ANTHRANILIC ACID HYDROXYLASE FROM ASPERGILLUS NIGER

P. V. Subba Rao, N. S. Sreeleela, R. Premkumar and C. S. Vaidyanathan
Department of Biochemistry, Indian Institute of Science,
Bangalore-12, India

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The in vivo conversion of radioactive tryptophan to anthranilic acid and 2,3-dihydroxybenzoic acid by submerged cultures of Claviceps paspali was shown by Groeger and his co-workers (1965). More recently, Subba Rao et al. (1967a) reported that washed mycelial felts of Aspergillus niger incorporate the radioactivity from DL-tryptophan-C^{14} (benzene ring-labeled) into anthranilic acid, 3-hydroxyanthranilic acid, 2,3-dihydroxybenzoic acid and catechol. However, the conversion of anthranilic acid to 2,3-dihydroxybenzoic acid by cell-free preparations has not been demonstrated. In the present paper we report the demonstration of a soluble anthranilic acid hydroxylase from Aspergillus niger which is different from the anthranilic acid hydroxylases reported so far from microbes and higher plants.

EXPERIMENTAL

Aspergillus niger (UBC 814) was grown for 48 hr. at 30° in 1 litre flasks on a synthetic medium (Byrde et al., 1956) supplemented with 0.1% anthranilic acid. The mycelia were harvested before the advent of sporulation, washed repeatedly with distilled water and stored at -20°.

Washed mycelium (10 g) was macerated at 4° with equal weight of glass powder and extracted with 0.025 M sodium phosphate buffer, pH 7 containing 0.001 M GSH (30 ml). After passing through a cheese cloth, the extract was centrifuged at 10000xg for 10 min. To the supernatant (27 ml) was added with stirring, 2% protamine sulfate solution (3 ml)

and the resulting precipitate was removed by centrifugation. Fifteen ml of the supernatant was stirred with 2 g (wet weight) of DEAE-cellulose which has been previously washed and adjusted to pH 7 with 0.025 M phosphate buffer (Peterson and Sober, 1962). After 30 min. the suspension was filtered through a Buchner funnel and the clear filtrate was used as the enzyme.

A reaction mixture (2 ml) containing Tris-HCl buffer, pH 8.2 (60 umoles), anthranilic acid (0.4 umole), NADP (0.4 umole), glucose-6-phosphate (0.5 umole), glucose-6-phosphate dehydrogenase (0.2 unit) and 1 ml enzyme was incubated for 20 min. at 30°. The reaction was terminated by the addition of 0.5 N HCl (0.2 ml) and the mixture extracted with 5 ml of peroxide-free ether. Suitable aliquots from the ether layer were used for the estimation of anthranilic acid (Venkataraman et al., 1948) or 2,3-dihydroxybenzoic acid (Nair and Vaidyanathan, 1964).

Isolation of the reaction product: For the isolation of the enzymic product from anthranilic acid, a large scale incubation mixture (400 ml) containing 100 ml of Tris-HCl buffer, 25 mg of anthranilic acid, 10 mg of NADPH, and 100 ml of enzyme were incubated for 1 hr. at 30°, acidified to pH 2 with 1 N HCl and extracted twice with equal volume of peroxide-free ether. After shaking with anhydrous Na_2SO_4, the solvent was taken to dryness at room temperature. The residue was dissolved in ethyl acetate and subjected to chromatography on Whatman No. 1 filter paper using 2% formic acid as the solvent. The fluorescent band corresponding to 2,3-dihydroxybenzoic acid was eluted with hot 95% ethanol. The compound was finally crystallized from hot water.

RESULTS AND DISCUSSION

Identification of the reaction product: The enzymic product formed from anthranilic acid in the presence of partially purified anthranilic acid hydroxylase was identified as 2,3-dihydroxybenzoic acid by comparing

its properties with those of an authentic sample. Chromatographically, the compound was indistinguishable from synthetic 2,3-dihydroxybenzoic acid in different solvents (Reio, 1958). Both synthetic and enzymic samples of 2,3-dihydroxybenzoic acid gave similar color reactions with p-nitroaniline, diazotized sulfanilic acid and ferric chloride (Ibrahim and Towers, 1960). The ultraviolet spectrum of the isolated product in ethanol was identical with that of authentic 2,3-dihydroxybenzoic acid showing peaks at 248 and 316 mu.

Incubation of the enzymically isolated product with the purified 2,3-dihydroxybenzoic acid carboxy-lyase from A. niger, resulted in its decarboxylation to catechol (Subba Rao et al., 1967b). The identity of the decarboxylation product was established by comparing with authentic catechol.

Optimum pH and cofactor requirement: Anthranilic acid hydroxylase had an optimum pH around 8.2 and showed an absolute requirement for NADPH. Various other cofactors like NADH, THFA, FMN and FAD could not replace NADPH either alone or in various combinations. Even in crude preparations, there was no activity without the addition of NADPH.

Time course and stoichiometry: Even though crude preparations further metabolized 2,3-dihydroxybenzoic acid, treatment with DEAE-cellulose resulted in the complete removal of 2,3-dihydroxybenzoic acid carboxy-lyase. From the data presented in Table I, it is apparent that there was a mole to mole relationship between the anthranilic acid disappeared and 2,3-dihydroxybenzoic acid formed in the presence of partially purified anthranilic acid hydroxylase.

Taniuchi et al. (1964) reported an anthranilic acid hydroxylase from Pseudomonas which catalyzed the direct conversion of anthranilic acid to catechol without apparently involving any detectable intermediate.

TABLE I

Time course and stoichiometry

Time in min.	Anthranilic acid disappeared (umole)	2,3-Dihydroxybenzoic acid formed (umole)
5	0.036	0.033
10	0.054	0.052
15	0.065	0.065
20	0.094	0.090
30	0.145	0.138

This enzyme showed a requirement for NADH and FAD. Anthranilic acid hydroxylase from Tecoma stans, on the other hand, is a simple nono-oxygenase which hydroxylates anthranilic acid to 3-hydroxyanthranilic acid in the presence of NADPH and THFA (Nair and Vaidyanathan, 1965). Partially purified anthranilic acid hydroxylase from A. niger represents a third type which is involved in the formation of 2,3-dihydroxybenzoic acid with a requirement for NADPH alone. Further studies on the purification and properties of this enzyme are in progress.

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REFERENCES

Byrde, R.J.W., Harris, J.F. and Woodcock, D., Biochem. J., 64, 154 (1956).
Groeger, D., Erge, D. and Floss, H.G., Z. Naturforsch., 20b, 856 (1965).
Ibrahim, R.K. and Towers, G.H.N., Arch. Biochem. Biophys., 87, 125 (1960).
Nair, P.M. and Vaidyanathan, C.S., Anal. Biochem., 7, 315 (1964).
Nair, P.M. and Vaidyanathan, C.S., Biochim. Biophys. Acta, 110, 521 (1965).
Peterson, E.A. and Sober, H.A., in Methods in Enzymology, Vol. 5, edited by Colowick, S.P. and Kaplan, N.O., Academic Press, New York, p.3 (1962).
Reio, L., J. Chromatography, 1, 338 (1958).
Subba Rao, P.V., Moore, K. and Towers, G.H.N., Biochem. Biophys. Res. Commun., 28, 1008 (1967a).
Subba Rao, P.V., Moore, K. and Towers, G.H.N., Arch. Biochem. Biophys. 122, 466 (1967b).
Taniuchi, H., Hatanaka, M., Kuno, S., Hayaishi, O., Nakazima, M., and Kurihara, N., J. Biol. Chem., 239, 2204 (1964).
Venkataraman, A., Venkataraman, P.R. and Lewis, H.B., J. Biol. Chem., 173, 641 (1948).