3 -HYDROXYBENZOATE 6-HYDROXYLASE FROM PSEUDOMONAS AERUGINOSA

EDYE E. GROSECLOSE and D. W. RIBBONS*
Department of Biochemistry
University of Miami School of Medicine
and
Howard Hughes Medical Institute
Miami, Florida, 33152, U.S.A.

Received October 22, 1973

SUMMARY: An inducible 3-hydroxybenzoate 6-hydroxylase has been purified to homogeneity from Pseudomonas aeruginosa. It contains FAD as a prosthetic group. 3-Hydroxybenzoate is quantitatively hydroxylated to give gentisate with equimolar consumptions of NADH and O_2. NADPH will substitute as an electron donor, and several aromatic analogues of 3-hydroxybenzoate stimulate reduced nucleotide oxidation by the enzyme with formation of both hydrogen peroxide and hydroxylated products. Of various analogues of 3-hydroxybenzoate, those substituted in 2,4,5 and 6-positions are competent substrates; partial uncoupling of electron flow from hydroxylation with concomitant formation of hydrogen peroxide and "gentisates" occurs. The "natural" product of the reaction, gentisate, is an effector in that it stimulates NADH oxidation with the formation of hydrogen peroxide. 3-Hydroxybenzoate 6-hydroxylase thus resembles other flavoprotein hydroxylases in the general regulatory properties dictated by their aromatic substrates, pseudosubstrates or effectors.

Two routes for the metabolism of 3-hydroxybenzoate by the aerobic pseudomonads have been described; namely, hydroxylation at the 6-position to give gentisate and hydroxylation at the 4-position to give protocatechuate (1, 2). The products in each case are the substrates for ring fission enzymes, gentisate oxygenase and protocatechuate 4,5-oxygenase, respectively. Yano and Arima (1) described the preparation of cell-free extracts of Pseudomonas desmolytica which, in the presence of NADH, or an NADH generating system, could hydroxylate 3-hydroxybenzoate and the product was further metabolized to fumarate and pyruvate. To our knowledge 3-hydroxybenzoate 6-hydroxylase has not been purified and characterized though gentisate has been shown to

* Howard Hughes Medical Institute Investigator

TABLE 1

PURIFICATION OF 3-HYDROXYBENZOATE 6-HYDROXYLASE FROM Ps. AERUGINOSA

Fraction	Volume (ml)	Protein (mg/ml)	Specific Activity (μmoles, min^{-1}, mg $protein^{-1}$)	Yield (%)
Crude Extract	1710	35.7	0.28	100
55,000g Supernatant	1500	30.3	0.34	89.5
Protamine Sulfate DEAE Cellulose	822	8.3	1.26	49.0
$(NH_4)_2SO_4$ Ppt	124	29.0	2.7	55.5
Sephadex G-100 $(NH_4)_2SO_4$ Ppt	31	33.5	4.9	29.6
Hydroxylapatite	150	1.05	24	22

be the product of 3-hydroxybenzoate oxidation in Ps. acidovorans (2).

This report gives a purification procedure for an inducible 3-hydroxybenzoate 6-hydroxylase from Ps. aeruginosa T1 (3) and demonstrates that this enzyme is very similar to the other flavoprotein hydroxylases, p-hydroxybenzoate hydroxylase (4, 5, 6), salicylate hydroxylase (7, 8, 9), orcinol hydroxylase (10) and melilotate hydroxylase (11, 12) prepared from pseudomonads. An accompanying paper describes the purification and properties of a 3-hydroxybenzoate 4-hydroxylase from Ps. testosteroni (13).

EXPERIMENTAL

Ps. aeruginosa strain T1 was grown in 10 l stirred fermentors in mineral salts media (3) supplemented with 0.25% 3-hydroxybenzoate. Cell pastes were suspended in 2 volumes of 20mM KH_2PO_4-NaOH buffer, pH 7.2 containing 0.3% mercaptoethanol and 1mM 3-hydroxybenzoate and disrupted in a French Press and centrifuged at 55,000g for 30 min. The supernatant was treated with protamine sulphate and after centrifugation applied directly to a DEAE cellulose column (5 x 50 cm); the hydroxylase was eluted with a linear KCl gradient (0-0.5M, 2 l). Yellow fractions containing the 3-hydroxybenzoate 6-hydroxylase activity were

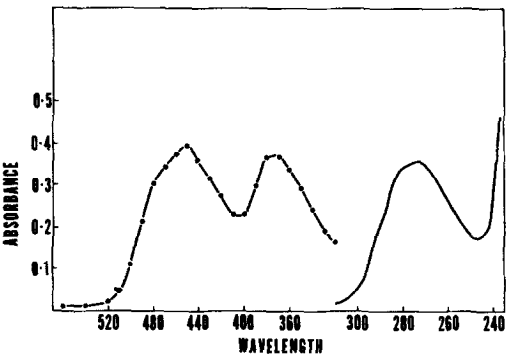

Figure 1. Absorption spectrum of 3-hydroxybenzoate 6-hydroxylase (3.3mg/ml for the visible and 0.33mg/ml for the u.v.).

pooled, precipitated with $(NH_4)_2SO_4$ and the redissolved precipitate was chromatographed on Sephadex G-100. Effluent fractions containing activity were again pooled. The enzyme was fractionated with $(NH_4)_2SO_4$ dialyzed and finally chromatographed on hydroxylapatite.

3-Hydroxybenzoate 6-hydroxylase was assayed by the consumption of oxygen, using a Clark O_2 electrode at 30°. Assay mixtures contained in 3ml; 20mM KH_2PO_4-NaOH buffer, pH 8.0 (2.8-2.9ml); 25mM NADH (40 l); 25mM 3-hydroxybenzoate (20 l) and enzyme (2-100 l).

Details of individual experiments appear in the legends.

RESULTS AND DISCUSSION

Prosthetic Group

3-Hydroxybenzoate 6-hydroxylase from Ps. aeruginosa was purified quite easily as outlined in Table 1. Even after the initial DEAE cellulose chromatography it became obvious that this enzyme was a flavoprotein and probably similar to the other flavin hydroxylases from pseudomonads. FAD, but not FMN, could stimulate oxygen consumption in standard assays with some preparations. The absorption spectrum of the purified enzyme is shown in Figure 1. The prosthetic group was identified as FAD by chromatography and its absorption spectrum (14). Disc gel electrophoresis of the preparation gave one major diffuse band which possessed the enzymic activity as indicated by the reduction of a tetrazolium salt in the assay. A molecular weight value

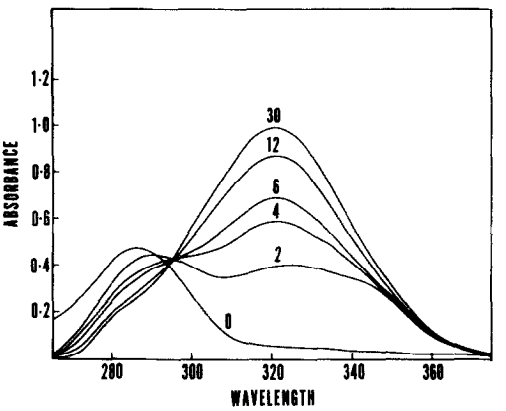

Figure 2. Absorption changes during the conversion of 3-hydroxybenzoate to gentisate by 3-hydroxybenzoate 6-hydroxylase. The cuvette contained: 20mM KH_2PO_4-NaOH buffer, pH 8.0 (2.5ml); Sigma alcohol dehydrogenase-NAD kit (0.2ml); 3-hydroxybenzoate 6-hydroxylase (40µl); ethanol (30µl) and 25mM 3-hydroxybenzoate (20µl). Reference cell lacked the aromatic substrate. Numbers on the curves refer to the times (in minutes) the spectrum was scanned, before (0) and after initiation of the reaction with ethanol. Temperature, 29°.

of approximately 85,000 was given from sedimentation equilibrium measurements, and velocity sedimentation gave an S_{20W} value of 5.3. From the absorbance at 450nm approximately 0.9moles of FAD are present per 85,000g of protein.

Stoichiometry of the Reaction

3-Hydroxybenzoate 6-hydroxylase catalyzes the conversion of 3-hydroxybenzoate to gentisate with equimolar consumption of both NADH and O_2. A slight excess of each were consumed per mole of 3-hydroxybenzoate supplied and gentisate was formed (Table 2). A small amount of hydrogen peroxide is also produced which probably occurs when gentisate accumulates and acts as a non-hydroxylatable effector for electron flow from NADH to oxygen. The spectral changes associated with hydroxylation of 3-hydroxybenzoate to gentisate in the presence of an NADH generating system are shown in Fig. 2. Gentisate was identified as the product by comparison with authentic gentisate by thin-layer chromatography by absorption, fluorescence and mass spectroscopy, and as a substrate for gentisate oxygenase (from Ps. aeruginosa). The hydroxylation of 3-hydroxybenzoate by the 6-hydroxylase then conforms to the general equation for mono-oxygenases:

TABLE 2

STOICHIOMETRY OF 3-HYDROXYBENZOATE 6-HYDROXYLASE REACTIONS

Aromatic substrate supplied	NADH supplied	O_2 consumed	NADH consumed	H_2O_2 formed	"Gentisate" formed
3-Hydroxybenzoate					
500	250	261	N.D.	10	238
250	500	280	290	24	242
4-Fluoro-3-hydroxybenzoate					
200	1000	201	220	<5	N.D.

All values are in nmoles. The standard assay was used for simultaneous measurements of O_2 and absorbance changes. N.D.-not determined.

TABLE 3

SUBSTRATE SPECIFICITY OF 3-HYDROXYBENZOATE 6-HYDROXYLASE

Aromatic Effector	Product	Relative rate	% Uncoupled
3-Hydroxybenzoate	2,5-Dihydroxybenzoate	100	0-5
2,3-Dihydroxybenzoate	2,3,5-Trihydroxybenzoate	10.5	0-10
3,4-Dihydroxybenzoate	2,4,5-Trihydroxybenzoate	9	0-5
3,5-Dihydroxybenzoate	2,3,5-Trihydroxybenzoate	7	0-5
Gentisate	--	5	100
2-Amino-3-hydroxybenzoate	N.I.	24	20-30
3-Hydroxy-5-methylbenzoate	2,5-Dihydroxy-3-methyl-benzoate *	31	0
4-Fluoro-3-hydroxybenzoate	4-Fluoro-2,5-dihydroxy-benzoate	36	0-5

Data in the last two columns were obtained with reaction mixtures containing: 50mM KH_2PO_4-NaOH buffer, pH 8.0 (2.9ml); 25mM substrate (20µl); 25mM NADH (20µl) and 3-hydroxybenzoate 6-hydroxylase (20µl containing 50µg of protein). H_2O_2 production was measured with catalase, and all products were identified by GC-MS of their trimethylsilyl derivatives by Dr. P. J. Chapman, University of Minnesota, St. Paul, and by chromatography absorption and fluorescense spectroscopy. N.I.- not identified. *GC-MS analysis not available.

3-Hydroxybenzoate + $NADH_2$ + O_2 → → gentisate + NAD + H_2O

Substrate Specificity

A common feature of all the flavin hydroxylases so far studied is the role of the aromatic substrate as a regulator of electron flow from reduced nicotinamide nucleotides to oxygen. This property is often shared by several analogues of the true substrate although they may not themselves be hydroxylated in the catalytic process; H_2O_2 is then the product of oxygen reduction. Such compounds have been called pseudo-substrates (9) or effectors (6). In addition certain analogues are also capable of being hydroxylated at lowered efficiency with formation of both a hydroxylated product and H_2O_2, i.e., electron flow is partially uncoupled from the hydroxylation reactions (13, 15, 16, 17). The effector role of the aromatic compounds appears to be two-fold; (a) the rate of flavin reduction by reduced nicotinamide nucleotides is greatly stimulated (8, 12, 15, 17, 18, 19) and (b) the apparent Km for the reduced nucleotide is considerably lowered.

Table 3 shows the results of a preliminary survey for aromatic substrates (or effectors) of 3-hydroxybenzoate 6-hydroxylase. The enzyme is versatile in that it can accept analogues of 3-hydroxybenzoate which are substituted in the 2,4,5 and 6- positions of the aromatic ring. The prerequisite structural requirements appear to be a carboxyl group and a hydroxyl group in a 1,3-orientation. It is not certain whether all of the substituted analogues tested, which stimulate respiration, are hydroxylated, or merely act as effectors for the facilitated reduction of flavin by NADH. The data of Hopper and Chapman (20) suggest that 3-hydroxybenzoate 6-hydroxylase from another pseudomonad also possesses a remarkable versatility for transforming alkyl analogues of 3-hydroxybenzoate but uncoupling of electron flow from hydroxylation was not investigated. Like salicylate and orcinol hydroxylases, NADH (apparent Km 0.16mM) is the preferred electron donor for 3-hydroxybenzoate 6-hydroxylase; NADPH (apparent Km 0.8mM) will substitute.

ACNOWLEDGEMENTS

The authors are grateful to Dr. P. J. Chapman for the GC-mass spectral data and for gifts of the fluoro- and methyl analogues of 3-hydroxybenzoate. The expert assistance of Mr. J. L. Michalover is appreciated.

REFERENCES

1. Yano, K. and Arima, K. J. Gen. Appl. Microbiol. 4, 241 (1958).
2. Wheelis, M. L., Palleroni, N. J. and Stanier, R. Y. Arch. Mikrobiol. 59, 302 (1967).
3. Ribbons, D. W. J. Gen. Microbiol. 44, 225 (1966).
4. Hosokawa, K. and Stanier, R. Y. J. Biol. Chem. 241, 2453 (1966).
5. Yano, K., Morimoto, M., Higashi, N. and Arima, K., in "Biological and Chemical Aspects of Oxygenases", Ed. by K. Bloch and O. Hayaishi, p. 329. Maruzen Co., Ltd.
6. Howell, L. G. and Massey, V. Biochem. Biophys. Res. Commun. 40, 887 (1970).
7. Katagiri, M., Maeno, H., Yamamoto, S. and Hayaishi, O. J. Biol. Chem. 240, 3414 (1965).
8. Katagiri, M. and Takemori, S., in Flavins and Flavoproteins, 3rd International Symposium, p. 447. Ed. H. Kamin, University Park Press, Baltimore (1971).
9. White-Stevens, R. H. and Kamin, H. Biochem. Biophys. Res. Commun. 38, 882 (1970).
10. Ohta, Y. and Ribbons, D. W. FEBS Letters 11, 189 (1970).
11. Levy, C. C. J. Biol. Chem. 242, 147 (1967).
12. Stickland, S. and Massey, V. J. Biol. Chem. 248, 2944 (1973).
13. Michalover, J. L. and Ribbons, D. W. Biochem. Biophys. Res. Commun. (accompanying paper) (1973).
14. Kilgour, G. L., Felton, S. P., Huennekens, F. M. J. Am. Chem. Soc. 79, 2254 (1957).
15. White-Stevens, R.H. and Kamin, H. J. Biol. Chem. 247, 2358 (1972).
16. Ribbons, D. W. and Ohta, Y. FEBS Letters 12, 105 (1970).
17. Howell, L. G., Spector, T. and Massey, V. J. Biol. Chem. 247, 4340 (1972).
18. Ribbons, D. W., Ohta, Y. and Higgins, I. J., in The Molecular Basis of Electron Transport, Miami Winter Symposia, Vol. 4, p. 251, Ed. by J. Schultz and B. F. Cameron, Academic Press, Inc., New York (1972).
19. Nakamura, S., Ogura, Y., Yano, K., Higashi, N. and Arima, K. Biochemistry 9, 3235 (1970).
20. Hopper, D. J. and Chapman, P. J. Biochem. J. 122, 19 (1971).