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Anthranilate Hydroxylase from *Aspergillus niger*: New Type of NADPH-Linked Nonheme Iron Monooxygenase

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Anthranilate hydroxylase from *Aspergillus niger* catalyzes the oxidative deamination and dihydroxylation of anthranilic acid to 2,3-dihydroxybenzoic acid. This enzyme has been purified to homogeneity and has a molecular weight of 89,000. The enzyme is composed of two subunits of 42,000 with 2 gram-atoms of nonheme iron per mol. Fe^{2+}-chelators like α,α′-dipyridyl and *o*-phenanthroline are potent inhibitors of the enzyme activity. Absorption and fluorescence spectra of the enzyme offer no evidence for the presence of other cofactors like flavin. Flavins and flavin-specific inhibitors like atebrin have no effect on the activity of the enzyme. The enzyme incorporates one atom of oxygen each from $^{18}O_2$ and $H_2{}^{18}O$ into the product 2,3-dihydroxybenzoic acid. Based on these studies, it is concluded that anthranilate hydroxylase from *A. niger* is a new type of NADPH-linked nonheme iron monooxygenase.

Anthranilic acid is metabolized via catechol in bacteria and fungi (1, 10, 13, 21, 23, 26). The initial reaction in bacteria is the oxidative decarboxylation and deamination of anthranilic acid to catechol (12, 13, 26). This reaction is catalyzed by anthranilate hydroxylase, which incorporates both the atoms of molecular oxygen into catechol and hence is a dioxygenase (14). Similar dioxygenases are involved in the bacterial oxidation of benzoic acid (28), benzene (3), toluene (25), and other aromatic hydrocarbons (7). These are multienzyme systems in which the component proteins are organized into an electron transport chain (3, 7, 9, 25, 28). Electrons are transferred from the primary donor, NADH, to the terminal protein component, which catalyzes the dihydroxylation of the substrate (3, 7, 9, 25, 28).

In fungi, anthranilic acid is converted to 2,3-dihydroxybenzoic acid (2,3-DHBA) and then decarboxylated to catechol (1, 10, 21). The initial reaction is catalyzed by anthranilate hydroxylase, which has previously been partially purified from *Aspergillus niger* and characterized as an iron-containing protein (17, 20, 22). Subsequently, we purified this enzyme to homogeneity and confirmed that it is a simple iron enzyme with molecular weight of 89,000 (24). In contrast, anthranilate hydroxylase from *Trichosporon cutaneum* is an NADPH-dependent flavoprotein monooxygenase of molecular weight 94,000 (16).

In the present paper, we provide further evidence that purified anthranilate hydroxylase from *A. niger* is an iron enzyme and does not contain any other cofactor. Experiments with $^{18}O_2$ and $H_2{}^{18}O$ indicate that this enzyme is a new type of NADPH-linked nonheme iron monooxygenase.

MATERIALS AND METHODS

Materials. Anthranilic acid, purchased from Eastman Kodak Co., Rochester, N.Y., was recrystallized from hot water. Ion-exchange resins and NADPH were obtained from Sigma Chemical Co., St. Louis, Mo. Biogel P-100, $^{18}O_2$ (99%), and $H_2{}^{18}O$ (99.5%, enriched) were obtained from Bio-Rad Laboratories, Richmond, Calif. Alumina and tricalcium phosphate gels were prepared by published procedures (5).

Organism. *Aspergillus niger* (UBC 814) was grown on Byrde medium (4) supplemented with 0.1% (wt/vol) anthranilic acid. The mycelia were harvested after 48 h of growth and used as an enzyme source.

$^{18}O_2$- and $H_2{}^{18}O$-incorporation studies. The $^{18}O_2$ experiment was performed in the apparatus shown in Fig. 1. The flask was flushed with nitrogen, and 200 ml of 0.05 M Tris–hydrochloride (pH 8.2) was introduced into chamber A. Anthranilic acid and NADPH (80 μmol each in 7 ml of 0.05 M Tris–hydrochloride) were placed in bulb B. The flask was alternately evacuated and flushed with nitrogen through a vacuum line at D and with the valve to bulb C open. Purified anthranilate hydroxylase (30 ml, 1,500 U) was introduced into chamber A by removal of the attachment B. The flask was evacuated once more, and stopper D was closed. A mixture of 40 ml of 99% $^{18}O_2$ and 15 ml of $^{16}O_2$ was introduced into the flask by piercing the rubber tube attached to stopper D. The apparatus was brought to atmospheric pressure with nitrogen, and contents were equilibrated for 10 min. The reaction was initiated by tilting the substrates from bulb B. After 20 min, stopper C was closed, and the reaction was terminated by the addition of 5 ml of concentrated HCl. The product 2,3-DHBA was extracted with ether and examined by mass spectroscopy. Bulb C was detached, and the ratio of $^{18}O_2$ to $^{16}O_2$ was determined.

The same reaction was examined in 0.05 M Tris–hydrochloride containing $H_2{}^{18}O$ (115 μl of 99.5% $H_2{}^{18}O$-enriched water), 1.2 μmol each of NADPH and anthranilic acid (60 μl) and 10 U of anthranilate hydroxylase. The product was isolated as described earlier for mass spectral analysis. For determination of any exchange of oxygen between 2,3-DHBA and water, the compound was incubated for 20 min in 1 ml of 0.05 M Tris–hydrochloride containing 15% $H_2{}^{18}O$-enriched water and 10 U of anthranilate hydroxylase. Mass spectral analysis was performed on a Finnigan Model 2100 mass spectrometer at an ionizing voltage of 70 eV with a solid probe. Spectra were recorded at a probe temperature of 240°C and an ion source temperature of 325°C. The Ramsay dipyridyl method (19) was used with appropriate controls for estimation of enzyme-bound iron. Standard iron at 0 to 20 μg/ml was used to construct a

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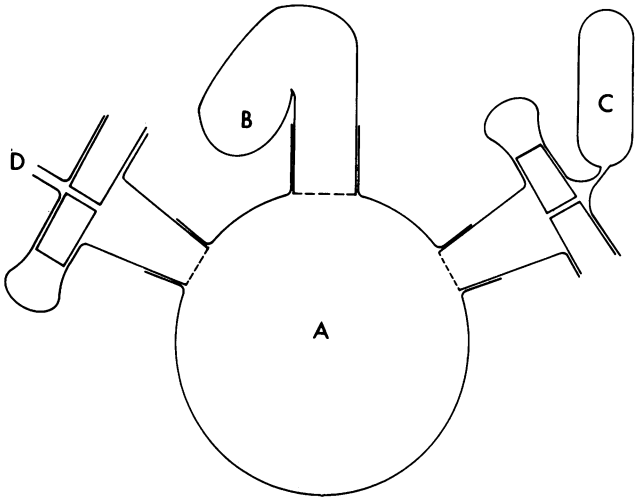

FIG. 1. Apparatus in which the incorporation of $^{18}O_2$ into 2,3-DHBA was performed. Reaction was carried out in the main chamber A while substrates anthranilic acid and NADPH were held in bulb B before the start of the reaction. Bulb C was used to analyze the atmosphere in the apparatus and D was attached to a rubber tubing and taken to a vacuum line.

calibration curve. Protein contents of various preparations were estimated by the method of Lowry et al. (15) with bovine serum albumin as standard.

Purification of anthranilate hydroxylase from *A. niger*. Mycelium (45 g) was ground with glass powder and extracted with 180 ml of 0.025 M sodium phosphate buffer (pH 7.0) containing 0.01 M reduced glutathione (GSH). The filtrate obtained from a cheese cloth was clarified at 12,000 × *g*, and the supernatant was subjected to protamine sulfate precipitation (1 mg/ml). The supernatant was suspended in DEAE-cellulose (25 g) for 15 min and filtered on a Büchner funnel. The 40 to 80% ammonium sulfate precipitate of the filtrate was dissolved in phosphate buffer containing GSH (6 ml) and fractionated on a Biogel P-100 column (65 by 2 cm). Active fractions were pooled and further purified by successive negative adsorption on alumina gel (0.16 g/ml), tricalcium phosphate gel (0.15 g/ml) and DEAE-cellulose column (6 by 1 cm). The enzyme was then precipitated at 80% ammonium sulfate and redissolved in 2 ml of phosphate buffer containing GSH. The final step was positive adsorption on a DEAE-Sephadex A-50 column (1.5 by 10 cm) from which the enzyme was eluted with a NaCl gradient (0 to 0.2 M) in phosphate buffer containing GSH.

RESULTS

It was reported by Sreeleela et al. (20, 22) that due to extreme instability of anthranilate hydroxylase from *A. niger*, only partial purification was achieved. Presently, this enzyme has been purified to homogeneity by a procedure that takes less than 8 h; the details of this process are presented in Table 1. Thiol agents like GSH, dithiothreitol, and 2-mercaptoethanol stabilized the enzyme during purification, yielding final preparations with specific activities of 193, 117, and 77 U, respectively (see footnote *a* of Table 1 for a definition of units). In the absence of thiols, the pure enzyme obtained had a specific activity of only 20 U. Purified anthranilate hydroxylase had a half-life of about 14 h at 4°C; hence, it had to be used for experiments immediately or stored as ammonium sulfate precipitate at −20°C. In the latter state, the enzyme was stable for over 3 months with no noticeable loss in activity. Analysis of the purified enzyme by polyacrylamide gel electrophoresis (6) revealed a single band. The molecular weight of the purified protein was found to be 89,000; however, on 10% sodium dodecyl sulfate–polyacrylamide gels (27), the enzyme migrated as a single band with a relative mobility that corresponded to a molecular weight of 42,000. The native enzyme is composed of two subunits.

Sreeleela et al. (17, 20) provided indirect evidence that partially purified anthranilate hydroxylase from *A. niger* is an iron enzyme. Indeed, the purified enzyme was estimated to contain 2 gram-atoms of iron per mol. Absorption spectra of the concentrated solution of the enzyme revealed a prominent peak at 412 nm which could be attributed to bound iron (24). Although *o*-phenanthroline and α,α'-dipyridyl were reported as inhibitors for the partially purified enzyme (20), the effects are more pronounced with the homogeneous preparation (Table 2). Flavin adenine dinucleotide, flavin mononucleotide, riboflavin, or flavin adenine dinucleotide analogs like atebrin and aminopterin had no effect on the enzyme activity. A fluorescence scan also offered no evidence for the presence of enzyme-bound flavin.

$^{18}O_2$ and $H_2{}^{18}O$ incorporation studies. Details of the experimental set up are provided above. A control reaction was performed under normal atmospheric conditions, and the product 2,3-DHBA was isolated and identified by the procedure described by Sreeleela et al. (20). Part of the mass spectrum of the isolated product is shown in Fig. 2a. The parent ion peak is at *m/e* 154, and the base peak attributable to a fragment formed by loss of water from the parent compound is at *m/e* 136 (Fig. 2a, inset). This spectrum is identical to that of authentic 2,3-DHBA. The mass spectrum of the product from the reaction conducted in the presence of $^{18}O_2$ is shown in Fig. 2b. The composition of the atmosphere was 80.8% N_2, 4.28% $^{16}O_2$, and 14.28% $^{18}O_2$. The major parent ion peak at *m/e* 156 (Fig. 2b) and the base peak at *m/e* 138 (Fig. 2b, inset) are consistent with the incorporation of one atom of ^{18}O into 2,3-DHBA. The mass spectrum of the product derived from the reaction conducted in the presence of $H_2{}^{18}O$ (shown in Fig. 3 and inset) further confirms the monooxygenase nature of the enzyme from *A. niger*. In a separate control experiment, no detectable ex-

TABLE 1. Purification of anthranilate hydroxylase from *A. niger*

Step	Total protein (mg)	Activity (U)[a]	Sp act (U/mg of protein)	Recovery (%)
1. Crude	405	324	1	
2. Protamine sulfate supernatant	243	485	3	
3. DEAE-cellulose treatment	150	3,165	21	100
4. $(NH_4)_2SO_4$, 40 to 80% Biogel P-100 column	22	1,549	70	49
5. Alumina-gel supernatant	16	1,469	93	46
6. Tricalcium phosphate gel supernatant	12	1,134	98	36
7. DEAE-cellulose column	6	915	150	28
8. DEAE-Sephadex A50 column chromatography	3.7	715	193	23

[a] The assay mixture in 1 ml of 0.05 M Tris–hydrochloride contained 1.2 μmol each of NADPH and anthranilic acid and the appropriate amount of the enzyme from various steps. After incubation for 20 min at 30°C, the amount of 2,3-DHBA formed was quantitated by the colorimetric method of Arnow (2). A unit is defined as the amount of enzyme required to produce 1 nmol of 2,3-DHBA per min.

TABLE 2. Effects of various agents on the activity of anthranilate hydroxylase from *A. niger*[a]

Reagent	Final concentration (mM)	Inhibition (%)
None		0
8-Hydroxyquinoline	0.5	30
	1.0	40
α,α'-Dipyridyl	0.5	50
	1.0	80
o-Phenanthroline	0.25	50
	0.50	80
	1.0	95
Atebrin	0.5	0
	1.0	5
Aminopterin	0.5	0
	1.0	0
Flavin adenine dinucleotide, flavin mononucleotide, or Riboflavin	0.1	0
	0.25	15

[a] The reaction mixture in 1 ml of the buffer contained 24 U of the enzyme, 1.2 μmol each of anthranilic acid and NADPH, and indicated amounts of listed compounds. The test compounds were incubated with the enzyme for 10 min before addition of substrates. The amount of 2,3-DHBA formed in the absence of test compounds (480 nmol) was considered 100% activity.

change occurred between the oxygen atoms of 2,3-DHBA and $H_2{}^{18}O$.

The ratios of the relative abundances of parent ion (156 to 154 *m/e*) and base peaks (138 to 136 *m/e*) of 2,3-DHBA isolated from various reaction conditions are summarized in Table 3. These values are much higher for the reactions conducted in the presence of $^{18}O_2$ and $H_2{}^{18}O$ than that for nonenzymatic exchange, indicative of incorporation of an atom of ^{18}O. Also, the initial enrichment of $H_2{}^{18}O$ and the extent of incorporation of ^{18}O into 2,3-DHBA are stoichiometric (Table 3). Such a correlation, however, was not obtained for the $^{18}O_2$ reaction, in which the ratio of the ion intensities is about 1.9 as opposed to the $^{18}O_2/^{16}O_2$ ratio of 3.35 (Table 3).

DISCUSSION

Catechol is the central metabolite in the degradation of anthranilic acid by both bacteria (13, 26) and fungi (1, 10, 21). However, there are significant differences in the enzymology of initial oxidation of anthranilic acid. Bacterial anthranilate hydroxylase is an NADH-dependent dioxygenase (12, 13, 26), and although it is not fully characterized, it is composed of at least two protein components (26). In contrast, fungi oxidize anthranilic acid to 2,3-DHBA, which is subsequently decarboxylated to catechol (1, 10, 18, 20,

TABLE 3. Ratios of the relative abundance of parent ion and base peaks of 2,3-DHBA isolated under various reaction conditions

Reaction condition	Ratio of relative abundance of:[a]	
	Parent ion	Base peak
$^{18}O_2/^{16}O_2$ ratio of 3.35	1.82	1.92
$H_2{}^{18}O/H_2{}^{16}O$ ratio of 0.114	0.11	0.112
Nonenzymatic exchange[b]	0.007	0.009

[a] The ratios of the relative abundance of the peaks were calculated from the peak intensities obtained as computer output. The parent ion peaks were 156 and 154 m/e, and the base peaks were 138 and 136 m/e.

[b] These values are comparable to those of authentic 2,3-DHBA.

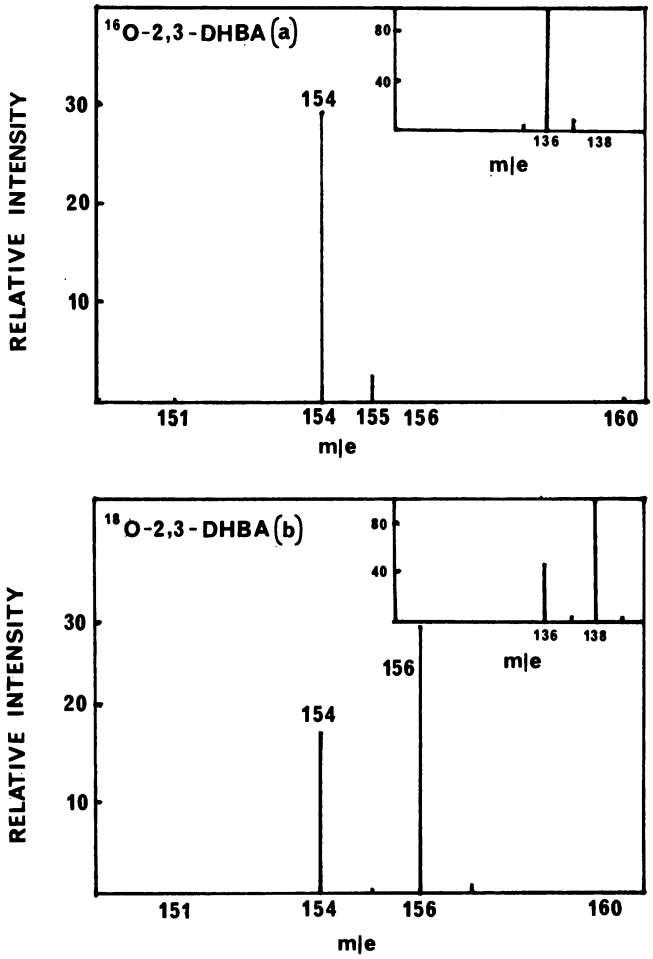

FIG. 2. Mass spectrum of 2,3-DHBA isolated from control reaction performed under normal atmospheric conditions (a) and from reaction conducted in the presence of $^{18}O_2$ (b).

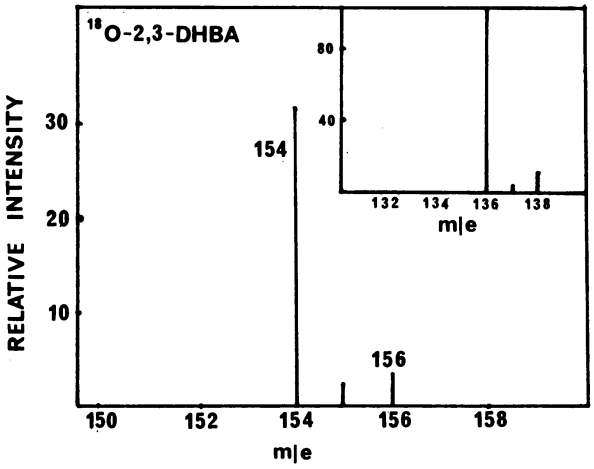

FIG. 3. Mass spectrum of 2,3-DHBA isolated from $H_2{}^{18}O$-reaction.

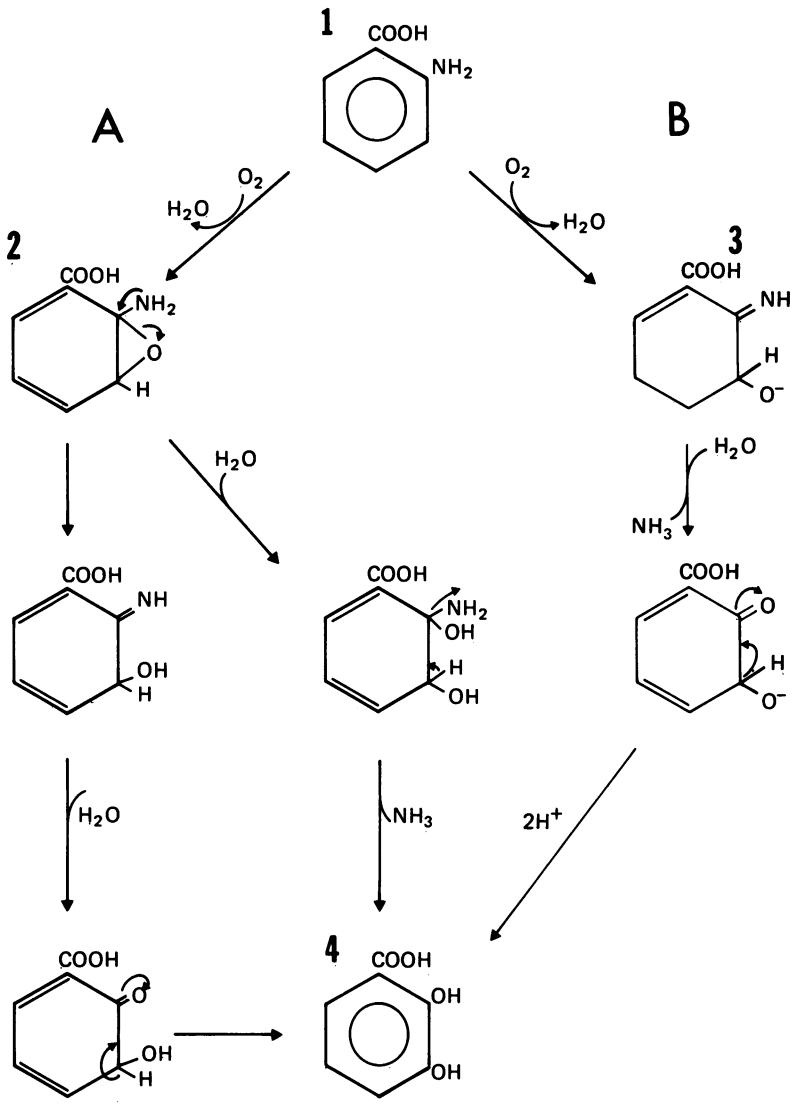

FIG. 4. Possible mechanisms for the enzymatic oxidation of anthranilic acid to 2,3-DHBA in *Aspergillus niger* and (B) *Trichosporon cutaneum* (16). 1, Anthranilic acid. 2, 'Epoxide' intermediate. 3, 'Imine' intermediate. 4, 2,3-DHBA.

21). Anthranilate hydroxylase from *A. niger* was partially purified and was characterized as an iron enzyme by indirect evidence, but ^{18}O incorporation studies were not performed (17, 20, 22). Floss et al. (8) showed that one atom of ^{18}O is incorporated into 2,3-DHBA during the growth of *Claviseps paspali* in a medium containing $H_2{}^{18}O$, but the experiment was not done with the isolated enzyme. Anderson and Dagley (1) provided conclusive evidence that anthranilate hydroxylase from *Trichosporon cutaneum* is a monooxygenase. This enzyme is a flavoprotein with a molecular weight of 94,000 but composed of two subunits with molecular weights of 50,000, each with 1 mol of flavin adenine dinucleotide (16).

Presently, anthranilate hydroxylase from *A. niger* has been purified to homogeneity (Table 1). Several lines of evidence indicate that this is an iron enzyme, containing 2 gram-atoms of bound iron per mol. Earlier we reported that the enzyme forms a pink complex with *o*-phenanthroline that is spectrally identical to Fe^{2+}-*o*-phenanthroline complex (24). Apoenzyme prepared by dialysis of enzyme-*o*-phenanthroline complex was reconstituted only with Fe^{2+} ions (24). Electron spin resonance studies with the purified enzyme showed that bound Fe^{2+} is oxidized to Fe^{3+} during catalysis (24). Although the partially purified anthranilate hydroxylase is slightly inhibited by Fe^{2+}-specific chelators (20), the purified enzyme was more susceptible (Table 2). Absorption and fluorescence spectra of the pure enzyme offered no evidence for the presence of bound flavin or iron-sulfur cluster. Added flavins or flavin-specific inhibitors also had no effect on the activity of the enzyme (Table 2). All of these observations clearly indicate that, unlike the one from *Trichosporon cutaneum* (16), anthranilate hydroxylase from *A. niger* is an iron enzyme.

Experiments with $^{18}O_2$ and $H_2{}^{18}O$ showed that anthranilate hydroxylase from *A. niger*, like analogous fungal enzymes (1, 8), is a monooxygenase (Fig. 2 and 3). There was stoichiometric incorporation of ^{18}O from $H_2{}^{18}O$ but not from $^{18}O_2$ (Table 3), which could be due to inadequate equilibration of the reaction mixture. However, the fact that there is no peak at *m/e* 158 (Fig. 2b) unequivocally establishes the monooxygenase nature of this enzyme.

The nonheme iron-containing anthranilate hydroxylase from *A. niger* is a new type of NADPH-dependent monooxygenase hitherto not described in the classification

of oxygenases (11). The various mechanisms for the hydroxylation of anthranilic acid to 2,3-DHBA are shown in Fig. 4. Powlowski and Dagley (16) showed that in the presence of sodium cyanoborohydride, anthranilate hydroxylase from *T. cutaneum* yielded both 2,3-DHBA and 3-hydroxyanthranilic acid as products. This observation led to the proposal of an imine intermediate and the reaction scheme in Fig. 4B. However, the imine intermediate was not detected in the reaction catalyzed by anthranilate hydroxylase from *A. niger*, although it cannot be disqualified. An alternate candidate is an epoxide (Fig. 4A) which is known to be formed in reactions catalyzed by eucaryotic monooxygenases (9). The possible electronic rearrangements the epoxide could undergo at the active site of anthranilate hydroxylase from *A. niger* are depicted in Fig. 4A.

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