HYDROXAMATE FORMATION BY ANTHRANILATE SYNTHETASE OF ESCHERICHIA COLI K12

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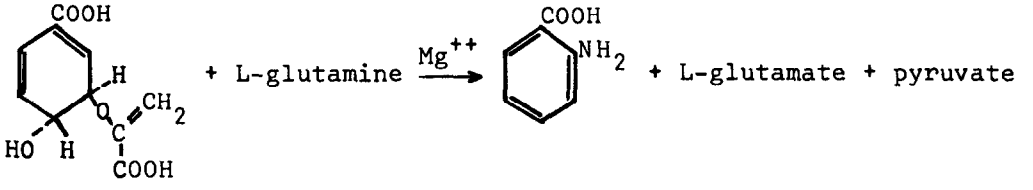
The first reaction specific to tryptophan biosynthesis is the conversion of chorismic acid to anthranilic acid (Gibson and Gibson, 1964). The stoichiometry of this reaction, catalyzed by the enzyme anthranilate synthetase, is



chorismic acid anthranilic acid

(Gibson and Gibson, 1964; Somerville, unpublished results). In Escherichia coli the catalytically active species is a protein complex formed by aggregation of the products of the E and D genes of the tryptophan operon (Ito and Yanofsky, 1966). Anthranilate synthetase is one of a class of amidotransferase enzymes where L-glutamine serves as the physiological nitrogen donor (Meister, 1962). Ammonia, added at relatively high concentrations, will serve as the nitrogen source for anthranilate formation in vitro (Srinivasan and Rivera, 1963; Edwards et al., 1964). Ammonia is also active in vivo (Gibson et al., 1967). Although the overall reaction leading from chorismate to anthranilate has been studied (Baker and Crawford, 1967), neither the structures of possible intermediates between chorismate and

anthranilate nor the details of the reaction mechanism are known. A number of speculative schemes have been advanced (Levin and Sprinson, 1964; Ratledge, 1965; Srinivasan, 1965).

RESULTS

We have found that anthranilate synthetase catalyzes the formation of an hydroxamic acid, as determined by the reactivity of the product in a modified $FeCl_3$ test for hydroxamates (Woolfolk et al., 1966). The basic requirements of the reaction are given in Table 1. In the complete system, all components were at saturating levels and the formation of hydroxamate proceeded linearly with time. It is clear that hydroxamate formation is strictly dependent on chorismate, glutamine, enzyme, and hydroxylamine. A surprising finding was the fact that the omission of Mg^{++} from the reaction mixture allowed hydroxamate formation to proceed at about 80% of the control rate. The addition of excess EDTA to the system likewise had little effect on hydroxamate formation. This is in striking contrast to the overall reaction leading to anthranilate, which is completely inhibited by EDTA and reversed by Mg^{++} (Somerville, unpublished results).

That hydroxylamine interacts with the anthranilate synthetase system was also shown using the standard spectrophotofluorometric assay for this enzyme. These data are presented in Table 2. It can be seen that hydroxylamine (0.2 M) inhibited anthranilate formation by about 30%. N-methyl hydroxylamine also inhibited anthranilate formation quite effectively (75% inhibition at 0.2 M inhibitor concentration) as did O-methyl hydroxylamine (70% inhibition at the same concentration). However, N,O-dimethyl hydroxylamine produced an effect comparable

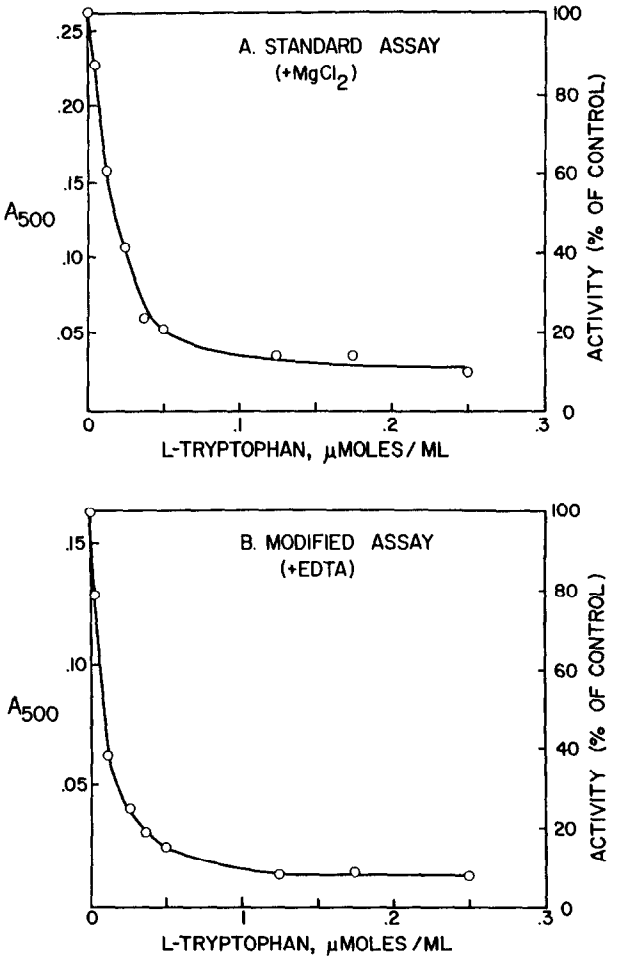


Figure 1. Inhibition by L-tryptophan of hydroxamate formation by anthranilate synthetase. The reaction mixtures are described in the legend to Table 1, except that L-tryptophan was included at the indicated concentration. In B, $MgCl_2$ was omitted and the mixture was supplemented with EDTA (5 μmoles). Reactions were initiated by the addition of enzyme (0.3 mg). Incubation was carried out for 30' at 25°, then stopped by the addition of $FeCl_3$ reagent, as previously described.

in magnitude to that of hydroxylamine (25% inhibition).

In view of the fact that L-tryptophan is a known feedback inhibitor of the anthranilate synthetase reaction (Gibson and Gibson, 1964; Edwards et al., 1964), it was of interest to see whether this amino acid could inhibit hydroxamic acid

Table 1

Hydroxamate Formation by Partially Purified *E. coli* Anthranilate Synthetase

	A_{500}
Complete System	0.208
-L-glutamine	0.012
-chorismate	0.000
-$MgCl_2$	0.163
-enzyme	0.000
-hydroxylamine	0.012

The complete reaction mixture (final volume 1 ml) contained chorismic acid (1 µmole), L-glutamine (100 µmoles), $MgCl_2$ (2 µmoles), EDTA (0.01 mµmole), freshly prepared neutral hydroxylamine (200 µmoles) Tris-HCl buffer, pH 7.8 (10 µmoles), and partially purified anthranilate synthetase (0.54 mg). The reaction was initiated by the addition of enzyme. After incubation at 25° for 30 minutes, the reaction was stopped by adding 0.25 ml of a mixture of equal volumes of 24% trichloroacetic acid, 6N HCl, and 10% $FeCl_3$ in .02$\bar{N}$ HCl (Woolfolk *et al.*, 1966). Denatured protein was removed by centrifugation, and the absorbancy of the supernatant solution at 500 mµ measured in 1.5 ml cuvettes (light path, 10 mm) on a Zeiss PMQ II spectrophotometer.

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formation. Figure 1 shows that enzyme activity is in fact progressively inhibited by increasing concentrations of L-tryptophan, measured either in the presence or absence of Mg^{++}. Both reactions were 50% inhibited at tryptophan concentrations of about 1×10^{-5} $\bar{M}$. This effect is quantitatively comparable to the inhibition observed in the overall reaction leading to the formation of anthranilic acid (Baker and Crawford, 1967; Somerville, unpublished results).

Another method used to show that hydroxylamine reacts in the anthranilate synthetase system was to follow the disappearance of hydroxylamine from reaction mixtures, by a

modification of the method of Frear and Burrell (1955). In this assay, hydroxylamine utilization was dependent upon enzyme, chorismate and glutamine, and was subject to inhibition by L-tryptophan in a similar manner to that shown for anthranilate or hydroxamic acid formation.

To study the effect of hydrogen ion concentration on hydroxamate formation, a series of 14 assays (see Legend to Table 1

Table 2

Effects of Hydroxylamine and Methyl Hydroxylamines on Anthranilate Formation

Expt	Inhibitor	Concentration (M)	Rate of Anthranilate Synthesis (mμmoles/min)
1	--	--	1.30
	NH_2OH	0.05	1.26
	"	0.10	0.95
	"	0.15	0.70
	"	0.20	0.90
2	--	--	2.60
	CH_3NHOH	.05	1.68
	"	.10	1.13
	"	.15	.65
	"	.20	.64
	NH_2OCH_3	.05	1.98
	"	.10	1.28
	"	.15	.91
	"	.20	.78
	CH_3NHOCH_3	.20	1.96

The complete reaction mixture (final volume, 1 ml) contained chorismic acid (100 mμmoles, L-glutamine (5 μmoles) $MgCl_2$ (2 μmoles), EDTA (0.33 mμmoles), Tris buffer, pH 7.8, 10 μmoles, partially purified anthranilate synthetase (11 μgrams in Expt 1, 55 μgrams in Expt 2), and freshly prepared neutral hydroxylamine or methyl hydroxylamine at the indicated concentration. The reaction was carried out at 25° C in an Aminco-Bowman spectrophotofluorimeter and was initiated by adding enzyme. The appearance of anthranilate as a function of time (activation wavelength, 320 mμ, emission wavelength, 400 mμ) was quantitated by reference to a previously prepared calibration curve. Values given are initial reaction velocities observed during the first 1-2 minutes after enzyme was added.

for method) were run in the range from pH 6 to pH 10.5. The buffers used were phosphate, Tris, and bicarbonate, depending on the pH of the assay. A typical pH dependency curve was obtained with a broad optimum in the range from pH 7.5 to 8.5. This is similar to that observed for anthranilate formation in the spectrophotofluorometric assay (Baker and Crawford, 1967; Somerville, unpublished results).

A number of try E mutants, blocked in the ability to convert chorismate to anthranilate, were tested for their ability to catalyze hydroxamic acid formation. No reaction took place when extracts of try E nonsense mutants were employed, but three CRM-forming try E mutants, out of a total of 8 tested,

Table 3

Hydroxamate Formation by Anthranilate Synthetase Mutants

Mutant	Type	Protein (mg)	A_{500} +chorismate	A_{500} -chorismate	A_{500} Δ
T3	E-CRM	2.52	.438	.136	.302
10220	"	.84	.52	.157	.363
7706	"	.84	.456	.129	.327
5947	"	.87	.105	.064	.041
E-4	"	.97	.072	.046	.026
E-6	"	1.22	.080	.100	----
E-7	"	.88	.068	.066	.002
7827	"	1.04	.105	.102	.003
7735	E-CRM-less	.86	.052	.038	.014

The assay system was as described in the legend to Table 1. Crude extracts contain factor(s) which cause a chorismate-independent formation of $FeCl_3$-reactive material, hence the need for a control tube lacking chorismate. Mutant 7735 has been provisionally designated as a CRM-less mutant on the basis of its ability to form phosphoribosyltransferase which is available for complexing with ASase component I from the try D nonsense mutant 9778 (Ito and Yanofsky, 1966). Mutant 7735 does not respond to amber or ochre suppressors.

catalyzed the formation of significant amounts of $FeCl_3$-reactive material (Table 3). Mutants T3 and 10220 have been previously described (Yanofsky, 1955; Yanofsky and Ito, 1966). Mapping experiments indicate that T3 and 10220 are located at or near the same site within the try E gene, while 7706 maps between the try E alleles T3 and T15 (Somerville, unpublished experiments). Mutant 5947 is known to map toward the try D side of the E gene (Somerville and Yanofsky, 1965). The remaining mutants listed in Table 3 have not been characterized genetically.

DISCUSSION

At present we are unable to specify the structure of the hydroxamic acid formed in our reaction mixtures. On electrophoresis and co-electrophoresis in 0.05 M Tris-borate buffer at pH 8.2, the product could be easily separated from authentic γ-L-glutamyl hydroxamate. Furthermore, γ-L-glutamyl hydroxamate was not hydrolyzed upon incubation with anthranilate synthetase in the presence or absence of chorismate, L-glutamine, or $MgCl_2$. Understanding how hydroxylamine or its methylated derivatives interact with anthranilate synthetase is complicated by the fact that at neutral pH, hydroxylamine reacts with activated acyl groups to yield O-acylhydroxylamines as well as hydroxamic acids (Jencks, 1958). Thus, the $FeCl_3$ test employed in the present work may not be a true measure of the degree to which hydroxylamine or its derivatives intervene in the anthranilate synthetase system. Additional work is required before we can begin to understand the chemistry of the system.

Certain CRM-forming try E mutants show reactivity in the presence of hydroxylamine, although they are unable to form

anthranilate from chorismate. Taken together with the observations on wild-type anthranilate synthetase, this tends to suggest that the reaction in wild-type cells consists of at least two steps:

1. A Mg^{++} independent, tryptophan inhibitable conversion of substrates to an intermediate reactive toward hydroxylamine.

2. The Mg^{++}-dependent conversion of intermediate(s) to anthranilate.

If one imagines that both steps are susceptible to mutational alteration, one interpretation of our findings could be that some E-CRM mutants are unable to form hydroxamic acid due to alteration of an amino acid residue critical for step 1 or steps 1 and 2.

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