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Anthranilate Hydroxylase from *Aspergillus niger*: Evidence for the Participation of Iron in the Double Hydroxylation Reaction

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Evidence was obtained for the participation of iron in the double hydroxylation reaction catalyzed by anthranilate hydroxylase from *Aspergillus niger* (UBC 814). Omission of iron from the growth medium gave inactive preparations of anthranilate hydroxylase which could be reactivated by incubating the enzyme preparations with ferric citrate. The enzyme was susceptible to inhibition by metal chelating agents. The K_i for *o*-phenanthroline, which inhibited the enzyme activity non-competitively with respect to anthranilate, was calculated to be 0.9 mM. The inhibition by *o*-phenanthroline was counteracted by ferric complexes such as ferric-ethylenediaminetetraacetic acid and ferric citrate. Anthranilate afforded protection against inhibition by *o*-phenanthroline.

Iron has often been implicated in the reaction catalyzed by several oxygenases. Whereas evidence for the participation of this metal ion in dioxygenase reactions leading to the fission of the aromatic ring of dihydroxy phenols is well documented (5), its involvement in the hydroxylation of aromatic compounds is not well established.

Anthranilate hydroxylase isolated from *Aspergillus niger* (UBC 814) catalyzes the conversion of anthranilate to 2,3-dihydroxybenzoate (7). The reaction represents a double hydroxylation. In the present paper, evidence is presented for the indispensability of iron in the anthranilate hydroxylase reaction.

MATERIALS AND METHODS

Organism. *A. niger* strain UBC 814 was the organism used. It was grown on a chemically defined medium supplemented with 0.1% anthranilic acid. Details of the medium and the conditions of cultivation of the organism are described in an earlier communication (8). The spores used for the inoculation of the medium deficient in copper or iron were obtained from the organism grown for three generations on a medium deprived of copper or iron, respectively.

Preparation of iron- or copper-deficient medium. Before the addition of micronutrients, the standard medium, which was prepared in deionized water, was shaken with 5% (wt/vol) solution of 8-hydroxyquinoline (resublimed before use) (or salicylaldoxime). Redistilled chloroform (phosgene-free) was used to extract the quinolinates and the excess of 8-hydroxyquinoline (or copper complex of salicylaldoxime and the excess of salicylaldoxime), and redistilled ether was used to remove traces of chloroform.

Preparation of anthranilate hydroxylase (8). All the operations were done at 0 to 4 C. Freshly harvested mycelium (10 g) were ground with an equal amount of glass powder for 15 min and extracted with 30 ml of 2.5 mM sodium phosphate buffer, pH 7, containing 1 mM reduced glutathione. The extract was passed through a cheese cloth and centrifuged at 12,000 × *g* for 20 min. Protamine sulfate (3 ml of 2% aqueous solution) was added, with gentle stirring, to 27 ml of crude extract. After standing for 15 min, the precipitate was discarded and the supernatant fluid (30 ml) was stirred for 30 min with 2 g (wet weight) of diethylaminoethyl-cellulose, which had been washed and adjusted to pH 7 with 2.5 mM phosphate buffer. The suspension was rapidly filtered through a Buchner funnel, and the clear filtrate was used as the partially purified anthranilate hydroxylase.

Enzyme assay. The reaction mixture (1 ml) contained tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.2 (80 μmol), anthranilic acid (0.2 μmol), nicotinamide adenine dinucleotide phosphate (0.2 μmol), glucose-6-phosphate (0.25 μmol), glucose-6-phosphate dehydrogenase (10 mU), and 0.5 ml of enzyme (4-8 mU). After incubating for 20 min at 30 C, the reaction was stopped by the addition of 0.2 ml of 0.5 N HCl, and the hydroxylase activity was determined as described previously (8).

One unit of anthranilate hydroxylase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of 2,3-dihydroxybenzoic acid per

min. Specific activity is expressed as units per milligram of protein.

Protein. The protein concentration was estimated colorimetrically by the method of Lowry et al. (6), with bovine serum albumin as standard.

RESULTS

Metal ion dependence of anthranilate hydroxylase. Partially purified anthranilate hydroxylase (native enzyme) was not activated by any metal ion. The metal dependence of the reaction, however, could be demonstrated by altering the cultural conditions of *A. niger*. When the organism was grown on a medium deprived of iron, there was considerable decrease in anthranilate hydroxylase activity (Table 1). There was no change in the levels of the enzyme activity when the medium was made copper deficient.

A study of the effect of various metal ions on their ability to restore the activity of the hydroxylase prepared from the mycelial felts of *A. niger* grown under iron-deficient conditions has shown that only ferric complexes such as ferric citrate and ferric-ethylenediaminetetraacetic acid (EDTA) could restore about 85% of original activity (observed with anthranilate hydroxylase preparations obtained from mycelia grown on normal medium) (Table 2).

The effect of preincubation time on the activation of the partially purified enzyme isolated from *A. niger* grown in the absence of iron, by ferric citrate, is shown in Table 3. A 15-min preincubation time was necessary for optimal activation.

Effect of metal chelating agents on enzyme activity. The results presented in Table 4 show the effect of various metal chelating agents on the activity of the enzyme. It can be seen that *o*-phenanthroline is the most potent inhibitor. The study of the degree of inhibition at various concentrations of anthranilic acid showed that the inhibition by *o*-phenanthroline was of the noncompetitive type. In Fig. 1, the reciprocal velocity of anthranilate hydroxylase reaction was plotted against the inhibitor concentration at two different concentrations of the substrate. From the Dixon's plot (3) thus obtained, K_i for *o*-phenanthroline was calculated to be 0.9 mM.

The inhibition by salicylaldoxime seems to be a nonspecific one, as the activity could not be restored by copper or iron in any of their forms.

Reversal of *o*-phenanthroline inhibition. Various metal ions were tried for the reversal of inhibition by *o*-phenanthroline of anthranilate hydroxylase. Surprisingly, none, including ferrous and ferric ions, was effective in counteracting the inhibitory effect of the metal chelating agent. However, it was interesting to find that ferric complexes could effect a reversal of the inhibition (Table 5).

TABLE 1. *Iron dependence of anthranilate hydroxylase*[a]

Medium	Specific activity
Normal	5.58
Iron deficient	0.28
Copper deficient	5.16

[a] *A. niger* was grown on the normal medium as well as on iron- or copper-deficient medium for 48 h. Details of culture conditions and preparation of enzyme and assay of the activity are given under Materials and Methods.

TABLE 2. *Reactivation of anthranilate hydroxylase from A. niger grown on iron-deficient medium*[a]

Addition (1 mM final concn)	Specific activity
None	0.28
Ferric citrate	4.90
Ferric-EDTA	4.60
Control[b]	5.60

[a] Conditions of growth of the organism and enzyme assay are as described in Materials and Methods, except that the enzyme was preincubated with the above components for 15 min before the addition of the other constituents of the reaction mixture.

[b] Control represents the specific activity of anthranilate hydroxylase grown on standard iron-sufficient medium.

TABLE 3. *Effect of preincubation time on the reactivation by ferric citrate of anthranilate hydroxylase from A. niger grown on iron-deficient medium*[a]

Time (min)	Specific activity
0	0.28
5	2.20
10	4.20
15	4.90
20	4.90
30	4.90

[a] The fungus was grown on the standard medium deprived of iron (see Materials and Methods). The enzyme was preincubated with ferric citrate (1 mM) for various time intervals prior to the addition of other constituents of the reaction mixture. Standard assay conditions were used to determine the enzyme activity.

TABLE 4. *Effect of metal chelating agents on anthranilate hydroxylase activity*[a]

Metal chelating agent	Concn (mM)	Inhibition (%)
EDTA	0.5	0
	1.0	7
8-Hydroxyquinoline	0.5	23
	1.0	37
α,α'-Dipyridyl	0.5	27
	1.0	41
o-Phenanthroline	0.5	46
	1.0	60
	3.0	80
Salicylaldoxime	0.5	40
	1.0	52

[a] The enzyme was preincubated with the test compound for 10 min before the addition of other constituents of the reaction mixture. The reaction was carried out under standard assay conditions. The organism was grown on standard medium containing iron.

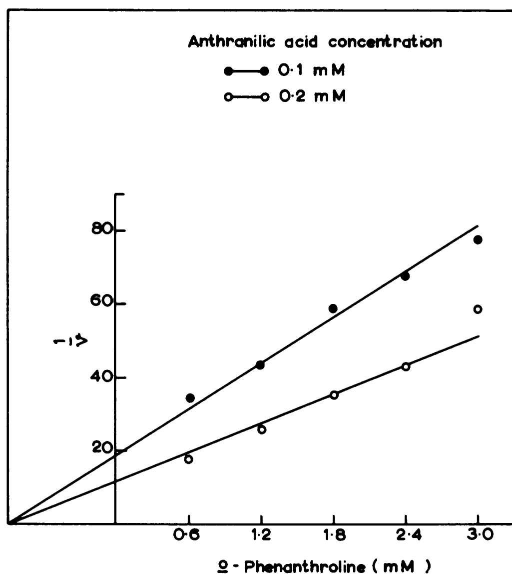

FIG. 1. *Dixon's plot for the determination of K_i for o-phenanthroline. The velocity of the reaction at different inhibitor concentrations was determined at two fixed concentrations of anthranilic acid. Except for the variation in the concentrations of o-phenanthroline and anthranilic acid, the other conditions were the same as described in Materials and Methods.*

Protection of enzyme against o-phenanthroline inhibition. Preincubation with substrates, anthranilic acid, and NADP, reduced form (NADPH), was tried, to test whether either of these substrates had any protective effect on the enzyme against inhibition by *o*-phenanthroline. It was found that only anthranilic acid protected the enzyme from inhibition by *o*-phenanthroline (Table 6).

DISCUSSION

Although iron dependence of double hydroxylation reactions catalyzed by anthranilic

TABLE 5. *Reversal of o-phenanthroline inhibition of anthranilate hydroxylase*[a]

Addition	Concn (mM)	Inhibition (%)
None		0
o-Phenanthroline	1	60
	3	80
o-Phenanthroline + ferric-EDTA	3	10
	1	
o-Phenanthroline + ferrous-EDTA	3	75
	1	
o-Phenanthroline + ferric citrate	3	8
	1	
o-Phenanthroline + siderochrome	3	78
	1	
o-Phenanthroline + cytochrome c	3	70
	1	

[a] The enzyme obtained from the mycelium grown on normal medium was incubated with *o*-phenanthroline and the iron compound, each for 10 min, before adding the other ingredients of the reaction mixture. Standard assay conditions were used to determine the enzyme activity.

TABLE 6. *Protection of anthranilate hydroxylase against o-phenanthroline inhibition*[a]

Addition	Concn (mM)	Inhibition (%)
Control		
o-Phenanthroline	1.0	50
Anthranilic acid + *o*-phenanthroline	0.2	15
	1.0	
NADPH + *o*-phenanthroline	0.2	53
	1.0	

[a] The enzyme from the mycelium grown on normal medium was preincubated with NADPH or anthranilic acid for 10 min and incubated with *o*-phenanthroline for another 10 min before starting the reaction by adding the remaining constituents of the reaction mixture. The formation of 2,3-dihydroxybenzoic acid was determined as described in Materials and Methods.

acid hydroxylase (9) and benzene oxygenase (4) from *Pseudomonas* has been reported, the obligatory participation of iron in these reactions has not been unequivocally established. The present studies with *A. niger* have provided definite evidence for the participation of iron in the double hydroxylation reaction catalyzed by anthranilate hydroxylase. Omission of iron in the growth medium gave inactive preparations of anthranilate hydroxylase which could readily be reactivated by ferrous or ferric ions or by ferric citrate. Chelates of trace metal ions play a fundamental role in the transport and metabolism of metal ions. Thus ferric-EDTA and ferric citrate are known to exchange iron with transferrin (1, 2). Although the specific inhibition by *o*-phenanthroline was not reversed by ferrous or ferric ions, ferric complexes such as ferric-EDTA and ferric citrate counteracted the inhibitory effect. The noncompetitive nature of inhibition of the hydroxylase activity by *o*-phenanthroline can be explained as being due either to the removal of iron from the metal site on the enzyme or to its strong binding to the enzyme to form a ternary complex of enzyme-iron-*o*-phenanthroline. It is interesting to find that anthranilic acid could afford protection against *o*-phenanthroline inhibition. It would appear that in the presence of substrate, the enzyme-bound iron involved in the catalytic process is not readily accessible to form a chelate with *o*-phenanthroline.

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