STUDIES ON pH DEPENDENCE OF *p*-HYDROXYBENZOATE HYDROXYLASE-CATALYZED REACTIONS: FUNCTIONAL GROUPS INVOLVED IN THE REACTIONS

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1. Introduction

p-Hydroxybenzoate hydroxylase is a flavoprotein external mono-oxygenase and has been purified from a few pseudomonad species [1–6]. The reaction mechanism of the enzyme has been extensively studied [5,7], but little is known about the roles of the protein moiety, such as essential functional groups in the active center.

In this regard, we investigated the pH-dependence of the enzyme catalyzed reactions and obtained some information on the functional groups involved in the catalytic activities.

Fluorometric titration showed that a basic group of the enzyme with a pK value of around 8.5, presumably the ϵ-amino group of lysine is participating in the binding with the substrate.

From stopped-flow experiments on the reduction step of the enzyme–substrate complex, together with the values of dissociation enthalpy, it was suggested that the ϵ-amino group of lysine (or the SH group of cysteine) and the phosphate group of NADPH are responsible for the enzyme–NADPH interaction.

Although no definite pK values were determined, the pH profile of the re-oxidation step of the reduced enzyme-substrate complex showed the optimum activity on the alkaline side, indicating that this step also regulates the pH dependence of the overall reaction.

The pH profile of the overall hydroxylation reaction is discussed in terms of those of the partial reactions.

2. Materials and methods

p-Hydroxybenzoate hydroxylase from *Pseudomonas desmolytica* IAM 1123 was purified according to Yano et al. [3]. NADPH was obtained from Kyowa Hakko Kogyo Co., Tokyo, and all other compounds were of the purest grade commercially available. Overall enzymatic reactions were assayed by oxygen uptake with a Clark oxygen electrode. The degree of hydroxylation of the substrate and substrate analogues was estimated by the method of White–Stevens and Kamin [8]. Dissociation constants were determined by fluorometric titration [5,9] using a Hitachi fluorospectrophotometer, Model MPF-3. The stopped-flow measurements of the reduction and re-oxidation reactions of the enzyme under anaerobic conditions were performed as previously reported [7] with the use of a Union Giken rapid analyzer, Model RA-1300. Measurement of pH was with a Toa Dempa pH-meter, Model HM-5. All experiments were done at 25°C unless otherwise stated.

3. Results

3.1. *Stability of the enzyme against pH*

The enzyme was stable in the pH range from 5.5 to 9.0 in a 0.05 M Tris–maleate buffer and from 5.5 to 8.5 in a 0.05 M phosphate buffer. 80% of the activity remained at pH 8.8 in the phosphate buffer after a 30 min incubation at 25°C. It is thus concluded that the enzyme is stable in the pH range studied.

3.2. *Degree of hydroxylation of the substrate and substrate analogues*

As it is known that the final product of the hydroxylation reaction is H_2O and that of the uncoupled reaction is H_2O_2 [8,10], the degree of hydroxylation of the substrate and substrate analogues was estimated by the use of the oxygen electrode by measuring oxygen evolution on adding catalase at the final stage of the NADPH oxidation. The results are shown in table I.

It was found that 2,4-DHBA*, as well as *p*-HBA, are good hydroxylation substrates, while 3,4-DHBA is a complete uncoupler. It is noteworthy that BA and 6-HNA were partially hydroxylated, and may be termed incomplete uncouplers. These results are somewhat in contrast to those reported with *P. fluorescens*-enzyme in that 6-HNA is a complete uncoupler [10,11].

3.3. *pH Profile of the overall oxygen uptake*

The results of the overall NADPH oxidation measured by oxygen uptake in the presence of the substrate and substrate analogues were as depicted in fig. 1. The reactions in the presence of *p*-HBA, 2,4-DHBA and 3,4-DHBA showed the optimum pH's in

Abbreviations used are: *p*-HBA, *p*-hydroxybenzoate; BA, benzoate; 2,4-DHBA, 2,4-dihydroxybenzoate; 3,4-DHBA, 3,4-dihydroxybenzoate; 6-HNA, 6-hydroxynicotinate; E_{ox} and E_{red}, the oxidized enzyme and the reduced enzyme; K_s^{pHBA} and K_s^{BA}, the dissociation constants of the enzyme–*p*-hydroxybenzoate and the enzyme–benzoate complexes; K_m^{NADPH} and $K_m^{O_2}$, the Michaelis constants for NADPH and molecular oxygen; and k_{red}^{max} and k_{ox}^{max}, the maximum reaction rates of the reduction step and the oxidation step, respectively, expressed as first-order rate constants.

Table 1
Effect of the substrate and substrate analogues on the oxygen uptake by *p*-hydroxybenzoate hydroxylase

Addition*	Relative rate of oxygen uptake (%) pH 6.0	pH 8.0	Degree of hydroxylation (%)**
none	0.009	< 0.006	
p-hydroxybenzoate	47.4	100	100
2,4-dihydroxybenzoate	0.97	2.2	> 95
3,4-dihydroxybenzoate	2.44	2.9	0
benzoate	0.41	0.075	20–30
6-hydroxynicotinate	0.97	0.22	20–30

The reaction mixture contained 0.15 mM NADPH, 5.7 mM substrate analogue with the exception of *p*-hydroxybenzoate (0.15 mM) in a 0.05 M phosphate buffer with a final volume of 3.5 ml. The reaction was initiated by adding the enzyme and the initial rate of the oxygen uptake was measured.

* The following compounds showed no detectable effect on the oxygen uptake: phenol, salicylate, *m*-hydroxybenzoate, *p*-methoxybenzoate, *p*-acetylphenol, and *p*-hydroxycyclohexanoate.

** Identical results were obtained at both pH 6.0 and 8.0.

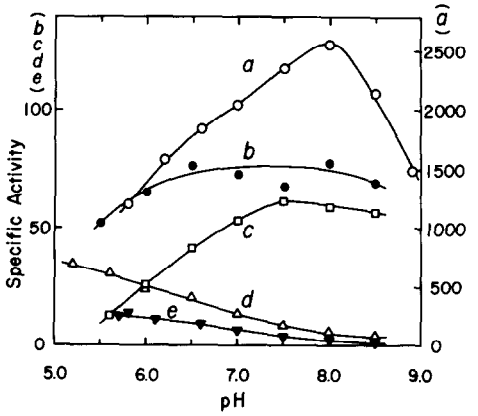

Fig. 1. pH dependence of the overall NADPH oxidation of *p*-hydroxybenzoate hydroxylase in the presence of the substrate and substrate analogues. The conditions were essentially the same as in table 1. Substrate analogue tested: a) *p*-HBA, b) 3,4-DHBA, c) 2,4-DHBA, d) 6-HNA and e) BA. Enzyme concentrations: a) 12 nM, b) 180 nM, c) 180 nM, d) 340 nM and e) 340 nM, respectively. Specific activities were expressed in terms of moles of O_2 uptake per minute per mole of the enzyme.

the alkaline region, but those in the presence of 6-HNA and BA in the acidic region.

It should be noted that the pH profile of the hydroxylation reaction with *p*-HBA as the substrate is not a simple 'bell-shaped' curve, implying that the reaction is controlled by many ionizable groups of both the substrate and the enzyme.

3.4. *pH Profiles of the partial reactions*

As previously reported [7,12], the overall hydroxylation is composed of three partial reactions: 1) the formation of the enzyme–substrate complex ($E_{ox} + S \rightleftharpoons E_{ox} \cdot S$); 2) the reduction of the flavin moiety by NADPH ($E_{ox} \cdot S + NADPH \longrightarrow E_{red} \cdot S + NADP$); and 3) the reoxidation of the reduced enzyme–substrate complex with molecular oxygen to produce the hydroxylated product and water ($E_{red} \cdot S + O_2 \longrightarrow E_{ox} + P + H_2O$).

The pH dependence of each of these reactions was investigated and the kinetic parameters were plotted according to Dixon and Webb [13]. As is seen in fig. 2a, both K_s^{pHBA} and K_s^{BA} increased markedly in the alkaline region, indicating a pK of around 8.5. The effect of pH change on the acid side caused different results on these parameters; K_s^{pHBA} increased considerably, while K_s^{BA} decreased as pH was lowered. These phenomena are in parallel with the pH profiles observed with the overall oxygen uptake in the presence of these compounds (fig. 1).

The effects of pH on the kinetic parameters of the reduction step of the enzyme in the presence of *p*-HBA are shown in fig. 2b. The k_{red}^{max} showed no pH dependence throughout the region studied, while K_m^{NADPH} was found to have two inflection points, at pH 6.5 and 8.2, suggesting that two dissociating groups are participating in the interaction of the enzyme and NADPH. For the purpose of identifying these two groups, the enthalpy of dissociation was also determined from the temperature dependence of the pK values (fig. 3). The values of the enthalpy were −3200 cal/mole for the group on the acidic side and +8800 cal/mole for that on the basic side. On the basis of these values, the dissociating group on the basic side is considered to be the ϵ-amino group of lysine or the SH group of cysteine [14,15]. However, no corresponding group to that of the acidic side (pK = 6.5, $\Delta H = -3200$ cal/mole) was found with any of the amino acid residues. On the other hand,

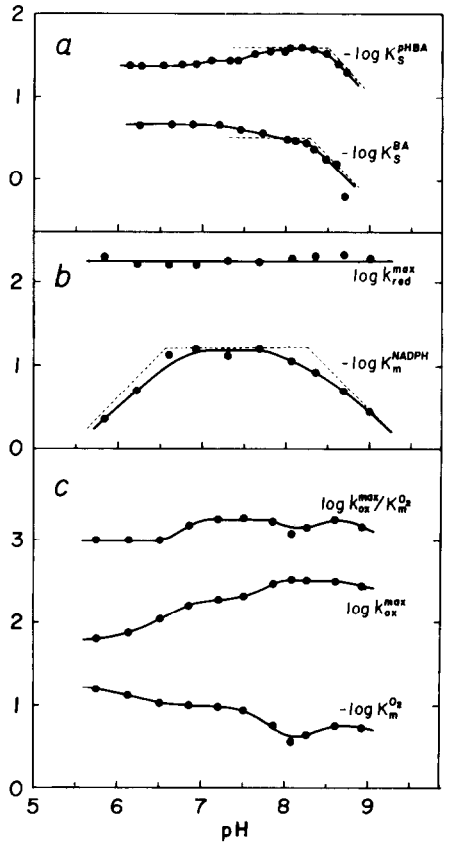

Fig. 2. pH dependence of the partial reactions. a) pH dependence of the dissociation constants of the enzyme–*p*-HBA and the enzyme–BA complexes measured by the fluorescence method. The enzyme solution was excited at 450 nm and the change in the emission at 520 nm was recorded. Enzyme concentration: 2.2 μM. b) pH dependence of the kinetic parameters of the reduction step. c) pH dependence of the kinetic parameters of the reoxidation step. Buffers: a) 0.05 M phosphate buffer; b) and c) 0.05 M Tris–maleate buffer.

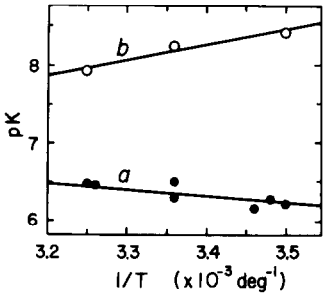

Fig. 3. Temperature dependence of pK values of the dissociating groups obtained in fig. 2b. Curves a and b are for the dissociating groups on the acid side and on the basic side, respectively.

many nucleotide compounds are known to possess a pK of around 7 with a negative value of the dissociation enthalpy [14,15]. It is thus likely that the phosphate group of NADPH is responsible for the pK of the acidic side as suggested by Steennis et al. [16].

Kinetic parameters obtained in the reoxidation step are plotted against pH as shown in fig. 2c. Since the pH profiles of $K_m^{O_2}$, k_{ox}^{max}, and $k_{ox}^{max}/K_m^{O_2}$ are all quite complex, no conclusion can be drawn from these results as to the functional groups relating to the oxidation step. Further studies are required.

4. Discussion

In the present study, the pH profiles of the overall reaction as well as the partial reactions of *p*-hydroxybenzoate hydroxylase of *P. desmolytica* were investigated.

From the pH dependence of the overall reactions and the complex formation with the enzyme, the substrate analogues are classified into two groups: one is typical with *p*-HBA, having the pH optimum in the alkaline region; and the other with 6-HNA, having the pH optimum in the acidic region. This phenomenon is irrespective of whether the substance used is hydroxylated or not, but is related to the structure of the aromatic compounds. In other words, all the compounds with the optimum pH in the alkaline region have the *p*-hydroxybenzoate structure in common.

It has been reported that the carboxylate group of the substrate plays an important role on binding with the enzyme [9,10]. Teng et al. have found an interesting phenomenon, i.e., that the electron-donating property of the para-substituent on the benzoate derivatives has an important effect on the ability of the compound to inhibit the enzymatic reaction [17]. It is thus conceivable that the negative charge of the carboxylate anion of the substrate has an ionic interaction with the positive charge of a basic amino acid residue in the protein moiety of the enzyme. This assumption would be supported by the fact that the carboxylate group of the substrate can be replaced by the carbonyl group, to some extent, for the binding of the compound with the enzyme [9,10]. The most probable amino-acid residue participating in the substrate binding is lysine (ε-amino group) judging from the pK value obtained from fig. 2a.

As described before, the lysine ε-amino group or the cysteine SH group of the protein moiety and the phosphate group of NADPH might be participating in the reduction of the enzyme–substrate complex.

Although no functional group was definitely demonstrated in the reoxidation step, it is apparent that this partial reaction also has an optimum pH in the alkaline region.

It is reasonable to conclude that the pH dependence of the overall hydroxylation with *p*-HBA as the substrate can be explained by a composite of those of the three partial reactions.

Taking into account the results of figs. 1 and 2, the overall reaction in the acidic region is controlled mainly by the partial reactions 2 and 3, i.e., the interaction of NADPH with the enzyme in the reduction step and the reoxidation of the reduced enzyme–substrate complex; while the reaction in the alkaline region is principally controlled by reactions 1 and 2, i.e., the formation of the enzyme–substrate complex and the interaction of NADPH with the enzyme. The formation of the enzyme–substrate complex does not seem to be a main factor in controlling the pH profile of the overall reaction in the acidic region.

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