ON THE INTERACTION OF *PARA*-HYDROXYBENZOATE HYDROXYLASE FROM *PSEUDOMONAS FLUORESCENS* WITH HALOGEN IONS

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1. Introduction

Inducible *p*-hydroxybenzoate hydroxylases from *Pseudomonas* species have been studied in detail by several groups [1–3]. During our investigations of *p*-hydroxybenzoate hydroxylase from *Ps. fluorescens* we observed inhibition of the enzyme by halogen ions. Although an inhibition of *p*-hydroxybenzoate hydroxylase from *Ps. putida* by chloride ion has been briefly mentioned [4], this inhibitory effect has not previously been studied in detail, and indeed chloride ion is routinely incorporated in assays for the enzyme [1, 2].

We have found that the inhibition is competitive with respect to NADH, and a mixture of competitive and non-competitive with respect to the substrate *p*-OHB[1] *. Binding of chloride ion to the protein quenches the flavin fluorescence and changes its polarisation. Addition of *p*-OHB or *p*-OHNA (an effector and inhibitor [5]) to the enzyme causes a decrease in the binding constant for halogen ions.

2. Materials and methods

NADPH was obtained from Sigma Chemical Co. All other chemicals used were of analytical grade and were purchased from Merck Chemical Co. Large scale production of the *Ps. fluorescens* was kindly performed by Diosynth B.V., Oss, The Netherlands.

The bacteria were cultured on the medium described by Howell et al. [1]. The enzyme has been purified according to the method of Howell et al. [1] except that the bacterial cells were broken with a M.S.E. 60kc. sonicator instead of passing them through a Manton Gaulin homogeniser. The enzyme was assayed according to Hosokawa and Stanier [2] except that EDTA and FAD were omitted and KP_i buffer (0.03 M, pH 8) was used in place of Tris–HCl. Assays were carried out with a Zeiss PMQII spectrophotometer. The reaction was started by the addition of enzyme. Initial velocities were measured. The specific activities of different preparations of the enzyme were in the range 30–40 μmoles NADP oxidized/min/mg. The fluorescence measurements were performed with a Hitachi Perkin Elmer MPF 2A fluorospectrophotometer equipped with a thermostated cell-holder. Fluorescence polarisation was measured with an apparatus similar to that described by Weber [6].

3. Results and discussion

The inhibition of *p*-hydroxybenzoate hydroxylase by F^-, Cl^-, Br^-, I^- and CNS^- is competitive with respect to NADPH. Kinetic data for Cl^- are summarized in fig. 1. The inhibition constants for these ions were derived from Lineweaver–Burk plots and calculated according to Dixon and Webb [7]. The data are collected in table 1.

It can be seen that, compared with the K_i values for Cl^-, I^- and CNS^-, the constants for F^- and Br^- are abnormally high. Effects similar to these have been observed with salicylate hydroxylase [8]. The inhibition constant for CN^-, an ion that resembles the halogen ions, was not determined, since this ion is

* *Abbreviations*: *p*-OHB = *p*-hydroxy benzoic acid; 6-OHNA = 6-hydroxy nicotinic acid; *p*-AB = *p*-amino benzoic acid.

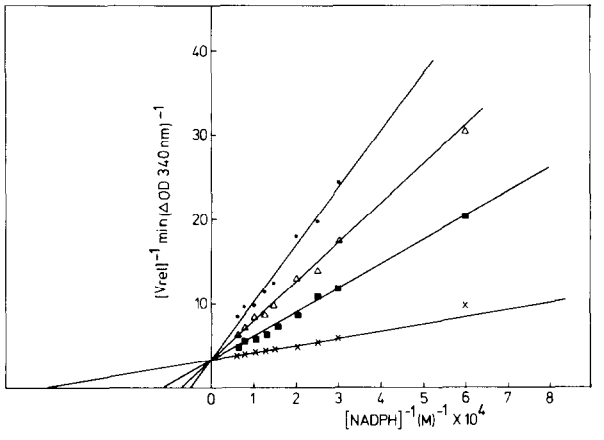

Fig. 1. Effect of chloride ions on the reaction of *p*-hydroxybenzoate hydroxylase in 0.03 M KP_i, pH 8.0. $1/V_{rel}$ is plotted against 1/[NADPH] at different $[Cl^-]$ and constant [*p*-OHB] (0.3 mM). $[Cl^-]$: (×—×—×), 0; (■—■—■), 13 mM; (△—△—△), 26 mM; (●—●—●), 39 mM. The temperature was 25°C.

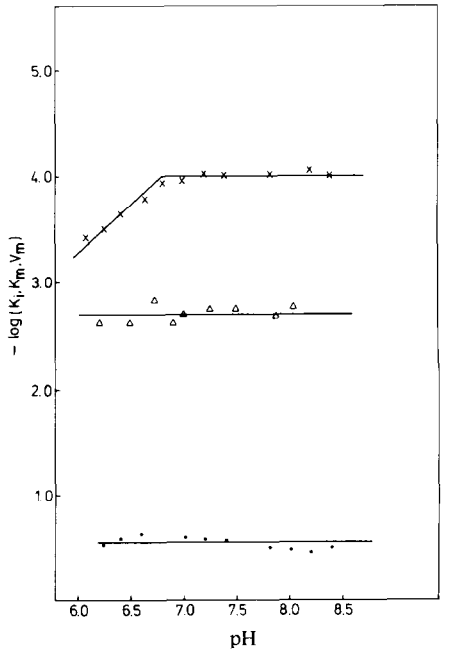

Fig. 2. Effect of pH on K_i, K_m and V_m in the presence of Cl^-. The negative log of each parameter is plotted against the pH. K_i for Cl^- was determined as described in the text, K_m and V_m for NADPH have been obtained from Lineweaver–Burk plots. (×—×—×), K_m; (△—△—△), K_i; (○—○—○), V_m. The temperature was 25°C.

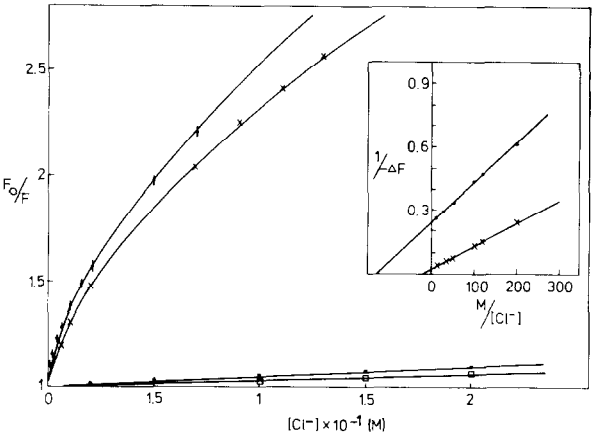

Fig. 3. The influence of chloride ions on the fluorescence emission of free and *p*-OHB-complexes *p*-hydroxybenzoate hydroxylase and of free coenzymes. The excitation wavelength was 450 nm and the fluorescence emission was observed at 520 nm. F_0/F is plotted against the Cl^- concentration (F and F_0 are the fluorescence intensities in the presence and absence of Cl^- respectively). Conditions: [FAD] = 50 μM; [FMN] = 50 μM; [*p*-OHB] = 0.5 mM; [enzyme] = 30 μM; all samples in 0.03 M KP_i, pH 8.0, 25°C (□—□—□), FMN; (●—●—●), FAD; (×—×—×), free enzyme; (◆—◆—◆), *p*-OHB-complexed enzyme. Inset: Plot of the fluorescence titration data of the enzyme solutions according to Benesi and Hildebrand [11] to obtain the dissociation constant of Cl^- for the free and *p*-OHB-complexed enzyme (×—×—×), free enzyme; (○—○—○), *p*-OHB-complexed enzyme).

known to complex with $NADP^+$, a product of the enzymic reaction. Initial kinetic observations suggested that the K_i value for Cl^- might be pH dependent. However, like the V_m for the ezyme, the K_i for Cl^- is not influenced by pH in the range 6–8 (fig. 2). It should be noted, that the K_m for NADPH does depend on pH (fig. 2); it shows a considerable increase below

Table 1
Inhibition constants (mM) of various halogen-ions, calculated from Lineweaver–Burk plots as demonstarted in fig. 1 for chloride ions, at pH 8.0, 25°C.

Ion	K_i (mM)
F^-	150
Cl^-	5.9
Br^-	65
I^-	9.7
CNS^-	6.0

pH 7, an effect that might be due to protonation of the phosphate of NADPH.

The inhibition by F^-, Cl^-, Br^-, I^- and CNS^- is a mixture, competitive and non-competitive with respect to *p*-OHB. The mixed inhibition might be due to a weak interaction of the halogen ions with the free enzyme and a stronger interaction with the enzyme–*p*-OHB complex. Nitrate and polyvalent ions such as sulphate and phosphate did not show any effect on the activity. With the latter ion the influence of the ionic strength on the enzyme activity has been investigated. No ionic strength effect was found.

3.1. *Influence of chloride ion on the flavin fluorescence of the enzyme*

The flavin of *p*-hydroxybenzoate hydroxylase shows about 65% of the fluorescence of FAD [5]. Binding of *p*-OHB and a variety of other effectors or inhibitor molecules causes a decrease in this fluorescence [5, 9]. A similar fluorescence quenching of both the free enzyme and enzyme-substrate (or effector) complexes is caused by halogen ions (fig. 3). The data are plotted according to Stern–Volmer [10]. This effect is not due to collisional quenching since at the concentrations used in the experiments of fig. 3, Cl^- has only a small effect on the fluorescence intensity of free FAD and FMN. In addition, the changes seen with the free coenzymes are linear with chloride ion concentration, in contrast to the relatively large non-linear changes observed with the enzyme. Dissociation constants for the complex of chloride and enzyme were calculated from the fluorescence titration data according to the method of Benesi and Hildebrand [11] (see inset to fig. 3). The values calculated were 110 mM for the free enzyme, 30 mM for the enzyme–6-OHNA complex and 8 mM for the enzyme–*p*-OHB complex. The latter value is in good agreement with the K_i value (5.9 mM). These results indicate that molecules which are effectors for the binding of NADPH to *p*-hydroxybenzoate hydroxylase also enhance the binding of chloride ion. It is interesting that the ratio of the dissociation constants for the binding of Cl^- in the presence and absence of *p*-OHB is similar to the ratio for the dissociation constants of NADPH to substrate-complexes and free enzyme [1].

From the kinetic and fluorescence data it might be concluded that halogen ions interact with the enzyme at the NADPH binding site. This idea is supported by fluorescence polarisation measurements of the enzyme in the presence and absence of various compounds. The results of these studies are summarized in table 2. The free enzyme has a high fluorescence polarisation, indicating that the flavin coenzyme is rather tightly bound to the apo-*p*-hydroxybenzoate hydroxylase. Addition of *p*-OHB, *p*-AB, NADPH or chloride ions to the enzyme leads to a small decrease of the fluorescence polarisation. Most striking is that the fluorescence polarisation is affected drastically upon addition of either chloride ions or NADPH to enzyme that is already complexed with *p*-OHB or *p*-AB. The large decrease of the fluorescence polarisation suggests a rather dramatic change of the microenvironment of the protein-bound FAD. These results and the fluorescence quenching data indicate that a conformational change occurs at the active center upon binding of chloride ions or NADPH to the appropriately complexed enzyme. Preliminary circular dichroism experiments support this interpretation. Determinations of the fluorescence lifetime of *p*-hydroxybenzoate hydroxylase under various conditions will provide a deeper insight into these phenomena. These studies are now in progress.

Table 2
The fluorescence polarisation of *p*-hydroxybenzoate hydroxylase in the presence and absence of various compounds.

Sample	Fluorescence polarisation
Free enzyme	0.43
Enzyme + Cl^-	0.43
Enzyme + *p*-OHB	0.42
Enzyme + *p*-OHB + Cl^-	0.38
Enzyme + *p*-AB	0.41
Enzyme + NADPH	0.42
Enzyme + *p*-AB + NADPH	0.34

Conditions: [enzyme] = 30 μM; [*p*-OHB] = 0.5 mM; [*p*-AB] = 1 mM; [NADPH] = 0.1 mM; [Cl^-] = 70 mM; all samples in 0.03 M KP_i, pH 8.0, 25°C.

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