On the Stable Enzyme-Substrate Complex of p-Hydroxybenzoate Hydroxylase

EVIDENCES FOR THE PROTON UPTAKE FROM THE SUBSTRATE

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-P-Hydroxybenzoate hydroxylase (EC 1.14.13.2) from Pseudomonas desmolytica is known to form a stable complex with its substrate. Investigations were made on properties of the complex by spectrophotometric measurements and photooxidation. The addition of p-hydroxybenzoate or benzoate to the oxidized (with respect to bound FAD) enzyme caused changes in absorption, fluorescence, and CD spectra, most of which could be ascribed to spectral changes of the enzyme by the complex formation. Among them, the change in the absorbance around 285 nm caused by p-hydroxybenzoate was pH-dependent with an enormous increase in the alkaline side, indicating the formation of the phenolate anion of the substrate above pH 7 (pK = 7.13, 20°C). On the other hand, the change caused by benzoate was smaller and pH-independent. p-Hydroxybenzoate hydroxylase was inactivated by the photooxidation mediated by methylene blue, following pseudo-first-order kinetics. The pH dependency of the inactivation rate showed the involvement of an ionizing group with a pK of 7.0 (25°C, in free enzyme) participates in the inactivation. The addition of p-hydroxybenzoate shifted the pK toward the alkaline side by more than 1 pH unit (to 8.20), while benzoate exerted little effect.

The pH dependency of the apparent dissociation constant of the enzyme-p-hydroxybenzoate complex also revealed a minor change around pH 7.

These results strongly suggest that an ionizing group with a pK of 7.0 (25°C, in free enzyme) participates in the enzyme-substrate complex formation of p-hydroxybenzoate hydroxylase, having an interaction with the hydroxyl group of the substrate. The ionizing group is judged to be most likely an imidazole of histidine, from the pK value and other evidences. The results also suggest that the proton uptake from the substrate occurs by the complex formation in the alkaline side resulting in the formation of a phenolate anion of the substrate and the protonation of the ionizing group. Discussion was made on the phenomenon together with its effects on the following steps of the enzymatic reaction, i.e., reactions with NADPH and molecular oxygen.

-p-Hydroxybenzoate hydroxylase (EC 1.14.13.2) from Pseudomonas desmolytica is one of the external flavoprotein monooxygenases and catalyzes the initial oxidation reaction in the catabolic degradation pathway of p-hydroxybenzoate in the microbe which has adapted to this aromatic compound. The same enzyme has been purified from a few pseudomonad species (1-4). The enzyme was shown to form a stable complex with its substrate (1) and the complex was proved to participate in the overall enzymatic reaction (5). We showed that the enzyme-substrate complex formation consists of at least two steps (6), and that an arginyl residue might be involved in the substrate-binding site by modification studies (7). An ordered addition mechanism for reactions with p-hydroxybenzoate, NADPH, and molecular oxygen has been proposed (5, 8). Studies on the mechanism of the reaction with molecular oxygen are also underway (9-12).

External flavoprotein monooxygenases including this enzyme share an intriguing phenomenon that the binding of the substrate to the enzyme stimulates the rate of the reduction of the enzyme-bound flavin with reduced pyridine nucleotide enormously (in the case of p-hydroxybenzoate hydroxylase, by more than 10^4-fold; see review, Ref. 8). The phenomenon seems to have a significant physiological meaning for the bacteria, so as to prevent the waste of reduced pyridine nucleotides when the substrate is absent. We also indicated that the substrate binding increases the affinity of the reduced enzyme for molecular oxygen (10). Thus, the substrate exerts regulatory effects on the following steps of the enzymatic reaction, with NADPH and possibly also with molecular oxygen. It is, therefore, of most interest and importance to investigate the properties and characteristics of the enzyme-substrate complex, for clarifying the elegant self-regulatory reaction mechanism of this enzyme. P-Hydroxybenzoate hydroxylase possesses a unique characteristic in that it forms a stable complex with the substrate in the absence of NADPH, allowing static measurements of the complex.

In the present report, we describe results of studies on the complex formation by two different methods, spectrophotometric measurements and photooxidation. Comparative studies were made on the complex formations with p-hydroxybenzoate and benzoate to discriminate the roles of the hydroxyl group of the substrate. Results by both methods were consistent with each other, indicating that proton uptake from the hydroxyl group of the substrate occurs by the complex formation and that a histidyl residue might participate in the interaction.

EXPERIMENTAL PROCEDURES

-p-Hydroxybenzoate hydroxylase was purified from P. desmolytica IAM 1123 as previously reported (1). The enzyme concentration was determined with respect to the enzyme-bound FAD from the absorbance at 450 nm, taking ε as 11,300 M^-1 cm^-1.

1 H. Shoun, N. Higashi, T. Beppu, and K. Arima, unpublished data.
p-Hydroxybenzoate and benzoate were purchased from Dai-Ichi Pure Chemicals, methylene blue was from Tokyo Kasei Kogyo Co., and NADPH was from Kyowa Hakko Kogyo Co., respectively. All other reagents used were of the purest commercial grade available.

Enzyme activity assays were made spectrophotometrically. The reaction mixture contained 0.2 μmol of p-hydroxybenzoate and the equivalent amount of NADPH and 0.15 mmol of potassium phosphate (pH 8.0) per 3.0 ml. The reaction was initiated by the addition of catalytic amounts of p-hydroxybenzoate hydroxylase and the initial rate of the decrease in absorbance at 340 nm was measured.

Spectrophotometric measurements were made with a Hitachi spectrophotometer, model 124, a Hitachi fluorospectrophotometer, model MPF-3, and JASCO automatic recording spectropolarimeter, model J-20. Difference absorption spectra of p-hydroxybenzoate hydroxylase caused by the substrate, which means the difference spectra of (oxidized (with respect to the bound FAD) enzyme plus substrate (p-hydroxybenzoate or benzoate) (mixed)) minus (oxidized enzyme plus substrate (separated)), were obtained at 20°C, using two pairs of 1-cm path quartz cuvettes. Each cuvette of the one pair was filled with an equivalent volume (2.2 ml) of the same enzyme solution, and to the other pair was added the equivalent volume of the buffer solution, respectively. 10 to 50 μl of the concentrated solution of the substrate (2.0 to 100 μM in potassium salt, pH 7) were added to the enzyme solution on the sample side and the buffer solution on the reference side simultaneously, and at each time the same volume of the buffer solution was added to the enzyme solution on the reference side. Thus, the difference absorption spectra at various substrate concentrations were obtained one after another. All spectra were indicated after being corrected for dilution.

The photooxidation of p-hydroxybenzoate hydroxylase was carried out as follows. Reaction mixtures containing 8.2 μM p-hydroxybenzoate hydroxylase and 50 μg/ml of methylene blue were kept and stirred in a glass cell and illuminated with a 500-watt tungsten light from a slide projector at a distance of 30 cm from the cell. The light was passed through a glass filter (Toshiba V-053, orange) to cut off the light with wavelength below 550 nm. The temperature of the mixture was kept at 25°C by circulating water of constant temperature around the outside of the cell. Aliquots (20 μl) were taken at adequate intervals, diluted by adding 1.0 ml of the phosphate buffer (pH 6) at 0°C, and then assayed for activity within 15 min. The remaining activity of the photooxidized enzyme was compared with that of a control which was exposed to the same treatment without irradiation.

All experiments were made using 50 mM potassium phosphate buffers at 25°C, unless otherwise stated. The data on pH titrations were curve fitted using a computer (Hitac 8800/7700, Tokyo University Computer Center) according to a least square method.

**RESULTS**

**Spectrophotometric Measurements of the Enzyme-Substrate Interaction of p-Hydroxybenzoate Hydroxylase**—The addition of p-hydroxybenzoate to an oxidized (with respect to bound FAD) p-hydroxybenzoate hydroxylase solution caused changes in absorption, fluorescence, and CD spectra of the enzyme, as shown in Figs. 1 to 3. They are attributable to the perturbation of FAD caused by the substrate binding, except for the change in UV absorption. Each spectral change affords an apparent dissociation constant of the enzyme-substrate complex, for example, as shown in Fig. 2B. The $K_{a, app}$ values obtained from UV absorption (28 μM, Fig. 1B) and fluorescence (26 μM, Fig. 2B) agree well with each other, in both cases, the enzyme concentration employed was smaller than the $K_{a, app}$ values. The effects of the substrate on CD spectrum are fairly different from those observed in the case of the same enzymes from other pseudomonad species (3, 13, 14). Benzoate, an effector of the enzyme (15), also caused spectral changes (only the fluorescence quenching was indicated in Fig. 2B).

**pH Dependency of the Enzyme-Substrate Interaction**—Difference absorption spectra of p-hydroxybenzoate

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The abbreviations used are: $K_{a, app}$, apparent dissociation constant; $A_{285 nm}$, molar absorption coefficient of the difference at 285 nm; $V_{max}$, the maximum rate of reduction.

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**FIG. 1.** Difference absorption spectra of [oxidized p-hydroxybenzoate hydroxylase plus p-hydroxybenzoate (mixed)] minus [oxidized p-hydroxybenzoate hydroxylase plus p-hydroxybenzoate (separated)], at pH 8.0, at 20°C. The enzyme concentration was 44.7 μM (A) and 9.45 μM (B), respectively. The substrate concentration was 17.9 μM, 30.8 μM (B), 51.7 μM, 94.4 μM (d), and 303 μM, respectively, in A; and 9.01 μM (a), 11.9 μM (b), 30.8 μM (c), 51.7 μM (d), and 94.4 μM (e), respectively, in B. Other details were described in the text ("Experimental Procedures").

**FIG. 2.** A, fluorescence spectra of p-hydroxybenzoate hydroxylase at pH 8 (25°C) in the presence of 0 μM (a), 13.4 μM (b), 53.3 μM (c), and 422 μM (d) p-hydroxybenzoate, excited at 450 nm. The enzyme concentration was 2.2 μM. B, plots of the amount of the fluorescence quenching at 520 nm against the concentration of p-hydroxybenzoate (a) or benzoate (b). Conditions were the same as in A.

**FIG. 3.** CD spectra of p-hydroxybenzoate hydroxylase in the presence of 0 μM (a), 26.6 μM (b), 53.2 μM (c), 106 μM (d), and 266 μM (e) p-hydroxybenzoate at pH 8.0. The enzyme concentration was 44.3 μM. Cell path, 10 mm, 25°C.
hydroxylase caused by the interaction with the substrate (see "Experimental Procedures") were obtained at pH values 6 (or 5.7) and 8, as depicted in Figs. 4 and 5. The extent of the difference in the UV region caused by p-hydroxybenzoate was pH-dependent, being much larger at pH 8 than at pH 6 (cf. curves a and b in Fig. 5). On the other hand, the amount of the difference due to the enzyme-benzoate interaction was pH-independent (curves c and d in the same figure). In the case of those spectra in the visible region, only the difference spectrum caused by p-hydroxybenzoate at pH 8 (curve a in Fig. 4) showed a hyperchromism in the region from 400 to 470 nm. The other three spectra were similar to each other. These results indicate that the interaction between the enzyme and the p-hydroxyl group of the substrate is pH-dependent in the region around pH 7.

Molar absorption coefficients of the difference at 285 nm ($\Delta_{285\text{nm}}$), where the difference maximum occurs in the change caused by p-hydroxybenzoate, were obtained at various pH values and plotted against pH in Fig. 6. The plots fitted well to a single protonic titration curve with a pK of 7.13 (20°C).

FIG. 4. Spectral perturbations of the FAD moiety of p-hydroxybenzoate hydroxylase caused by: p-hydroxybenzoate at pH 8.0 (a), p-hydroxybenzoate at pH 5.7 (b), benzoate at pH 8.0 (c), and benzoate at pH 5.7 (d). Conditions and methods were the same as those in Fig. 1A (except pH). Each spectrum was expressed as the molecular absorption coefficient of the difference (Ar) with respect to the enzyme (FAD), which was obtained by extrapolation of the spectral change to infinite substrate concentration (cf. Fig. 1).

FIG. 5. Difference absorption spectra of p-hydroxybenzoate hydroxylase in the UV region caused by: p-hydroxybenzoate at pH 8.0 (a), p-hydroxybenzoate at pH 6.0 (b), benzoate at pH 8.0 (c), and benzoate at pH 6.0 (d), obtained by the same methods as in Fig. 4. Conditions were the same as in Fig. 1B (except pH).

FIG. 6. pH dependency of the absorption difference at 285 nm induced by the interaction of p-hydroxybenzoate hydroxylase with p-hydroxybenzoate. Conditions and methods were the same as in Fig. 5. Solid line represents a theoretical curve obtained by the calculation.

The minimum value of the $\Delta_{285\text{nm}}$ in the acidic side obtained from the calculation was 3,950 M$^{-1}$ cm$^{-1}$, which is, to a similar extent, comparable with those of the differences by benzoate (cf. Fig. 5). However, the maximum value observed in the alkaline side (15,400 M$^{-1}$ cm$^{-1}$) seems too enormous to be merely the perturbation of aromatic amino acid residues in the protein moiety. On the other hand, the phenol of the substrate ionizes in the extremely high pH region to form its phenolate anion (pK = 9.3, 25°C; Ref. 16), which accompanies a drastic change in the absorption spectrum in this region as shown in Fig. 7. The $\Delta$ around 280 nm of free p-hydroxybenzoate between pH 12 and pH 7 (16,300 M$^{-1}$ cm$^{-1}$ (curve c)) was comparable with the maximum value of the $\Delta_{285\text{nm}}$ in Fig. 6.

From the results above, it is clear that the enormous increase of the $\Delta_{285\text{nm}}$ in the alkaline side observed in the enzyme-p-hydroxybenzoate interaction is due to the change in the substrate induced by the complex formation, possibly forming the phenolate anion. The pK of the phenol might thus be lowered substantially (7.13, 20°C) by the complex formation with the oxidized enzyme. Effects of the ionization of the carboxyl group of the substrate (pK = 4.6; Ref. 16) were...
postulated to be able to be excluded in the pH range studied.

The pH dependency of the $K_{d, app}$ for $p$-hydroxybenzoate or benzoate was also investigated. The $K_{d, app}$ values were obtained utilizing fluorescence quenching as shown in Fig. 2 and plotted against pH in Fig. 8 according to Dixon and Webb (17). In both cases, a minor change appeared around pH 7 to 8. Reliable values could not be obtained at pH values above 8.5, since the intensity of the fluorescence became weaker and the amount of quenching by the substrate became smaller. The $K_{d, app}$ for $p$-hydroxybenzoate at pH 6.1 (42 mM) was larger than that at pH 8.0 (26 mM) only by less than 2-fold. This indicates that the change in the ionizing state of the interaction involving the phenol, which is expected to occur around pH 7 as noted above, does not affect so much the binding of the substrate to the enzyme. This is consistent with the observation that the carboxyl group of the substrate is more important than the hydroxyl group merely for the binding, since benzoate binds to the enzyme fairly well as shown above ($K_{d, app} = 220$ mM to $320$ mM, at pH 6 to 8) but phenol hardly binds.

Photooxidation of $p$-Hydroxybenzoate Hydroxylase—$p$-Hydroxybenzoate hydroxylase was inactivated by the photooxidation mediated by methylene blue following pseudofirst order kinetics. Typical time courses of the inactivation in the presence or absence of saturated amounts of $p$-hydroxybenzoate or benzoate were indicated in Fig. 9A. The addition of $p$-hydroxybenzoate decreased the inactivation rate below pH 8.5, but increased it at higher pH (8.6). Benzoate exerted little effects on the inactivation. Oxygen consumption was determined in a closed reaction system (see "Experimental Procedures") at pH 8.2, and the time courses for both the oxygen consumption and the inactivation were shown in Fig. 9B. It required 6 or 7 mol of oxygen consumption per mol of the enzyme for the 90% inactivation in the absence of the substrate, whereas $p$-hydroxybenzoate and benzoate repressed the oxygen consumption by about 1.5 and 0.5 mol, respectively, at the same reaction time. The inactivation was pH-dependent and the rates obtained at various pH values (5.5 to 8.6) were plotted against pH in Fig. 10. The pH profile of the inactivation rate without substrate represented a typical pH titration curve of a single ionizing group with a pK of 7.02 (25°C). The presence of saturated amounts of benzoate during the treatment gave no effect on the profile, whereas $p$-hydroxybenzoate drastically changed it. The whole profile could not be obtained experimentally in the latter case because of the instability of the enzyme at higher pH; however, the calculation gave a well fitted curve (single protonic) with a pK of 8.20 (25°C). The expected maximum inactivation rate in the absence of $p$-hydroxybenzoate was 0.313 and 0.459 min$^{-1}$, respectively. Thus, the substrate ($p$-hydroxybenzoate) showed the effects to increase the maximum inactivation rate by about 1.5-fold, as well as to shift the pK value of the ionizing group which is expected to be involved in the inactivation.

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1 H. Shoun, T. Beppu, and K. Arima, unpublished observation.
Dislocation of FAD from the enzyme was observed in the latter stage of the reaction, which was examined by gel filtration of the reaction mixture after irradiation with a Sephadex G-25 column equilibrated by the phosphate buffer (pH 6.0) at 5°C. However, the dislocation lagged behind the inactivation, indicating that it possibly occurs after the inactivation by further reactions.

p-Hydroxybenzoate hydroxylase from P. desmolytica possesses a rather high molecular weight value per FAD (68,000; Ref. 1) and contains, for example, more than 15 mol of histidine/mole, therefore, making it difficult to detect a decrease in small amounts of amino acid residues caused by the treatment. On the other hand, it is well established that the pH dependency of the photooxidation of histidine reflects the ionization of its imidazole group; only the unprotonated species (7.02) is close to that of the normal ionization of the pH dependency of the photooxidation of histidine reflects the treatment. On the other hand, it is well established that the pH dependency of the dissociation constants (Fig. 8), and the pK in equilibrium II is from the pH dependency of the ΔE290 nm (Fig. 6). The two pK values in equilibria I and II (7.02 and 7.13) are judged to agree very well with each other when the difference in the temperature at which the values were obtained is taken into consideration. This indicates that the pK of equilibrium II depends on intrinsic pK value of the ionizing group which participates in the interaction, although the equilibrium reflects the ionization of the phenol of the substrate. The ionizing group by itself acts as a base in equilibrium I and III are those obtained by the photooxidation (Fig. 10), and the pK in equilibrium II is from the pH dependency of the substrate, possibly by some configurational change around the group.

**DISCUSSION**

On comparing the interactions of p-hydroxybenzoate hydroxylase with two effectors, p-hydroxybenzoate and benzoate, which were observed as spectral changes (Figs. 4 to 6), the pH dependency of the dissociation constants (Fig. 8), and effects on the photooxidation (Fig. 10), it is evident that only the interaction of the enzyme with the p-hydroxybenzoate group could be elucidated so that the deprotonated species of the ionizing group becomes more accessible to the reaction with the activated oxygen by the complex formation with the substrate, possibly by some configurational change around the group.

![Fig. 11. A possible scheme for the equilibrium of the enzyme-substrate complex formation of p-hydroxybenzoate hydroxylase. H, Ar, and S represent the ionizing group, a basic amino acid residue, aromatic amino acid residue(s), and p-hydroxybenzoate, respectively.](http://www.jbc.org/)

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On the basis of the results of studies on the interaction of p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* with an unusual substrate, p-mercaptobenzoate, Entsch *et al.* (20) tentatively postulated that the diazin of the substrate is involved in the ternary complex of oxidized enzyme, substrate, and NADPH, which provides the driving force to stimulate the rate of the reduction of flavin with NADPH. On the other hand, we previously showed that the maximum rate of the reduction of the oxidized enzyme-p-hydroxybenzoate complex with NADPH ($k_{\text{max}}^{\text{red}}$) is almost constant in the pH range from 5.8 to 9.1, while the $K_m$ for NADPH in the reaction is variable with two inflection points at pH about 6.5 and 8.2 (at 25°C; Ref. 15). And our present results suggest that there exist three ionizing states of the enzyme-substrate complex in the pH range and that the diazin of the substrate is formed only above pH 7 (Fig. 11). These observations show that the $K_m^{\text{red}}$ is independent of the ionizing state of the enzyme-substrate complex. It is not clear whether or not the diazination provides the driving force to stimulate the reduction rate, since the ionizing equilibria concerning the enzyme-substrate interaction in the ternary complex (oxidized enzyme, substrate, and NADPH) is not known. Recently, we also showed that oxidized p-hydroxybenzoate hydroxylase forms a fairly stable complex with NADPH in the absence of the substrate, and that the pH dependency of the interaction, which was observed as the pH profile of the dissociation constant of the complex, is quite different (with the optimum below pH 6) from that of the $K_m$ for NADPH in the overall enzymatic reaction or in the reduction of the oxidized enzyme-substrate complex with NADPH, where p-hydroxybenzoate participates as a substrate or an effector (21). The results indicate that p-hydroxybenzoate dramatically changes the pH profile for the binding of NADPH to the enzyme. On the basis of these facts including present findings, we tentatively conclude that the ionizing equilibria of the enzyme-substrate complex, represented as II and III in Fig. 11, are reflected on the pH dependency of the $K_m$ for NADPH in the reduction of the oxidized enzyme-substrate complex which reveals approximate pK values of 6.5 and 8.2 as previously observed (15) and not on the pH profile for the $k_{\text{max}}^{\text{red}}$. The hypothesis could elucidate our previous observation that effectors of p-hydroxybenzoate hydroxylase can be divided into two groups from the pH profile of the overall enzymatic reaction stimulated by them (15). Effectors such as p-hydroxybenzoate, 2,4-dihydroxybenzoate, and 3,4-dihydroxybenzoate might change the pH profile for the NADPH binding, which seems to have an intimate correlation with the phenomenon of proton uptake suggested in the present report. Benzate or 6-hydroxynicotinate might not change the pH profile, since they cannot provide a proton to the enzyme by the complex formation and show the effect only to increase the reduction rate ($k_{\text{red}}^{\text{max}}$).

We observed that the enzyme-substrate complex formation consists of at least two steps (at pH 8.2), by rapid reaction analyses of the UV absorption change which accompanies the complex formation (6). Upon consideration of previous as well as present results, it could be elucidated so that the change includes those derived from the proton uptake (possibly very fast) and from the perturbation of aromatic amino acid residue(s) in the protein moiety (observable).

In the present studies, we obtained for the first time direct evidences for the proton uptake involved in the enzyme-substrate complex formation of p-hydroxybenzoate hydroxylase. We also suggested that the phenomenon affects significantly the binding of NADPH to the enzyme. This shows another role of the substrate as an effector, i.e. not only to stimulate the reduction rate ($k_{\text{red}}^{\text{max}}$) but also to regulate the binding of NADPH to the enzyme. The phenomenon is also suggestive on considering the mode of the reaction of the reduced enzyme-substrate complex with molecular oxygen, as tentatively proposed by Entsch *et al.* (11). Thus, the formation of the phenolate anion intermediate of the substrate should be important for the following processes of the enzymatic reaction, reactions with NADPH and molecular oxygen.

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