction in $H_2^{18}O$ and analyzing the products, anthranilate and pyruvate, for incorporation of $^{18}O$. A large scale incubation was therefore carried out with chorismate, glutamine, and Mg$^{2+}$ in the presence of excess anthranilate synthase in $^{18}O$ water. p-Bromophenylhydrazine was present in the reaction mixture to remove the pyruvate formed. A control experiment containing enzyme, pyruvate, anthranilate, and p-bromophenylhydrazine was also included to examine the nonspecific incorporation of $^{18}O$ into the products. The results presented in Table I show clearly that there was negligible incorporation of $^{18}O$ into the products, anthranilate and pyruvate. This in turn ruled out the activation of either of the two carboxyls of chorismate through an acyl enzyme complex.

Attempts were therefore made to isolate the hydroxamate that was produced by $\text{CH}_3\text{NHOH}$ with chorismate and anthranilate synthase. The most suitable method was found to be elution from a Dowex 1-acetate column with increasing concentrations of pyridine-acetic acid buffer, pH 5.05. A typical separation pattern of an incubation mixture inhibited by $\text{CH}_3\text{NHOH}$ is shown in Fig. 3. Uniformly labeled $^{14}$C-chorismate was used to facilitate the identification of different fractions eluted from the column. All the radioactive fractions were analyzed for chorismate and anthranilic acid after removal of the pyridine-acetic acid buffer by lyophilization, and were found to be negative. Only the fraction that was eluted with 0.2 M pyridine-acetic acid buffer reacted with FeCl₃ in the manner typical of hydroxamates, and this fraction contained 10 to 15% of the total radioactivity placed on the column. This fraction was lyophilized to remove the buffer and a mass spectral analysis was performed. The major fragments are shown in Fig. 4. The highest molecular ion had a mass of 117. The fragmentation pattern suggests that the...
hydroxamate is an adduct of CH$_2$-NHOH and pyruvic acid, probably of the following structure

\[
\begin{align*}
\text{CH}_2&=\text{C}-\text{COOH} & \text{CH}_2&=\text{C}-\text{COOH} \\
\text{CH}-&\text{N}-\text{OH} & \text{CH}-&\text{N}-\text{O}
\end{align*}
\]

The ultraviolet absorption spectra of the enzymatic product had a peak at 240 nm and the infrared spectra had a strong absorption at 1175 cm$^{-1}$, a stretching frequency that is considered to be due to N$^+$-O$^-$ (14). These spectral properties, characteristic of nitrones, are in accordance with the structure of $\alpha$-carboxy-$\alpha$-N-dimethylhydroxime.

Further evidence for the involvement of pyruvate was sought by performing experiments using chorismic acid labeled only in the ring, CH$_3$-NHOH, and enzyme. Again, Dowex 1-acetate column and pyridine-acetic acid buffers were used to separate the different components of the reaction mixture. The fractions were counted and analyzed for hydroxamate with FeCl$_3$. The results of this experiment are shown in Fig. 5. Because the labeled chorismate has been crystallized only once, a control experiment in which ring-$^{14}$C-chorismate, unlabeled chorismate, and CH$_3$-NHOH were incubated in the absence of enzyme and run through a column identical with the one used for the experiment. A small amount of radioactive impurity was eluted with both the experimental and control runs. With the incubation mixture in which enzyme was present, only Fractions 16 to 18 from the Dowex column contained all the FeCl$_3$-positive material and were not radioactive. The radioactivity was eluted between 0.5 to 1 M pyridine-acetic acid and these fractions gave no color with FeCl$_3$. In control experiments none of the fractions gave a color with FeCl$_3$. The fractions were analyzed for hydroxamate by reacting with FeCl$_3$. When $^{14}$C-glutamine (O$\cdot$O$\cdot$O$\cdot$) was used at a specific activity of 0.06 µCi per µmole, $^{14}$C-Chorismate (O$\cdot$O$\cdot$) had an activity of 3 x $10^3$ cpm per mg.

Studies with NH$_2$OH—The formation of hydroxamate by anthranilate synthase with NH$_2$OH required the addition of both chorismate and glutamine. Increasing the concentration of chorismate above its $K_m$ had no effect on the extent or the rate of hydroxamate formation. A large scale reaction mixture containing anthranilate synthase, chorismate, glutamine, and hydroxylamine was incubated for 30 min at 37$^\circ$ and placed on a Dowex 1-acetate column for separation and identification of the hydroxamate (Fig. 6). All fractions were analyzed for hydroxamate by reacting with FeCl$_3$. When $^{14}$C-glutamine was included in the reaction mixture, no radioactivity was associated with the water eluate, which gave a positive color reaction with FeCl$_3$. This observation, as well as the finding that $\gamma$-glutamylhydroxamate is not bound to the Dowex 1-acetate column, suggested that the hydroxamate formed in the reaction with NH$_2$OH is probably $\gamma$-glutamylhydroxamate. This was confirmed by employing $^{14}$C-glutamine in the reaction mixture. The components of the incubation mixture consisting
was subjected to ninhydrin spray. In all cases the enzymatic reagent and a second chromatogram from each solvent system was sprayed with FeC13. y-glutamylhydroxamate was included as a reference. A chromatogram from each solvent system was sprayed with FeC13. y-glutamylhydroxamate was further analyzed by paper chromatography in two solvent systems: (a) n-butyl alcohol, acetic acid-H2O (60:15:25) and (b) ethanol-NH4OH-H2O (80:5:15). Authentic y-glutamylhydroxamate was eluted with water and the glutamic acid was eluted with 0.1 M pyridine-acetic acid buffer, pH 5.85.

Fig. 8. Scheme for the various reactions catalyzed by anthranilate synthase. E, enzyme.

| TABLE II |
|---|
| Effect of NH4OH and CH3-NHOH on glutaminase activity |
| The reaction mixture (final volume 1.0 ml) contained Tris-chloride, pH 8.2 (50 μmoles), EDTA (5 μmoles), 14C-l-glutamine (5 μmoles; 0.63 μCi per μ mole), freshly prepared NH4OH or CH3-NHOH (200 μmoles), anthranilate synthase (0.4 units, specific activity 85). The incubation was carried out at room temperature for 30 min after which the components were separated on a Dowex 1-acetate column, 0.7 × 9.0 cm. The y-glutamylhydroxamate was eluted with water and the glutamic acid was eluted with 0.1 M pyridine-acetic acid buffer, pH 5.85.

| Addition | Chorismate concentration (μmoles) | Glutaminase activity (μmoles) |
|---|---|---|
| None | 1 | 3.25 |
| NH4OH | 1 | 2.65 |
| NH4OH | 10 | 1.85 |
| CH3-NHOH | 1 | 3.25 |
| CH3-NHOH | 10 | 4.0 |

of enzyme, chorismate, 14C-glutamine, and NH4OH were placed on a Dowex 1 column and separated by elution with H2O followed by pyridine-acetic acid. Radioactivity was found to be located in the fractions eluted with H2O as well as in fractions eluted with 0.1 M pyridine-acetic acid. The latter was identified as glutamic acid. However, only the water eluate gave a color reaction with acidic FeCl3. This fraction which contained the hydroxamate was further analyzed by paper chromatography in two solvent systems: (a) n-butyl alcohol, acetic acid-H2O (60:15:25) and (b) ethanol-NH4OH-H2O (80:5:15). Authentic y-glutamylhydroxamate was included as a reference. A chromatogram from each solvent system was sprayed with FeCl3 reagent and a second chromatogram from each solvent system was subjected to ninhydrin spray. In all cases the enzymatic product had the same mobility as authentic y-glutamylhydroxamate. Furthermore, the infrared spectra of the enzymatic product and of the authentic y-glutamylhydroxamate were identical in all respects.

Recently Nagano, Zalkin, and Henderson (13) have demonstrated glutaminase activity in a partially purified preparation of anthranilate synthase in the absence of Mg++ and in the presence of chorismate. We have confirmed this observation with the homogenous anthranilate synthase used in the experiments outlined above (Fig. 7). Glutaminase activity can be demonstrated only in the presence of chorismate. Invariably, a lag of approximately 1 to 3 min was observed in the appearance of glutamic acid. This delay could not be abolished by preliminary incubation of chorismate and enzyme before the addition of glutamine or by using a very large excess of anthranilate synthase. The inset in Fig. 7 demonstrates that, as the chorismate concentration is increased, the rate of glutaminase activity exhibited by anthranilate synthase does not follow linear kinetics.

The effect of NH4OH and CH3-NHOH on the glutaminase activity of anthranilate synthase is shown in Table II. Whereas NH4OH inhibits the glutaminase activity, CH3-NHOH had no effect on the rate or the extent of hydrolysis of glutamine.

DISCUSSION

The results presented demonstrate that although CH3-NHOH is a methyl derivative of NH4OH, its mechanism of inhibition of anthranilate synthase is different from that of hydroxylamine. The formation of hydroxamate with NH4OH required the presence of enzyme, chorismate, and glutamine, whereas only enzyme and chorismate were needed for reaction with CH3-NHOH. The evidence presented indicate that the reaction product is an adduct of pyruvate and CH3-NHOH, α-carboxy-α,N-dimethylnitronitrosyl. Nitrones are generally prepared by reaction of aldehydes and ketones with substituted hydroxylamines and have been extensively studied as useful intermediates in chemical syntheses (14, 15). We have observed that concentrated solutions of α-keto acids react with CH3-NHOH and FeCl3 in strong acids to yield a chromogenic product with an absorption maximum around 500 μm. It should be stressed that even at high concentrations of chorismate the formation of FeCl3-positive material with chorismate and CH3-NHOH required the presence of anthranilate synthase.

The formation of the adduct of pyruvate and CH3-NHOH suggests that a binding site of the enzyme to chorismate may exist through the enolpyruvate side chain. This is supported by the finding that pyruvate can partially substitute for chorismate in eliciting a glutaminase activity and in overcoming the inhibition of anthranilate synthase by tryptophan (13).
Moreover, when the anthranilate synthase reaction was carried out in D_2O, the pyruvate formed not only contained close to 1 atom of deuterium in the methyl group, but also a small yet significant amount of —CHD_2 species because the enzyme-chorismate complex underwent a limited exchange with water (4).

The formation of a hydroxamate with hydroxylamine required both chorismate and glutamine. The identification of the reaction product with NH_2OH as γ-glutamylhydroxamate suggests that anthranilate synthase in the presence of chorismate can form an acylenzyme complex through the γ-carboxyl of glutamic acid and can exhibit glutaminase activity. Evidence for such an activity has recently been provided by Nagano et al. (13). We have confirmed the finding with our homogenous anthranilate synthase preparation. Glutaminase activity can be demonstrated only in the presence of chorismate. Addition of NH_2OH to the reaction mixture containing enzyme, chorismate, and glutamine, results in a decrease of glutamic acid formed with the concomitant production of γ-glutamylhydroxamate. Thus, hydroxylamine competes with water in the hydrolysis of the acyl enzyme.

Although chorismate is essential for demonstrating glutaminase activity of Mg^{++} free-anthranilate synthase, increasing the chorismate concentration had a nonlinear effect on the glutaminase activity. A similar positive cooperative effect could be shown to occur with anthranilate synthase only when studying the effect of chorismate concentration on the inhibition of the over-all reaction (i.e. formation of anthranilic acid) by tryptophan (7). Chorismate, in the absence of tryptophan, does not exhibit a homotropic effect normally observed with regulatory enzymes in which the allosteric ligand affects the affinity for the substrate.

On the basis of the present work the following scheme for the enzymatic reactions catalyzed by the anthranilate synthase can be advanced (Fig. 8). Anthranilate synthase reacts with chorismate to form enzyme-chorismate complex. This complex can combine with ammonia in the presence of Mg^{++} to give anthranilate, pyruvate, and enzyme or react with CH_2NHOH to yield the adduct shown in the figure. The enzyme-chorismate complex also interacts with glutamine. This new complex can undergo hydrolysis with H_2O to give glutamate or give γ-glutamylhydroxamate with NH_2OH or form anthranilate, pyruvate, and glutamine in the presence of Mg^{++}. Further studies to obtain direct evidence for the existence of an enzyme intermediate in anthranilate synthase reaction are in progress.

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