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Kinetic Studies on the Reaction Mechanism of *p*-Hydroxybenzoate Hydroxylase*

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ABSTRACT: The reaction mechanism of *p*-hydroxybenzoate hydroxylase has been investigated by the "flow methods" as well as by the overall reaction measurements. No intermediate enzyme species, such as a semiquinoid flavin, is detected at the steady state of the reaction. The reaction scheme of this enzyme is: (1) the holoenzyme is activated by binding with the substrate (*p*-hydroxybenzoate), (2) the enzyme–substrate complex is then reduced by NADPH to yield the reduced enzyme–substrate complex and NADP through transitory formation of a ternary complex such as an oxidized enzyme–substrate–NADPH complex, and (3) the reduced enzyme–substrate complex forms another ternary complex with molecular oxygen and it breaks down to the oxidized enzyme, product (protocatechuate), and water. The binding of the substrate to the enzyme is assumed to occur by a two-step process, which facilitates an interpretation of the inhibition mechanism of the reaction under excess substrate conditions.

Many investigators reported on the purification and properties of oxygenases, including both dioxygenases and monooxygenases (Nozaki *et al.*, 1963; Kita *et al.*, 1965; Fujisawa and Hayaishi, 1968; Yamamoto *et al.*, 1965; Hosokawa and Stanier, 1966), but only a few reports which contain kinetic studies have been published (Goldstein *et al.*, 1968; Sparrow *et al.*, 1969).

In previous papers (Yano *et al.*, 1966, 1969a,b,c), it was reported that *p*-hydroxybenzoate hydroxylase, a monooxygenase, can be induced by adding *p*-hydroxybenzoate as a sole carbon source in the culture medium of *Pseudomonas desmolytica* IAM 1123, and that the enzyme can be crystallized in two forms, *i.e.*, the holoenzyme–*p*-hydroxybenzoate complex and the free holoenzyme. This enzyme contains 1 mole of FAD per mole of enzyme, whose molecular weight was estimated to be 68,000, and it catalyzes the hydroxylation of *p*-hydroxybenzoate (usually referred to as the substrate) to yield protocatechuate (the product) in the presence of NADPH and molecular oxygen. It was also reported that one molecule of *p*-hydroxybenzoate could activate one enzyme molecule, so that the anaerobic reduction rate, v_{red}, of the bound FAD of the activated enzyme was increased about 10^4 times over that of the holoenzyme. The activation could be attributed to the conformational changes in the active site, which was caused by binding with the substrate.

By stoichiometric analyses and spectrophotometric investigations, the following reaction sequence was postulated for the overall reaction of the enzyme, where E_{ox}, E_{ox}–S, E_{red}, and E_{red}–S stand for oxidized enzyme, oxidized enzyme–*p*-hydroxybenzoate complex, re-

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$$E_{ox} + S \longrightarrow E_{ox}\text{-}S$$

$$E_{ox}\text{-}S + NADPH \longrightarrow E_{red}\text{-}S + NADP$$

$$E_{red}\text{-}S + O_2 \longrightarrow E_{ox} + P + H_2O$$

duced enzyme, and reduced enzyme–*p*-hydroxybenzoate complex, respectively; and S and P represent *p*-hydroxybenzoate and protocatechuate, respectively. Similar reaction schemes have been reported on other hydroxylases: salicylate hydroxylase of a pseudomonas (Katagiri *et al.*, 1966) and *p*-hydroxybenzoate hydroxylase of *Pseudomonas putida* (Hosokawa and Stanier, 1966; Hesp *et al.*, 1969). But inadequate kinetic data to support the scheme have been presented.

From the viewpoint of enzyme kinetics, the reactions catalyzed by these hydroxylases are three-substrate reactions: the enzyme requires the aromatic substrate to be hydroxylated, NAD(P)H as the electron donor, and oxygen as the electron acceptor. Very few papers have described extensive kinetic investigations of a three-substrate enzyme reaction with a highly purified enzyme, although such types of enzymes are rather common. Especially, in the case of an oxygen-linked enzyme, such as an oxidase or an oxygenase, the effect of oxygen concentration on the reaction has sometimes been neglected, so that the important information on the interactions of the enzyme with oxygen might have possibly been overlooked.

The present paper describes the reaction mechanism of *p*-hydroxybenzoate hydroxylase purified from *P. desmolytica*, which is based on the results obtained by kinetic investigations. Studies were done with steady-state kinetics by measuring overall oxygen uptake at several concentrations of *p*-hydroxybenzoate, NADPH, and oxygen, and with flow methods measuring the absorbance change of the enzyme-bound FAD.

Some of the results were presented at Duke University, N. C. (Nakamura *et al.*, 1970).

Materials and Methods

p-Hydroxybenzoate Hydroxylase. *p*-Hydroxybenzoate hydroxylase was purified from *P. desmolytica* IAM 1123 and obtained in a crystalline state of the (holo)enzyme–*p*-hydroxybenzoate complex as previously reported (Yano *et al.*, 1969a). The enzyme–substrate complex was dissolved in a Tris-maleate buffer, pH 6.0, and the solution was passed through a Sephadex G-25 column to remove the substrate. The active fractions were collected and used as the free (holo)enzyme. For the sake of convenience, the concentration of the enzyme was expressed as that of enzyme-bound FAD, which was calculated with the aid of the millimolar extinction coefficient of free FAD (Whitby, 1953).

Chemical Reagents. The reagents used were of the purest commercial grade available and were used without further purification unless otherwise stated. *p*-Hydroxybenzoate was recrystallized from water. NADPH was purchased from Böhringer und Söhne Co., Mannheim, and its concentration was determined spectrophotometrically by measuring the absorption at 340 mμ (Horecker and Kornberg, 1948).

Spectrophotometric Measurements. Spectrophotometric measurements were made with a Hitachi double-beam recording spectrophotometer, Model 124, or with a Hitachi-Perkin-Elmer spectrophotometer, Model 139.

Measurement of Oxygen Uptake. The rate of the overall reaction was estimated by measuring the consumption of molecular oxygen dissolved in the reaction medium using a Clark oxygen electrode from Yellow Springs Instruments Co., Ohio. The details of the apparatus were as reported previously (Nakamura and Ogura, 1968). The activity was assayed at 25°, pH 8.2. The reaction was initiated by introducing 0.1 ml of an enzyme solution into 2.4 ml of a buffer containing known amounts of *p*-hydroxybenzoate and NADPH. Oxygen concentration was calculated from the table of its solubility.

Flow Experiments. Flow experiments were performed either with a Chance–Legallais type flow apparatus attached with a San-ei oscillograph, Model 101, as previously reported (Nakamura and Ogura, 1962), or with a Yanaco Stopped Flow SPS-1 from Yanagimoto Manufacturing Co., Kyoto (Hiromi *et al.*, 1968), the dead time of which was 2 msec when 1.0-cm light path length was used. Anaerobic flow experiments were carried out with essentially the same methods as reported (Nakamura and Ogura, 1968). The anaerobic reduction rate, v_{red}, of the FAD moiety was obtained by one of the following ways. When Yanaco SPS-1 was used, the rate was calculated from the stopped-flow trace recorded on the oscilloscope, assuming the reduction follows the first-order kinetics; when the Chance–Legallais type apparatus was used, the value of the slow reaction was obtained similarly from the stopped-flow trace, and that of the fast reaction was from the continuous-flow trace of the oscillograph using the following formula (Chance, 1951):

$$v_{red} = \frac{2.3}{t} \log \frac{e_0}{e_0 - e_t} \qquad (1)$$

where e_0 is the concentration of the activated enzyme present, and e_t the amount of reduced enzyme in t seconds after mixing.

The rate of oxidation, v_{ox}, was also calculated similarly using Yanaco SPS-1.

Results

Absorption Spectrum of the Enzyme at the Steady State. The stopped-flow method was used to determine whether any transitory intermediate species of the enzyme would appear before and at the steady state of the reaction. The enzyme solution reduced with 0.78 mM NADPH in the presence of 1.0 mM *p*-hydroxybenzoate was mixed with an equal volume of the buffer containing molecular oxygen. The results are shown in Figure 1. Although measurements could not be performed in the wavelength region shorter than 430 mμ because of the high absorbance of NADPH in this region, no evidence was obtained from the steady state absorption spectrum to indicate the presence of an intermediate, such as a flavin semiquinone or a charge-transfer complex. Furthermore, any spectral intermediates were not detected in the presteady state. Therefore, it does not seem likely that any intermediate species are formed in the catalyzed reaction; the reaction will proceed by the turnover of the enzyme between the oxidized form and the reduced form.

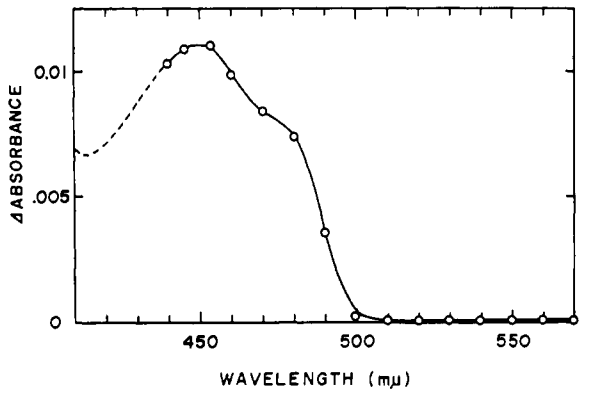

FIGURE 1: Difference absorption spectrum of the flavin moiety (steady-state minus reduced level) measured by the stopped-flow method: enzyme concentration, 4.8 μM; *p*-hydroxybenzoate concentration, 1.0 mM; NADPH concentration, 0.78 mM; oxygen concentration, 0.12 mM; buffer, 0.05 M Tris-maleate, pH 6.0; temperature, 30°.

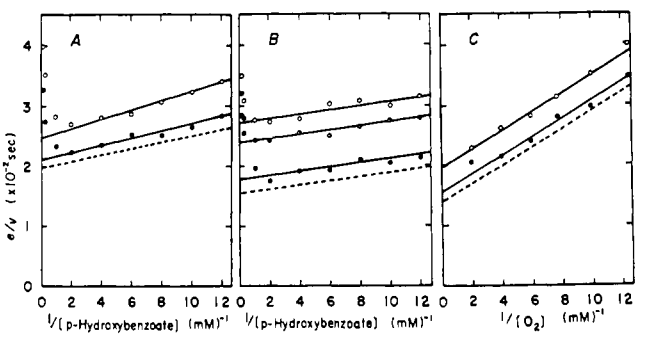

FIGURE 2: Lineweaver–Burk plots of the initial velocity measured with a Clark oxygen electrode at 25°, pH 8.2. (A) Effect of NADPH on the substrate–velocity relationship for *p*-hydroxybenzoate in the presence of 0.26 mM oxygen: enzyme concentration, 4.4 × 10^{-8} M; NADPH concentration, 0.14 mM (○), 0.56 mM (●), and infinite (--). (B) Effect of oxygen on the substrate–velocity relationship for *p*-hydroxybenzoate in the presence of 0.30 mM NADPH: enzyme concentration, 5.6 × 10^{-8} M; oxygen concentration, 0.13 mM (○), 0.24 mM (◑), 0.67 mM (●), and infinite (--). (C) Effect of NADPH on the substrate–velocity relationship for oxygen in the presence of 0.50 mM *p*-hydroxybenzoate: enzyme concentration, 4.4 × 10^{-8} M; NADPH concentration, 0.14 mM (○), 0.55 mM (●), and infinite (--).

Rate of Overall Reaction. The overall reaction was followed by measuring oxygen uptake with a Clark oxygen electrode. Since the reaction catalyzed by this enzyme is a three-substrate reaction from the kinetic point of view, it is necessary to investigate the relationship between each combination of *p*-hydroxybenzoate with NADPH, *p*-hydroxybenzoate with oxygen, or NADPH with oxygen. The results are shown in Figure 2. Figure 2A is the Lineweaver–Burk plot of the data obtained with NADPH as the changing fixed substrate[1] and *p*-hydroxybenzoate as the variable substrate in the presence of a given amount of oxygen. An inhibition of the reaction by *p*-hydroxybenzoate is seen in the concentration range higher than 1 mM. However, most of the points appear to fit straight lines, which may intersect each other. This suggests that a ternary complex, such as an oxidized enzyme–*p*-hydroxybenzoate–NADPH complex, may be formed as an intermediate of the enzymic reaction. In Figures 2B and 2C, the relationships of *p*-hydroxybenzoate with oxygen and oxygen with NADPH are shown, respectively, in the form of the Lineweaver–Burk plot. Each figure can be expressed as a series of parallel lines. It is evident that the equation to describe these experimental results is:

$$\frac{e}{v} = \frac{e}{V_{max}} + \frac{K_1}{[S]} + \frac{K_{12}}{[S][N]} + \frac{K_2}{[N]} + \frac{K_3}{[O_2]} \qquad (2)$$

where S and N denote *p*-hydroxybenzoate and NADPH, respectively, e stands for total enzyme concentration, V_{max} for the maximal reaction velocity, and K's are constants obtained graphically from the figures. For the sake of simplicity, the terms for excess substrate inhibition are omitted here. These constants are listed in Table I. A more detailed picture of the constants will be obtained by the flow experiments.

Rate of Enzyme Reduction by NADPH. As reported in the previous communications (Yano *et al.*, 1969b,c), the reduction rate, v_{red}, of the FAD moiety of the enzyme by NADPH under anaerobic conditions was markedly increased by the addition of *p*-hydroxybenzoate. The relationship between the anaerobic reduction and the amount of *p*-hydroxybenzoate added was investigated with flow methods. Previously deoxygenated solution of the enzyme with various amounts of *p*-hydroxybenzoate and that of NADPH were mixed in the flow apparatus under strictly anaerobic conditions. Figure 3 shows a typical flow pattern obtained with a smaller amount of *p*-hydroxybenzoate than that of the enzyme-bound FAD. As can be seen in the figure, the trace curve is biphasic with a definite inflection point, which reveals that the reduction consists of a fast reaction and a slow one. This indicates that both activated and nonactivated enzyme species were present under the given conditions. The amount of the activated enzyme species could be calculated from the absorbance difference between the original oxidized level of the enzyme and that of the inflection point. The v_{red} value of each reaction was calculated by the way stated in Methods. The results are listed in Table II. As seen in the

TABLE I: Kinetic Constants Obtained by Overall Reaction Measurements.

K_1[a]	5.0×10^{-7} M sec
K_{12}	3.3×10^{-11} M^2 sec
K_2	7.0×10^{-7} M sec
K_3	1.3×10^{-6} M sec
V_{max}/e[b]	72 sec^{-1}

[a] The values of K's were calculated from the slopes in Figure 2. [b] V_{max}/e was calculated by the use of an apparent maximal velocity, which was obtained as an intercept on the ordinate of the dotted (extrapolated) line in each plot of Figure 2 (A, B, and C) with the aid of a corresponding K value listed in this table. The value listed here is the average of those three values thus calculated.

[1] The terminology used in this paper is that of Cleland (1963a,b).

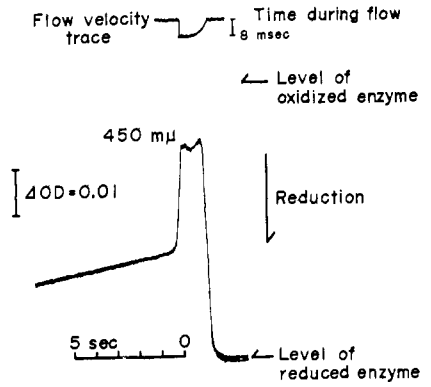

FIGURE 3: Trace of the absorbance change at 450 mμ obtained by an anaerobic flow experiment with a less amount of *p*-hydroxybenzoate: enzyme concentration, 7.0 μM; *p*-hydroxybenzoate concentration, 4.3 μM; NADPH concentration, 0.21 mM. Measurement was done with a Chance-Legallais type stopped-flow apparatus under anaerobic conditions at 25°, pH 8.2.

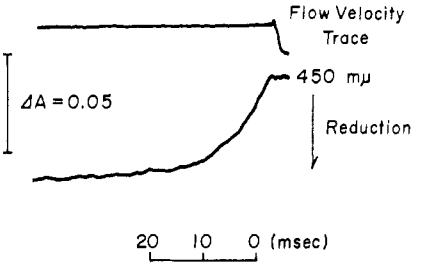

FIGURE 4: Trace of the absorbance change at 450 mμ obtained by an anaerobic flow experiment with a sufficient amount of *p*-hydroxybenzoate: enzyme concentration, 4.6 μM; *p*-hydroxybenzoate concentration, 0.22 mM; NADPH concentration, 0.15 mM. Measurement was done with a Yanaco Stopped Flow, SPS-1, under anaerobic conditions, at 25°, pH 8.2; dead time, 2.0 msec.

table, an equimolar relationship was found between the amount of the activated enzyme and that of *p*-hydroxybenzoate added when the latter was smaller than the former. The v_{red} values of the fast part of the reaction were large enough to explain the overall reaction velocity (Table I); on the other hand, the reaction of the holoenzyme with NADPH, v_{red} = 0.010 sec^{-1}, was too slow to be included in the enzymic reaction sequence.

The effect of NADPH concentration on the reduction rate was also investigated by flow methods under anaerobic conditions in the presence of a sufficient amount of *p*-hydroxybenzoate. A typical flow trace on the oscillograph is shown in Figure 4. Since the reaction was recognized to follow the pseudo-first-order kinetics, v_{red} was expressed as the first-order rate constant. Figure 5 shows a linear relationship between the reciprocal of v_{red} and that of NADPH. The extrapolation of NADPH concentration to infinity shows a definite intercept on the ordinate, which gives the v_{red}^{max} value of 200 sec^{-1}. This indicates that a rate-determining enzyme species is formed in the reduction step, which may be the oxidized enzyme–substrate–NADPH complex, E_{ox}–S–NADPH, as suggested from the overall reaction kinetics.

Rate of Enzyme Oxidation by Molecular Oxygen. A similar flow experiment was performed to investigate the oxidation reaction of the enzyme with Yanaco SPS-1. In the presence of a known amount of *p*-hydroxybenzoate, a solution of the enzyme reduced by NADPH was mixed with an equal volume of buffer containing oxygen. The reaction was followed by measuring the absorbance change at 450 mμ. Figure 6 shows the plot of the reciprocal values of v_{ox}, the oxidation rate expressed as the first-order rate constant, against those of oxygen concentration. The data again fit a straight line and the v_{ox}^{max} value was calculated to be 400 sec^{-1} from the intercept on the ordinate. This suggests that the oxidation step also has a rate-determining enzyme species which might be considered as an oxygenated form of the reduced enzyme–substrate complex, E_{red}–S–O_2, although the physicochemical nature of the oxygenated flavoprotein is so far uncertain.

Inhibition by Excess Substrate. As stated before, the overall oxygen uptake was inhibited in the presence of an excess amount of *p*-hydroxybenzoate (above 1 mM). The anaerobic flow experiment revealed that two enzyme species were

TABLE II: Activation of the Enzyme Reduction by *p*-Hydroxybenzoate.[a]

p-Hydroxybenzoate Added (μM)	Activated[b] Enzyme (μM)	v_{red} (sec^{-1})[c] Fast Reaction	Slow Reaction
0	0		0.010
2.1	2.7	60	0.05
4.3	4.4	66	0.14
6.5	5.2	77	0.23
233	7	96	

[a] Measurements were done with a Chance–Legallais type stopped-flow apparatus under anaerobic conditions at 25°, pH 8.2. Enzyme concentration: 7.0 μM. NADPH concentration: 210 μM. [b] The amount of the activated enzyme was calculated from the absorbance difference between the original oxidized level and the inflection point of the flow trace. [c] Calculated as stated in Methods.

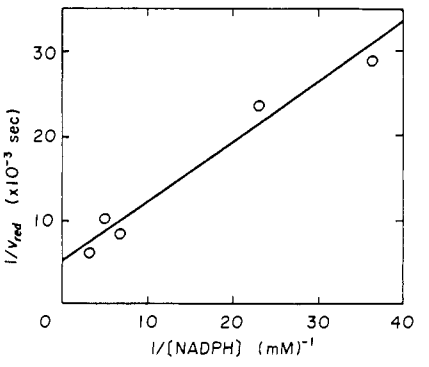

FIGURE 5: Plot of $1/v_{red}$ against 1/[NADPH]. The values of v_{red} were measured by anaerobic stopped-flow experiments with a Yanaco Stopped Flow, SPS-1: enzyme concentration, 4.6 μM; *p*-hydroxybenzoate concentration, 0.22 mM; temperature, 25°; pH 8.2.

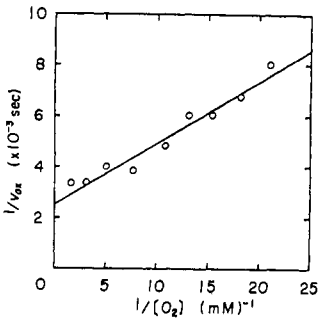

FIGURE 6: Plot of $1/v_{ox}$ against $1/[O_2]$. Measurements were done with a Yanaco Stopped Flow, SPS-1: enzyme concentration, 4.6 μM. *p*-hydroxybenzoate concentration, 0.22 mM; NADPH concentration, 0.61 mM; temperature, 25°; pH 8.2.

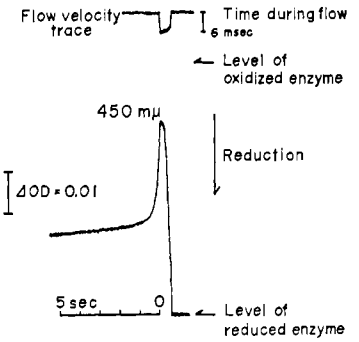

FIGURE 7: Trace of the absorbance change at 450 mμ obtained by an anaerobic flow experiment with an excess amount of *p*-hydroxybenzoate. Measurement was done with a Chance–Legallais type stopped-flow apparatus under anaerobic conditions at 25°, pH 8.2: enzyme concentration, 7.0 μM; *p*-hydroxybenzoate concentration, 7.0 mM; NADPH concentration, 0.21 mM.

present under excess substrate conditions, since the reduction proceeded biphasically as shown in Figure 7. These species are considered as activated and inactivated enzyme–substrate complexes. This may be interpreted in the following manner. At a higher concentration of the substrate, the active site of the enzyme combines with two molecules of the substrate in an antiparallel manner, so that they may form an ineffective complex, E_{ox}–S_aS_b, where S_a and S_b represent the substrate molecules bound to functional groups A and B, respectively, although these groups are not identified yet. From the investigations on the interactions of the enzyme with the substrate or substrate analogs, it was suggested that the active site of *p*-hydroxybenzoate hydroxylase contains, at least, two functional groups for the substrate binding: one for the hydroxyl group and the other for the carboxyl group of the substrate (Higashi *et al.*, 1970a). This assumption is quite compatible with the excess substrate inhibition (Dixon and Webb, 1964). The results of overall reaction experiments performed under excess substrate conditions are shown in Figure 8 in the double reciprocal form with respect to NADPH concentration. As can be seen in the figure, the inhibition pattern is a noncompetitive type with respect to NADPH. This will be discussed in the next paragraph.

Reaction Mechanism of p-Hydroxybenzoate Hydroxylase. On the basis of the results of kinetic investigations presented in this paper together with those reported previously (Yano *et al.*, 1969b), a reaction mechanism of *p*-hydroxybenzoate hydroxylase will be proposed.

By the stopped-flow measurements of the absorption spectra of the bound FAD at the steady state as well as in presteady state of the reaction, only two spectrophotometrically distinguishable enzyme species, *i.e.*, the oxidized form and the reduced form of the FAD moiety, were recognized to be involved in the catalytic reaction, and accordingly, any other intermediate spectral species can be excluded from the reaction mechanism.

Activation effect of *p*-hydroxybenzoate on the reduction of the enzyme indicates that the enzymic reaction proceeds by an ordered addition mechanism with *p*-hydroxybenzoate as the first ligand rather than that of random addition between the oxidized enzyme, *p*-hydroxybenzoate, and NADPH. As stoichiometric analyses have shown, however, *p*-hydroxybenzoate molecule still remained unchanged even after the bound FAD was reduced by NADPH unless oxygen was present. So the role of *p*-hydroxybenzoate here is simply regarded as that of an activator of the reduction step.

Flow experiments introduced a rate-determining species in each of the reduction and oxidation steps, whose rate constants are expressed as v_{red}^{max} (about 200 sec^{-1}) and v_{ox}^{max} (about 400 sec^{-1}), respectively. Since the maximal velocity obtained from the overall reaction measurements (V_{max}/e = 72 sec^{-1}, Table I) is smaller than that expected from $v_{red}^{max} \times v_{ox}^{max}/(v_{red}^{max} + v_{ox}^{max})$, another first-order step must therefore be introduced. This step can be assumed to be in the process of binding of the oxidized enzyme with the substrate. As stated in the preceding paragraph, the substrate binding probably occurs at two functional groups of the enzyme; in other words, the formation of the effective enzyme–substrate complex may consist of, at least, two steps:

$$E_{ox} + S \rightleftharpoons E_{ox}\text{–}S_a \rightleftharpoons E_{ox}\text{–}S_{ab}$$

where E_{ox}–S_{ab} represents the effective (active) enzyme–substrate complex in which the substrate molecule is bound to the enzyme at functional groups A and B properly. The latter step of the binding process will provide another first-

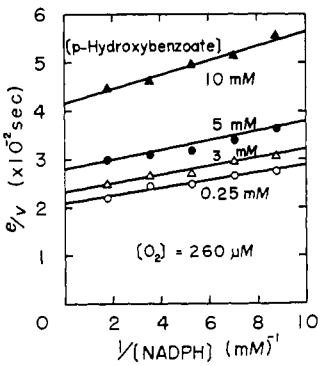

FIGURE 8: Lineweaver–Burk plot for NADPH under excess substrate conditions. Measurements were done with a Clark oxygen electrode at 25°, pH 8.2: enzyme concentration, 4.4 $\times$ 10^{-5} M. oxygen concentration, 0.26 mM; *p*-hydroxybenzoate concentrations are as indicated in the figure.

SCHEME I

$$E_{ox} + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}\text{-}S_a \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E_{ox}\text{-}S_{ab}\ (= E_{ox}\text{-}S)$$

$$E_{ox}\text{-}S_a + S \overset{Ks'}{\rightleftharpoons} E_{ox}\text{-}S_aS_b$$

$$E_{ox}\text{-}S + NADPH \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_{ox}\text{-}S\text{-}NADPH \xrightarrow{k_{+4}} E_{red}\text{-}S + NADP$$

$$E_{red}\text{-}S + O_2 \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} E_{red}\text{-}S\text{-}O_2 \xrightarrow{k_{+6}} E_{ox} + P + H_2O$$

order rate constant required above.[2] This two-step binding assumption, furthermore, facillitates the explanation for the excess substrate inhibition mechanism, since the ineffective complex, E_{ox}-S_aS_b, may be formed by the addition of the second substrate molecule to the intermediate E_{ox}-S_a species as follows:

$$E_{ox}\text{-}S_a + S \rightleftharpoons E_{ox}\text{-}S_aS_b$$

A complete scheme derived from these considerations is shown in Scheme I. The steady-state rate equation (Hearon *et al.*, 1959) calculated on the basis of the scheme is:

$$\frac{e}{v} = \frac{1}{k_{+2}}\left(1 + \frac{[S]}{K_s'}\right) + \frac{1}{k_{+4}} + \frac{1}{k_{+6}} + \frac{k_{-1} + k_{+2}}{k_{+1}k_{+2}[S]} + \frac{k_{-1}k_{-2}(k_{-3} + k_{+4})}{k_{+1}k_{+2}k_{+3}k_{+4}[S][N]} + \frac{k_{-2}(k_{-3} + k_{+4})}{k_{+2}k_{+3}k_{+4}[N]}\left(\frac{k_{-2} + k_{+2}}{k_{-2}} + \frac{[S]}{K_s'}\right) + \frac{k_{-5} + k_{+6}}{k_{+5}k_{+6}[O_2]} \quad (3)$$

where K_s' is the dissociation constant of the ineffective complex. As can be seen in the equation, the terms for the excess substrate inhibition, $[S]/K_s'$, appear only in the maximal velocity and apparent coefficient term of 1/[NADPH]. Then, if e/v is plotted against reciprocal NADPH concentration, the pattern of the plot under excess substrate conditions will show an apparent noncompetitive type of inhibition with respect to NADPH. This was already shown in Figure 8.

The scheme presented here accounts for all experimental results so far obtained.

Available kinetic constants based on the scheme are shown in Table III.

TABLE III: Kinetic Constants Calculated on the Basis of the Reaction Mechanism Presented in Scheme I.

Definition or Assumption	Estimated Value	
$V_{max}/e = k_{+2} \times k_{+4} \times k_{+6}/(k_{+2}k_{-4} + k_{+2}k_{+6} + k_{+4}k_{+6})$	72 sec^{-1}	*a*
k_{+2}	160 sec^{-1}	*b*
$k_{+4} = v_{red}^{max}$	200 sec^{-1}	*c*
$k_{+6} = v_{ox}^{max}$	400 sec^{-1}	*c*
$K_m^{O_2} = (k_{-5} + k_{+6})/k_{+5}$	0.52 mM	*d*
K_s'	3.6 mM	*e*

[a] Cited from Table I. [b] Calculated from the values of V_{max}/e, k_{+4}, and k_{+6}. [c] Data obtained by stopped-flow experiments. [d] Calcnlated by using the values of k_{+6} and K_3 in Table I. [e] Obtained from the increase in the intercept of Figure 8 by use of the values listed in Tables I and III.

Discussion

Some similarities were found between the present *P. desmolytica* enzyme and *P. putida* enzyme which was purified by Hosokawa and Stanier (1966) or by Hesp *et al.* (1969). Both enzymes contain 1 mole of FAD per mole of enzyme, and do not contain any significant amount of metal ion. These enzymes are induced by adding *p*-hydroxybenzoate to the culture medium as a sole carbon source. Optimal pH region of these enzymes is almost the same. The enzymic activities were inhibited by an excess amount of *p*-hydroxybenzoate and were quite specific for NADPH and could not be replaced by NADH. The activation of overall reaction was also found on *putida* enzyme (130 times), but not so significant as compared with our present enzyme (10^4 times). But, since the difference in magnitude of activation ratio seems to be mainly due to the limitation of technique they used (Cary 14 was used for measurement of activity), the activation phenomenon might be essentially the same with both of these enzymes (K. Hosokawa, 1969, personal communication).

On the other hand, some discrepancies were recognized. The molecular weight of *desmolytica* enzyme was estimated to be 68,000 (Yano *et al.*, 1969a), while that of *putida* enzyme was reported as 83,000 (Hosokawa and Stanier, 1966) or 93,600 (Hesp *et al.*, 1969). *p*-Aminobenzoate and protocatechuate have been reported to be noncompetitive inhibitors for the *putida* enzyme (Hosokawa and Stanier, 1966); on the other hand, these compounds were found to be competitive inhibitors with respect to *p*-hydroxybenzoate in the case of *desmolytica* enzyme (Nakamura *et al.*, 1970). Our present enzyme could be crystallized in the form of the enzyme-*p*-hydroxybenzoate complex more easily than in the form of free enzyme, while their enzyme was crystallized only in the form of free enzyme as judged from the absorption spectrum they reported.

Crystallization of an enzyme-substrate complex has been reported with D-amino acid oxidase (Yagi and Ozawa, 1964), which was achieved under anaerobic conditions, and the kinetic data to support the view that the complex is an intermediate of the catalyzed reaction were presented recently (Yagi *et al.*, 1969). Since an enzyme-substrate complex, *per se*, is transient and unstable, it is very difficult to obtain such a complex in a crystalline form. Fortunately, in the case of *p*-hydroxybenzoate hydroxylase, since the oxidized enzyme-substrate complex does not proceed to the next reaction step unless NADPH is present, the complex is

[2] Recent studies on the enzyme-substrate complex formation by a relaxation method have shown that the binding occurs *via* a two-step process in accord with the present assumption. However, the value of the first-order rate constant of the latter step was higher than the k_{+2} value of the present calculation (Higashi *et al.*, 1970b).

obtained in a stable crystalline state rather easily. The anaerobic reduction rate, v_{red}, obtained with a solution of the crystalline enzyme–substrate complex was the same as that of the enzyme activated by the substrate just before the measurement. As stated in the text, one molecule of *p*-hydroxybenzoate activates one enzyme molecule, and thereafter the bound substrate is hydroxylated to yield protocatechuate quantitatively (Yano *et al.*, 1969b). These results clearly indicate that the crystal of the complex is that of a kinetically significant intermediate of the enzymic reaction, and that the activator site and the substrate-binding site are one and the same; in other words, an activator site or an allosteric site need not be assumed. It is very important to point out here that the activation of the catalyzed reaction caused by the aromatic substrate is seen not only with *p*-hydroxybenzoate hydroxylase but also with at least two other FAD-containing oxygenases: salicylate hydroxylase (Katagiri *et al.*, 1965) and 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase (Sparrow *et al.*, 1969). This suggests a possibility that a general activation mechanism may be shared with all these enzymes in common.

In the paper of Hesp *et al.* (1969), the circular dichroism spectra were reported in the presence of the substrate or its analogs using *p*-hydroxybenzoate hydroxylase purified from *P. putida* M-6. Distinct changes in the circular dichroism spectrum was recognized in the ultraviolet wavelength region on binding with the substrate. This finding may correspond to our earlier observations that the physicochemical properties of the enzyme, such as sedimentation velocity, optical rotatory dispersion spectrum in the ultraviolet wavelength region, and absorption spectrum of the FAD moiety, were subject to change on binding with the substrate. All these changes together with the change in anaerobic reduction rate of the FAD moiety may suggest a conformational change in the protein moiety. As reported elsewhere (Nakamura *et al.*, 1970), kinetic investigations on protocatechuate inhibition showed that the binding sites for *p*-hydroxybenzoate and NADPH are kinetically different. These facts seem to correspond to those of an allosteric protein (Monod *et al.*, 1963). However, with this enzyme, no data have been obtained so far to indicate the subunit–subunit interactions which are usually cited for an allosteric phenomenon (Monod *et al.*, 1965). The plot of the reaction rate[3] against NADPH concentration in the absence of *p*-hydroxybenzoate gave a normal hyperbolic curve instead of a sigmoidal curve of a higher order. As reported (Yano *et al.*, 1969a), this enzyme contains 1 mole of FAD per mole of enzyme protein. This is a strong evidence for the enzyme to be a monomer in terms of the allosteric theory (Monod *et al.*, 1965). It is, thus, unlikely that this enzyme is composed of several identical subunits. However, the activation of the reduction caused by *p*-hydroxybenzoate could still be regarded as an allosteric phenomenon; because the conformational change of the enzyme protein was caused on binding with *p*-hydroxybenzoate (which should be regarded as the effector here), the binding site for *p*-hydroxybenzoate is different from that of NADPH (the substrate of the reduction step), and *p*-hydroxybenzoate and NADPH are, of course, quite different from each other in chemical structure (Monod *et al.*, 1963).

In the present paper, a relevant reaction scheme of *p*-hydroxybenzoate hydroxylase is postulated. The scheme is consistent with all the results of kinetic experiments, including both steady-state experiments and flow experiments, as well as with those of the stoichiometric analyses reported previously. The presented scheme is an ordered addition mechanism of *p*-hydroxybenzoate, NADPH, and oxygen in this order, involving three first-order reaction steps. As the rate-determining species, one binary, E_{ox}–S_a, and two ternary complexes, E_{ox}–S–NADPH and E_{red}–S–O_2, are proposed. The proposal of E_{ox}–S_a species is supported by the data on the excess substrate inhibition and enzyme–substrate (analog) interaction. The ternary species, E_{ox}–S–NADPH and E_{red}–S–O_2, result directly from the stopped-flow experiments on reductive and oxidative half-reactions (Figures 5 and 6), respectively. The oxygenated intermediate assumed in the scheme may require some comments. In the case of flavoprotein oxidases, such as D-amino acid oxidase or glucose oxidase, the reaction of oxygen molecule with the enzyme is so fast as compared with other reaction steps that no rate-determining intermediate species has been assumed in the oxidation reaction (Nakamura *et al.*, 1964; Nakamura and Ogura, 1968). The reaction of oxygen with an oxygenase might be another case. According to Ishimura *et al.* (1967), an oxygenated intermediate was observed spectrophotometrically with tryptophan pyrrolase, a heme-containing dioxygenase. Katagiri *et al.* (1968) also assumed an oxygenated form of cytochrome P-450 in the reaction of 5-*exo*-hydroxylation of camphor. Thus it seems useful to assume an oxygenated enzyme species as a rate-determining intermediate of oxygenase-catalyzed reactions, including those reactions catalyzed by flavin-containing monooxygenases. Recently, Massey *et al.* (1969) proposed a suggestive scheme concerning the reaction of flavin compounds with molecular oxygen, where reduced flavin–oxygen adducts were presented. In the case of *p*-hydroxybenzoate hydroxylase, however, the protein moiety may also have an additional role for the reaction with oxygen molecule, since stimulation effect by the aromatic substrate on the oxidation reaction of the FAD moiety was observed with this enzyme (N. Higashi, 1969, unpublished data).

Further studies are now in progress.

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[3] The reaction rate was measured by NADPH oxidation at 340 mμ spectrophotometrically with a Hitachi double-beam spectrophotometer, Model 124.

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