3-HYDROXYBENZOATE 4-HYDROXYLASE FROM PSEUDOMONAS TESTOSTERONI

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SUMMARY: 3-Hydroxybenzoate 4-hydroxylase has been purified to homogeneity from extracts pf Ps. testosteroni. It is a flavoprotein (FAD) which catalyzes the transformation of 3-hydroxybenzoate to protocatechuate with equimolar consumption of NADPH and O_2. NADH is a poor substitute for NADPH. Several analogues of 3-hydroxybenzoate substituted in the 2,4,5 and 6 positions, act as effectors and substrates for NADPH oxidation but with varying efficiencies of hydroxylation. 2,3-, 2,5-, 3,5-dihydroxybenzoates, 3-hydroxyanthranilate, 2-fluoro-5-hydroxybenzoate and 4-fluoro-3-hydroxybenzoate are competent substrates.

Of the two established pathways for the metabolism of 3-hydroxybenzoate by aerobic bacteria (1, 2), the possession of the route via protocatechuate has been suggested as a significant taxonomic character for the positive identification of the species testosteroni in the acidovorans group of non-fluorescent pseudomonads (2, 3). 3-Hydroxybenzoate 4-hydroxylase is the first enzyme of the pathway that enables Pseudomonas testosteroni to grow on 3-hydroxybenzoate as sole source of carbon (2). Subsequent ring fission, of the protocatechuate formed, by a 4,5-oxygenase yields from each mole, two moles of pyruvate and one mole of formate (4, 5) or pyruvate and oxaloacetate (6). 3-Hydroxybenzoate 4-hydroxylase has only previously been described as an activity in crude extracts of pseudomonads (1, 2). This report summarizes the purification and some general characteristics of the enzyme from Ps. testosteroni. volume, 4 l. Pooled active fractions were free from protocatechuate

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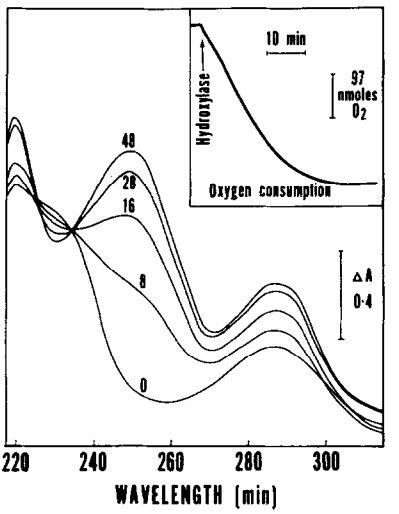

Figure 1. Simultaneous measurement of oxygen consumption and the spectral changes that occur during 3-hydroxybenzoate oxidation by 3-hydroxybenzoate 4-hydroxylase. The reaction mixture contained: 50mM KH_2PO_4-NaOH buffer, pH 7.5 (3ml); 10mM 6-phosphogluconate (0.1ml); 10mM NADP (4µl); 6-phosphogluconate dehydrogenase (10µl); 25mM 3-hydroxybenzoate (20µl) and 3-hydroxybenzoate 4-hydroxylase (15µl containing 82µg of protein). Temperature, 29°. The reference cuvet contained the same less 3-hydroxybenzoate. The numbers relate to the times (in minutes) at which spectra were recorded. There was no change in the spectrum after 40 minutes.

4,5-oxygenase and showed an apparent loss of 40% of the original activity. Ammonium sulphate fractionation between 40 and 60 per cent saturation and dissolution of the precipitate gave specific activities between 3 and 6µmoles of O_2 consumed, min^{-1}, $(mg\ of\ protein)^{-1}$. Three independent preparations purified in a similar way were diluted to 175ml and dialysed against 5mM KH_2PO_4-NaOH buffer, pH 7.5, before application to a hydroxylapatite column. 3-Hydroxybenzoate 4-hydroxylase was not absorbed and the first 188ml of effluent contained all the activity. The effluent was applied to a DEAE cellulose column as before and eluted with a 0.15-0.35M KCl gradient, total volume 1 l. The yellow fractions that eluted with a specific activity of between 10 and 12 were pooled, concentrated with ammonium sulphate and stored on ice. All operations were conducted between 4-10°. The purification is summarized in Table 1.

RESULTS AND DISCUSSION

Properties of 3-Hydroxybenzoate 4-hydroxylase.

TABLE 1

PURIFICATION PROCEDURE FOR 3-HYDROXYBENZOATE 4-HYDROXYLASE

	Fraction	Volume (ml)	Protein concn (mg/ml)	Specific Activity†	Total Activity (units)	Recovery %
1.	Crude extract	370	18.7	0.25	1730	100
2.	DEAE cellulose $(NH_4)_2SO_4$ Sephadex G-100	555	9.4	3.0	1535	89
3.	DEAE cellulose	120	1.9	3.0	690	40
	$(NH_4)_2SO_4$ ppt	4.8	12.7	5.67	345	20
4.	Hydroxylapatite*	188	1.4	7.0	1850	22
5.	DEAE cellulose	45	2.4	11.8	1280	16

* Three independent purifications of the enzyme up to step 3 were pooled, and used for steps 4 and 5. Total activity applied to the hydroxylapatite column was 1600 units, contained in 175ml of 5mM KH_2PO_4-NaOH buffer, pH 7.5 with substrate and mercaptoethanol. † units/mg of protein. A unit of activity is defined as the consumption of one μmoles of oxygen, min^{-1}.

The purification gave 110 mg of 3-hydroxybenzoate 4-hydroxylase from 1.1kg wet weight of cells. SDS Disc-gel electrophoresis revealed one major protein component and one minor band, and ultracentrifugation with Schlieren optics gave a single symmetrical peak with an S_{20w} value of 7.2. Sedimentation equilibrium centrifugation measurements provided a molecular weight of approximately 145,000. The absorption spectrum of 3-hydroxybenzoate 4-hydroxylase is typical of other flavoprotein mono-oxygenases and the flavin prosthetic group was shown to be FAD by chromatography and visible absorption spectroscopy of the protein free nucleotide. The flavin spectrum of the enzyme is bleached by dithionite, and by NADPH in the presence of 3-hydroxybenzoate under anaerobic conditions.

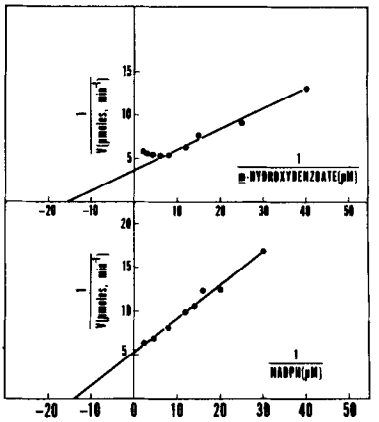

Figure 2. Lineweaver-Burke plots of the effect of 3-hydroxybenzoate and NADPH concentration on reaction velocity, measured by oxygen consumption.

Its properties are compared with 3-hydroxybenzoate 6-hydroxylase from a fluorescent pseudomonad, Ps. aeruginosa described in an accompanying contribution (7), and to other flavin hydroxylases (8, 9, 10, 11, 12, 13 and references cited in 7). A 3-hydroxybenzoate 4-hydroxylase has recently been partially purified from Aspergillus niger (15 and Subba Rao, personal communication), and shown to be a flavoprotein.

EXPERIMENTAL

The enzyme was assayed polarographically by measuring the 3-hydroxybenzoate dependent oxidation of NADPH, using a Clark oxygen electrode. The usual assay contained: 50mM KH_2PO_4-NaOH buffer, pH 7.5 (2.9ml); enzyme solution (5-50μl); 25mM NADPH (40μl) and 25mM 3-hydroxybenzoate (20μl); temperature, 30°C. 3-Hydroxybenzoate 4-hydroxylase was purified from the strain of Ps. testosteroni of Dagley and Patel (16), ATCC 17454 [Stanier #79 (3)] as outlined below: Cells were harvested from minimal media supplemented with 0.5% 3-hydroxybenzoate as sole carbon source. Cell pastes were suspended in 2 volumes of 50mM KH_2PO_4, pH 7.5 containing 0.3% mercaptoethanol and 1mM 3-hydroxybenzoate and disrupted by one passage through a French Press. All subsequent solutions contained mercaptoethanol and substrate.

The supernatant after centrifugation for 20 min. at 37,000g was diluted,

TABLE 2

STOICHIOMETRY OF HYDROXYLATION OF 3-HYDROXYBENZOATE(S)

Aromatic substrate supplied	NADPH supplied	O_2 consumed	NADPH consumed	"Protocatechuates" formed	H_2O_2 formed
3-Hydroxybenzoate					
500	1000	487	510	481	13
2-Fluoro-5-hydroxybenzoate					
350	1000	384	386	N.D.	16
3,5-Dihydroxybenzoate					
1250	125	140	110	137	16

All values are nmoles, and were obtained by simultaneous measurements of O_2 and NADH consumption; "protocatechuates" were determined with protocatechuate 4,5-oxygenase and H_2O_2 with catalase. N.D.--not determined.

with respect, to phosphate 2.5-fold, and the enzyme was adsorbed on to DEAE cellulose column (5 x 25cm) which was flushed with 20mM KH_2PO_4-NaOH buffer pH 7.5 until protein stopped emerging in the eluate. A steep 0-0.5M KCl gradient (total vol. 1 l) eluted the 3-hydroxybenzoate 4-hydroxylase along with protocatechuate 4,5-oxygenase. Active fractions were concentrated with solid $(NH_4)_2SO_4$ to 66 per cent saturation and the redissolved precipitate applied to a Sephadex G-100 column. Active fractions were pooled and applied to a DEAE-cellulose column (2.5 x 25cm) and eluted with a shallow 0-0.5M KCl gradient, total

Conversion of 3-Hydroxybenzoate to Protocatechuate.

The formation of protocatechuate from 3-hydroxybenzoate was demonstrated by u.v. spectroscopy. Figure 1 shows the spectral changes that occur during 3-hydroxybenzoate oxidation catalyzed by 3-hydroxybenzoate 4-hydroxylase in the presence of an NADPH generating system. The final spectrum achieved is identical to that of protocatechuate. It can be calculated from absorbance changes that occur at 250nm and 280nm that 3-hydroxybenzoate is quantitatively

TABLE 3

SUBSTRATE SPECIFICITY OF 3-HYDROXYBENZOATE 4-HYDROXYLASE FROM PS. TESTOSTERONI

Effector	Apparent Km (effector) (mM)	Apparent Km (NADPH) (mM)	Relative Activity Vmax (units/mg of protein)	% Uncoupled
3-Hydroxybenzoate	0.03	0.07	5	0-5
2,3-Dihydroxybenzoate	0.06	0.07	5	20-30
2,5-Dihydroxybenzoate	0.5	0.14	7	20-30
3,5-Dihydroxybenzoate	0.04	0.05	7	10-20
3-Hydroxyanthranilate	0.08	0.05	5	30-40
2-Fluoro-5-hydroxybenzoate	0.03	0.02	N.D.	0-5
4-Fluoro-3-hydroxybenzoate	0.12	0.15	N.D.	60-70

N.D.--not determined.

converted to protocatechuate (Table 2). Other experiments using the simultaneous assay of O_2 and NADPH consumption (Table 2) show that the stoichiometric relationships of the reactions are those expected of a mono-oxygenase (mixed-function oxidase) reaction:

3-hydroxybenzoate + $NADPH_2$ + O_2 → → protocatechuate + NADP + H_2O

Hydrogen peroxide is not formed in significant amounts during hydroxylation. The identity of the product of hydroxylation (and its quantitative formation) with protocatechuate, was confirmed by thin-layer chromatography, and by estimation with protocatechuate 3,4- and 4,5-oxygenases. The apparent Km values for 3-hydroxybenzoate and NADPH are 0.06 and 0.07mM respectively; Lineweaver-Burke plots for these determinations are shown in Figure 2. When NADH is substituted for NADPH, the apparent Km for NADH is 3.0mM.

Catalytic Properties of 3-Hydroxybenzoate 4-hydroxylase.

As anticipated from previous studies of flavoprotein hydroxylases (11, 12,

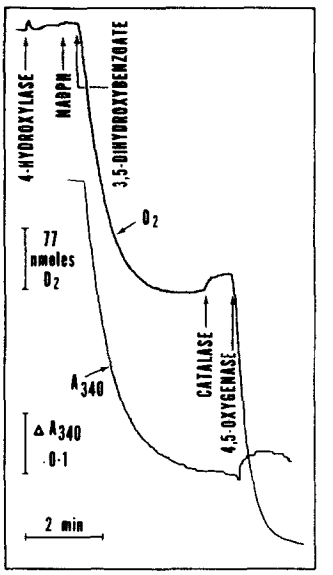

Figure 3. Oxidation of 3,5-dihydroxybenzoate by 3-hydroxybenzoate 4-hydroxylase. The reaction mixture contained: 50mM KH_2PO_4-NaOH buffer, pH 7.5 (2.9ml); 3-hydroxybenzoate 4-hydroxylase (20µl containing 112µg of protein); 25mM NADPH (40µl); 25mM 3,5-dihydroxybenzoate (15µl); catalase (5µl); and protocatechuate 4,5-oxygenase (10µl) as indicated. Temperature, 29°. Progress of the reaction was followed by simultaneous measurements of oxygen consumption and ΔA_{340}. Gallate the product of 3,5-dihydroxybenzoate is known to be a substrate of protocatechuate 4,5-oxygenase (6, 17).

13, 14) the aromatic compound acts as both a substrate and effector of flavin reduction by a nicotinamide nucleotide. This phenomenon is illustrated by the use of analogues of the "natural" substrate, some of which completely dissociate the two roles of substrate and effector. Some analogues of the true substrates e.g., benzoate with salicylate hydroxylase (11), 6-hydroxynicotinate with p-hydroxybenzoate hydroxylase (13) or m-cresol with orcinol hydroxylase (12) act only as effectors for the facilitated transfer of electrons from a reduced nucleotide to oxygen: hydrogen peroxide is then the only product of oxygen reduction and the aromatic substrates remain unmodified. Investigation of this phenomenon with 3,5-dihydroxybenzoate as effector for 3-hydroxybenzoate 4-hydroxylase indicates that it is a partial uncoupler of electron flow to oxygen from hydroxylation (Fig. 3), since both gallate (3,4,5-trihydroxybenzoate)

and H_2O_2 are formed. The results shown in Table 3 give other examples of the substrate-effector roles of several analogues. The data indicate, that of the substrate analogues tested, none completely uncouple electron flow from hydroxylation of the aromatic compound. Instead, those substrate analogues that facilitate NADPH oxidation by molecular oxygen are universally hydroxylated, but the efficiency of the reactions is variable. This is indicated by the ratio of O_2 consumed (or NADPH consumed) to the amount of hydrogen peroxide formed (which is a measure of the extent of uncoupling of electron flow from the oxygenation of the substrate supplied).

One interesting facet of these studies is the use of gentisate as a substrate for 3-hydroxybenzoate 4-hydroxylase. While gentisate is a partial uncoupler of electron flow, 2,4,5-trihydroxybenzoate has been tentatively identified as the product of the enzymic reaction. This compound is also the product of protocatechuate oxidation by 3-hydroxybenzoate 6-hydroxylase (7). Consequently, an alternative route for gentisate oxidation may exist in Ps. testosteroni. Gentisate oxygenase, 3-hydroxybenzoate 4-hydroxylase and protocatechuate 4,5-oxygenase are all induced during growth on gentisate, and 2,4,5-trihydroxybenzoate, which has been firmly identified as a metabolite of gentisate in cultures, is readily oxidized by extracts of these cells.

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