INTERACTIONS OF SUBSTRATE AND NON-SUBSTRATE EFFECTORS WITH p-HYDROXYBENZOATE HYDROXYLASE FROM PSUEDOMONAS FLUORESCENS‡‡

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SUMMARY

3,4-Dihydroxybenzoate (3,4-DOHB), 2,4-dihydroxybenzoate (2,4-DOHB), and benzoate facilitate the interaction of p-hydroxybenzoate hydroxylase with TPNH. The two dihydroxybenzoate effectors form 1:1 complexes with the enzyme, inducing large spectral perturbations and fluorescence quenching. The dissociation constants for 2,4-DOHB and 3,4-DOHB are 0.15 and 0.50 mM respectively. During the reaction of enzyme with TPNH and oxygen, all the 2,4-DOHB, <5% of the benzoate, and none of the 3,4-DOHB is hydroxylated.

A feature common to a number of flavoprotein-hydroxylases is the ability of their hydroxylatable substrates to induce a facilitated interaction of the enzyme with its specific pyridine nucleotide. The substrate thereby acting as an effector, causes a marked stimulation of the rates of the oxidation of the pyridine nucleotide, the uptake of oxygen, and the anaerobic reduction of the enzyme-bound flavin moiety by the pyridine nucleotide. This feature has been demonstrated for salicylate hydroxylase (1,2), melilotate hydroxylase (3), orcinol hydroxylase (4), and p-hydroxybenzoate hydroxylase (5-7). Recently, certain substrate-analogues, which are not hydroxylated during the enzymatic reaction, were also demonstrated to behave as effectors for their corresponding hydroxylase enzyme. The non-substrate effectors include benzoate for salicylate hydroxylase (2), m-cresol for orcinol hydroxylase (8), and 6-hydroxy nicotinate for p-hydroxybenzoate hydroxylase (7). A third situation in which only a fraction of the effector is hydroxylated was reported for resorcinol with orcinol hydroxylase (8). In general, non-substrate effectors are said to "uncouple" the hydroxylation reaction (2,7,8).

In the case of p-hydroxybenzoate hydroxylase, a number of compounds have been shown to be effectors for the various species of this enzyme isolated from different bacterial strains (5,7,9). However, with the exception of the substrate, p-hydroxybenzoate, and the non-substrate, 6-hydroxynicotinate, no

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attempt has been made to distinguish which of these effectors are hydroxylatable substrates and which are uncouplers of the hydroxylation reaction.

In the studies presented here a number of compounds were screened for their ability to act as effectors of p-hyroxybenzoate hydroxylase isolated from Pseudomonas fluorescens. Two of the compounds, 2,4-dihydroxybenzoate (2,4-DOHB) and 3,4-dihydroxybenzoate (3,4-DOHB), were determined to be effectors, and were capable of inducing marked changes in the environment of the enzyme. Spectrophotometric and fluorescence studies were performed to detect the effector-induced changes, and to determine dissociation constants for the enzyme-effector complexes. Product analysis revealed that 2,4-DOHB is a substrate and effector whereas, 3,4-DOHB is a non-substrate effector, i.e., its binding to the enzyme results in more rapid oxidation of TPNH, but it itself is not hydroxylated. Benzoate, the third effector, is mainly a non-substrate effector, being hydroxylated less then 5%.

RESULTS

Screening Assay for Effectors: The initial screening of potential

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF TPNH OXIDATION

COMPOUND	OBSERVED CATALYTIC VELOCITY	PERCENT HYDROXYLATION	INHIBITION AT HIGH CONCENTRATIONS
p-Hydroxybenzoate	1,900	100 (5,6)	yes (5,10,11)
3,4-Dihydroxybenzoate	197	0	yes
2,4-Dihydroxybenzoate	31	100	no
6-Hydroxynicotinate	12	0 (7)	yes (7)
Benzoate	2	< 5	?
None	< 0.5	-	-

The following compounds showed no detectable effect: 2,5-Dihydroxybenzoate; 2,6-Dihydroxybenzoate; 3,5-Dihydroxybenzoate; m-Hydroxybenzoate; salicylate; p-Cl-Benzoate; p-Br-Benzoate; p-F-Benzoate; p-I-Benzoate; o-I-Benzoate; p-Nitrobenzoate; p-Methoxybenzoate; p-Aminobenzoate; Benzamine; p-Toluate; Benzene sulfonate and Phenol.

Assay conditions: 1.0 µmole of the above compounds were individually added to cuvettes containing 100 µmoles Tris-HCl buffer, pH 8.0, 0.45 µmole TPNH, and various catalytic amounts of p-hydroxybenzoate hydroxylase. Total volume of 3.0 ml. The reaction was observed as a decrease in optical density at 340 mµ, 25°. Catalytic velocity is expressed as moles TPNH oxidized per minute per mole enzyme.

effectors of p-hydroxybenzoate hydroxylase was performed by observing the effect of each compound on the rate of TPNH oxidation in the presence of enzyme. The results, given in Table I, show the narrow specificity of this enzyme.

It is of interest that no inhibition of the rates of TPNH oxidation is seen when the concentration of 2,4-DOHB is increased to 2 mM. This finding is in contrast to the inhibition seen with 3,4-DOHB in concentrations above 1 mM and the similar inhibition reported at high concentrations of p-hydroxybenzoate (5,10,11) and 6-hydroxynicotinate (7).

Measuring the stoichiometric relationship between the amount of dihydroxybenzoate compound added and the amount of TPNH oxidized revealed interesting differences between the effectors. When TPNH was present in excess, it was rapidly oxidized until the amount consumed reached a 1:1 stoichiometry with

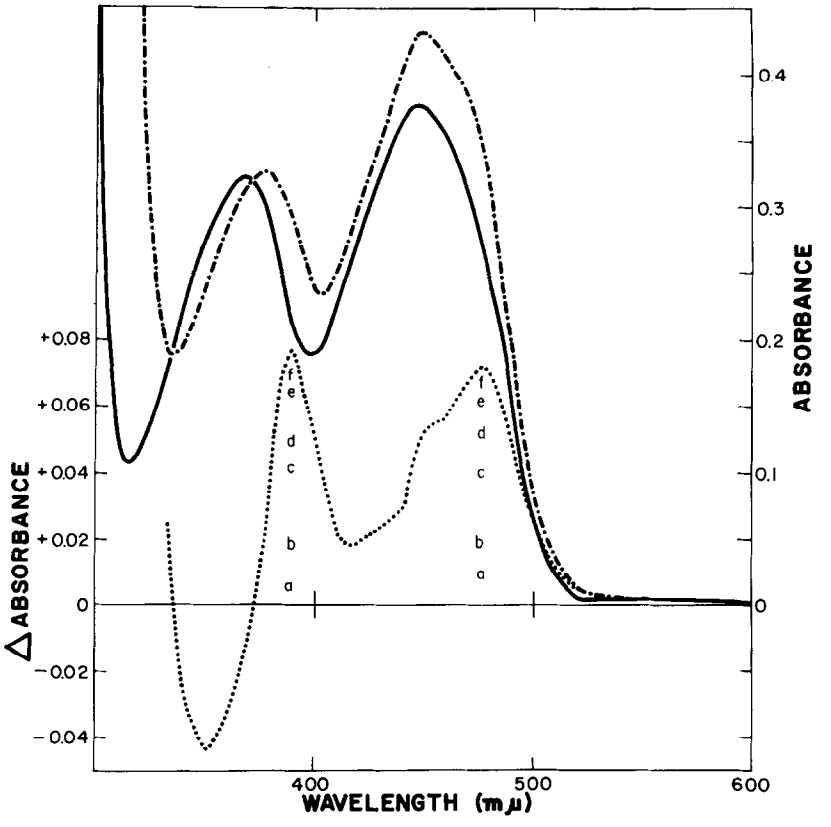

Fig. 1. Spectrophotometric titration of p-hydroxybenzoate hydroxylase with 2,4-DOHB. Left ordinate; difference spectrum. Both reference and sample cuvettes contained 1.0 ml of 34.7 μM enzyme, 0.033 M tris HCl, pH 8.0, and 0.3 mM EDTA. Points a-f and the final curve (····) were recorded after adding the following concentrations of 2,4-DOHB to the sample cuvette; 0.03, 0.06, 0.21, 0.45, 0.70, 1.18 and 1.65 mM. The absolute spectra (right ordinate) were then obtained by recording the sample (-·-·-·) against buffer, and the reference against buffer (——).

the amount of 2,4-DOHB initially present. However, the oxidation of TPNH then continued at a very slow rate beyond that point. When varying amounts of 3,4-DOHB were added to an excess of TPNH, all the TPNH was always readily oxidized. The slowness of the rate of TPNH oxidation in the presence of benzoate rendered stoichiometric relationships difficult to evaluate.

Evidence for an Enzyme-Effector Complex

Effector-Induced Alterations in the Enzyme's Absorption Spectrum: The addition of either 2,4-DOHB or 3,4-DOHB to p-hydroxybenzoate hydroxylase was found to result in pronounced spectral changes. The absolute and difference spectra obtained by titrating the free enzyme with 2,4-DOHB and 3,4-DOHB are shown in Figs. 1 and 2 respectively. The method of Benesi and Hildebrand (13) was used to determine by extrapolation the extinction coefficients of the enzyme-effector complexes. From such plots, dissociation

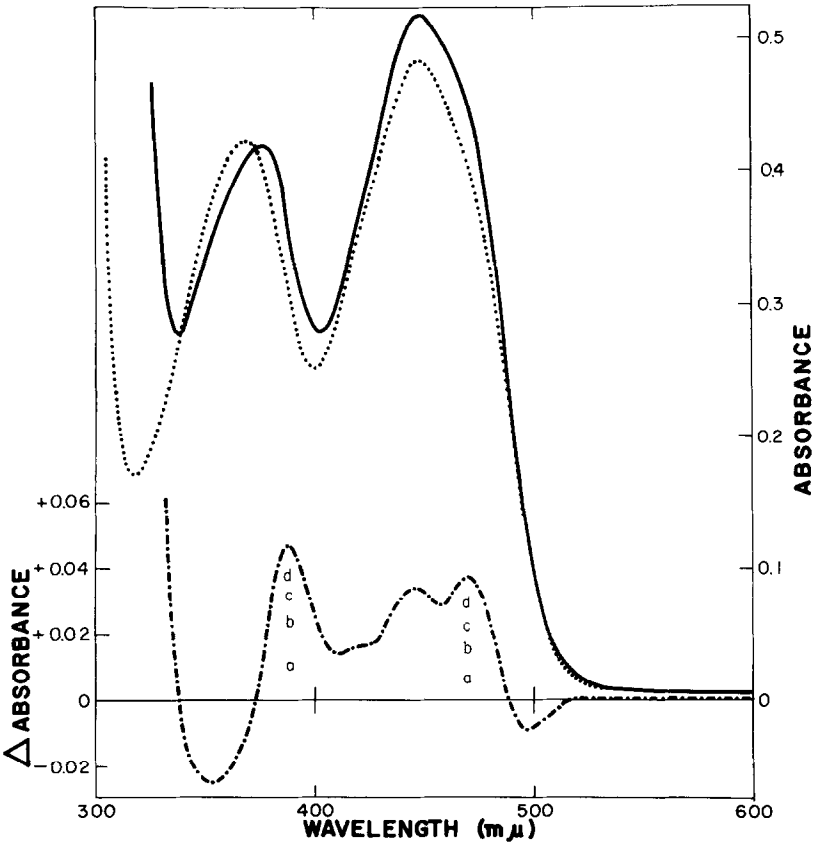

Fig. 2. Spectrophotometric titration of p-hydroxybenzoate hydroxylase with 3,4-DOHB. Procedure was the same as in Fig. 1. Enzyme concentration; 42.9 μM. Sequential concentrations of 3,4-DOHB; 0.10, 0.25, 0.40, 0.64, and 1.13 mM. Difference spectrum (-·-·-). Absolute spectra: complexed enzyme (——), uncomplexed enzyme (·····).

constants were determined and the actual data points were compared to theoretical curves drawn for those dissociation constants. The method is shown in Fig. 3 for the titration with 2,4-DOHB. The increase in extinction coefficient for this enzyme-effector complex over that of uncomplexed enzyme was 2,340 $\underline{M}^{-1}$ cm^{-1} at 477 mμ, and the data points fit a curve drawn for a dissociation constant of 0.17 mM with 1:1 binding with the enzyme. The increase in extinction coefficient similarly determined for 3,4-DOHB at 470 mμ was 1,400 $\underline{M}^{-1}$ cm^{-1}. The binding was also 1:1 with a K_D of 0.54 mM.

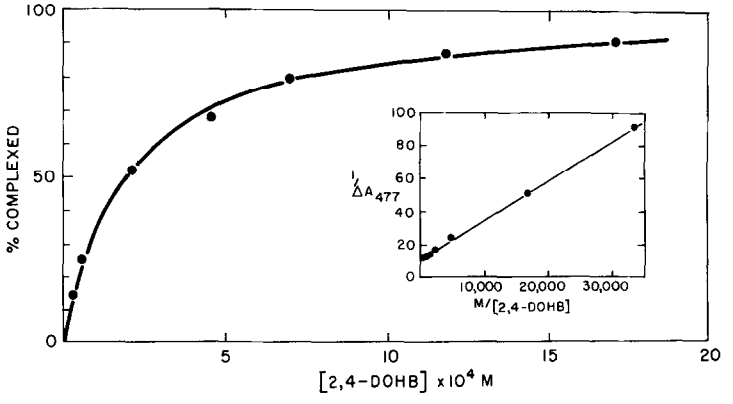

Fig. 3. Spectrophotometric determination of binding constants for 2,4-DOHB. Insert; treatment of data points (477 mμ) of Fig. 1 to obtain maximum absorbance change. Main fig.; ——— theoretical curve calculated for a dissociation constant of 0.17 mM, 1:1 binding with the enzyme. (●), actual data points.

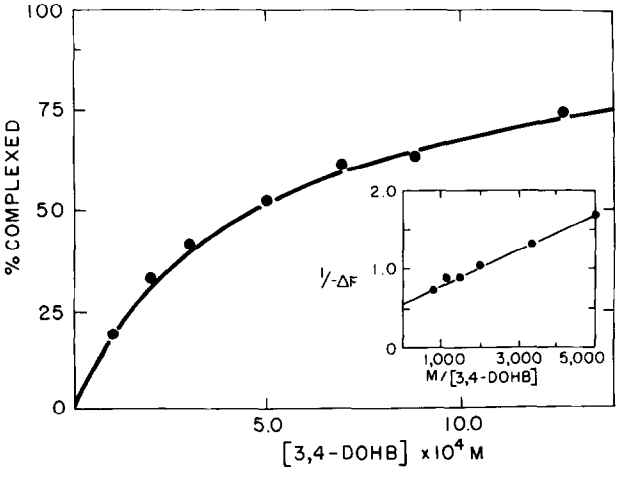

Fig. 4. Fluorometric determination of the binding constants for 3,4-DOHB. Insert; treatment of data obtained from fluorescence titration to obtain maximum quenching by 3,4-DOHB. Conditions: 5.9 μM p-hydroxybenzoate hydroxylase, excitation at 450 mμ, emission at 530 mμ. Main fig.; ——— theoretical curve calculated for a dissociation constant of 0.47 mM, 1:1 binding with the enzyme. (●), actual data points.

Quenching of the Protein-bound FAD Fluorescence: Both 2,4-DOHB and 3,4-DOHB produced a near complete quenching of the enzyme's flavin fluorescence. The total extent of fluorescence quenching, the stoichiometry of the binding, and the dissociation constants were determined in the same manner as demonstrated in Fig. 3. The maximal quenching was determined to be 80% for 2,4-DOHB, and 99% for 3,4-DOHB. Their respective dissociation constants were 0.13 mM and 0.47 mM. In both cases the data points fit the theoretical curve drawn for a 1:1 binding ratio of effector : enzyme. Fig. 4 shows the treatment of the data obtained from the titration with 3,4-DOHB.

Product Analysis

Fate of Effectors: Each effector was allowed to react with p-hydroxybenzoate hydroxylase and excess TPNH. The oxidation of TPNH was followed to completion spectrophotometrically. The benzoate compounds were extracted in ether at acid pH, and analyzed as their silylated derivatives by gas-liquid chromatography. The product of the 2,4-DOHB reaction appeared as one peak which was identical to that for 2,3,4-trihydroxybenzoate. In addition, the isolated product of this reaction had a UV spectrum identical to authentic 2,3,4-trihydroxybenzoate. 3,4-DOHB remained unchanged, eluting identically as a sample of the unreacted compound with no other peaks that would correspond to a trihydroxylated product. Benzoate remained mainly unchanged, with less than 5% eluting at a peak corresponding to either m-hydroxybenzoate or salicylate. The latter compounds were indistinguishable by this technique. Thin layer chromatography (benzene:propionate:H_2O; 2:2:1) with ferric chloride indicator was used to distinguish the trace amounts of hydroxylated product as m-hydroxybenzoate.

Role of Oxygen: It has been shown for salicylate hydroxylase (2), and orcinol hydroxylase (8) that in the presence of non-substrate effectors, the product of the reaction with O_2 if H_2O_2. In such a reaction, if catalase is added at any time during the reaction, 50% of the consumed oxygen should be returned to solution. Catalase present at the start of the reaction returns the oxygen as the reactions proceeds, thereby halving the observed rate of oxygen uptake.

Catalase was used here to demonstrate the lack of H_2O_2 production by the known substrate, p-hydroxybenzoate, and the formation of H_2O_2 by the known non-substrate effector, 6-hydroxynicotinate. Similarly, H_2O_2 was detected in the reaction with 3,4-DOHB, but was not produced in the reaction with 2,4-DOHB.

DISCUSSION

The physical techniques employed in this study demonstrate that both 2,4-DOHB and 3,4-DOHB elicit notable changes in the environment of this

enzyme. The alterations in the visible absorption spectrum of the native enzyme were fairly similar when produced by either effector. The alterations were also similar to those produced by 6-hydroxynicotinate, (7) but quite dissimilar to those of p-hydroxybenzoate (5,10,12). However, the difference spectra of the enzyme-effector complexes in the case of the dihydroxybenzoic acids were of greater magnitude than those of the other effectors.

The ability of 2,4-DOHB and 3,4-DOHB to quench the flavin fluorescence of native p-hydroxybenzoate hydroxylase is also shared by p-hydroxybenzoate (9,10). In contrast, 6-hydroxynicotinate causes a 1.8 fold enhancement of the fluorescence (7).

It becomes obvious that while these techniques can be used to demonstrate the formation of an enzyme-effector complex, they cannot be used to predict which effector will be hydroxylated when TPNH is added to the aerobic solution. Neither does the observance of the stoichiometry between the initial concentration of effector and the final concentration of TPNH oxidized during the reaction serve to make this distinction. This is because the products of the two hydroxylated substrates also cause the oxidation of TPNH. In the case of p-hydroxybenzoate, its product is 3,4-DOHB, which has been shown above to be itself an effector. 2,3,4-trihydroxybenzoate, the product of reaction with 2,4-DOHB, when added to a solution of TPNH under the standard assay conditions described above, slowly oxidizes the TPNH in the absence of enzyme. An apparent 1:1 stoichiometry can be seen if the concentration of enzyme is manipulated so that the rate of oxidation of TPNH occurs significantly faster with the substrate than the product, and differential rate extrapolation is used to determine the end point. However, the most definitive method for distinguishing which effector is also a substrate is clearly product analysis.

The results indicate that the enzyme will only hydroxylate efficiently the 3 position of a para-hydroxy-substituted benzoate compound. 6-Hydroxynicotinate presumably binds to the enzyme in such a way that the non-hydroxylatable nitrogen simulates the number 3 carbon of the benzoate compounds. Otherwise, it too would likely be hydroxylated. Although the para-hydroxyl group seems necessary for efficient binding and the induced facilitation of the enzyme-TPNH interaction, very weak facilitation and partial hydroxylation can be seen in its absence in the case of benzoate.

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