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A Study of *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*: Chemical Modification of Histidine Residues†

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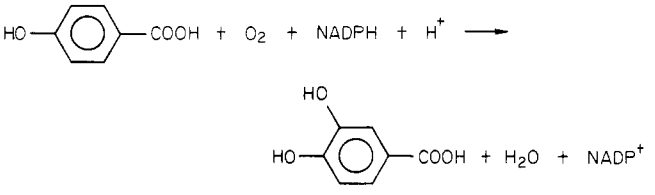
ABSTRACT: The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is inactivated by diethyl pyrocarbonate. Below pH 7, diethyl pyrocarbonate reacts specifically with histidine residues. The inactivation reaction is biphasic and follows pseudo-first-order kinetics. Four of the nine histidine residues of the enzyme are modified. During the first phase of the reaction, one histidine residue is modified and leads to a loss of about 30% of the activity. Modification of the additional three histidine residues during the second phase leads to complete loss of activity. Two of the latter histidine residues are essential for activity and are involved in the binding of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The activity can be restored almost quantitatively upon treatment of modified enzyme with hydroxylamine. The modified enzyme is still capable of binding NADPH. The dissociation constant of the enzyme–NADPH complex is larger by a factor of 10 for the modified enzyme as compared to that for the native enzyme. The modification does not affect the affinity of the enzyme for the substrate, although effectors protect two histidine residues from chemical modification by diethyl pyrocarbonate. The rate of inactivation of the enzyme is pH dependent and increases with increasing pH values. From the pH dependence of the rate constant, it is calculated that two cooperative histidine residues participate in the reaction with diethyl pyrocarbonate. Both histidine residues possess a pK_a' value of 6.2. At pH >7, other reactions take place which are completely abolished in the presence of an effector (substrate) of the enzyme.

The inducible enzyme *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) belongs to the class of external flavoprotein monooxygenases. It can be obtained from four different species of *Pseudomonas*, but the enzyme present in *Pseudomonas fluorescens* is the most stable one. It catalyzes the following reaction:



Significant progress has been made in recent years concerning the structure and catalytic mechanism of *p*-hydroxybenzoate hydroxylase (Shoun et al., 1979a; Müller et al., 1979; Husain & Massey, 1979). We have devoted ourselves to the study of the chemical modification of amino acid residues important for the catalytic activity of the enzyme. This is now a promising approach since the entire sequence of the enzyme is known (Hofsteenge et al., 1980; Vereijken et al., 1980; Weijer et al., 1982). In addition, the existing three-dimensional model of the enzyme–substrate complex at a resolution of 0.25 nm (Wierenga et al., 1979) allows a more detailed interpretation of the data of chemical modification. Furthermore, modification studies will yield results important for understanding the catalytic mechanism of the enzyme.

The work that has been done in the field of chemical modification so far includes arginine and histidine modification of the enzyme from *Pseudomonas desmolytica* (Shoun et al., 1979b, 1980) and cysteine modification of the enzyme from *P. fluorescens* (F. Müller and W. J. H. Van Berkel, unpublished experiments). Histidine modification by Shoun and his colleagues (Shoun et al., 1979b) was carried out by photooxidation at pH 6, and their conclusion was that a histidine residue with a pK_a' of 7.0 is involved in substrate binding by forming a hydrogen bridge with the phenolic OH of *p*-hydroxybenzoate. However, from the low-resolution three-dimensional model of the enzyme, it can be concluded that histidine is not involved directly in the binding of the substrate, at least not as far as the enzyme from *P. fluorescens* is concerned. This apparent discrepancy could be related to the fact that photooxidation reactions are not very specific, as tyrosine, methionine, and tryptophan can also be modified by photooxidation (Westhead, 1972).

The aim of this paper is to elucidate the role of histidine residues in *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Our results clearly indicate that histidine residues are not involved in the binding of the substrate *p*-hydroxybenzoate, but rather in the binding of the pyridine nucleotide.

Materials and Methods

Diethyl pyrocarbonate was from Fluka, L-histidine monochloride was from the British Drug Houses Ltd., reduced nicotinamide adenine dinucleotide phosphate (NADPH)[1] was

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from Boehringer, Mes, Hepes, and Tris were from Sigma, and all other chemicals were from Merck.

Spectrophotometric measurements were done on a Zeiss PMQII, a Cary 16, or an Aminco DW2A spectrophotometer, and fluorometric measurements were performed on an Aminco SPF-500 spectrofluorometer.

p-Hydroxybenzoate hydroxylase was purified from *P. fluorescens* as described previously (Müller et al., 1979). The purified enzyme was kept as an ammonium sulfate precipitate (70%) at 4 °C, pH 6–7. Prior to use, a desired amount of the precipitate was dialyzed against 20 mM Mes buffer, pH 6, ionic strength 20 mM, unless stated otherwise. The enzyme concentration was determined spectrophotometrically on the basis of the FAD content by assuming a molar absorption coefficient of 11 300 M^{-1} cm^{-1} at 450 nm (Müller et al., 1979).

The enzyme activity was assayed by adding 4 μL of a 20 μM enzyme solution to 1 mL of 0.1 M Tris–H_2SO_4, pH 8.0. The buffer solution contained 0.15 mM *p*-hydroxybenzoate and 0.15 mM NADPH. The activity was determined by recording the decrease of absorbance of NADPH at 340 nm vs. time. The temperature was 25 °C.

Ethoxyformylation of the enzyme was achieved by treating a solution of 20 μM *p*-hydroxybenzoate hydroxylase with 0.2–0.8 mM diethyl pyrocarbonate at 4 or 25 °C. The diethyl pyrocarbonate was added as an ethanol solution (15–25 mM). This stock solution was kept at −20 °C and before use checked for its diethyl pyrocarbonate content by adding 10 μL to 1 mL of a 10 mM histidine solution, pH 6.0. A molar difference absorption coefficient of 3200 M^{-1} cm^{-1} at 240 nm for ethoxyformylhistidine was used to calculate the concentration of diethyl pyrocarbonate (Ovádi et al., 1967). The reaction of diethyl pyrocarbonate with the enzyme was stopped at desired times either by addition of a solution of 10 mM imidazole to the reaction mixture or by dilution (100–500-fold) of an aliquot of the reaction mixture into the assay mixture.

The pH dependence studies of inactivation were conducted similarly with the pH values adjusted from 5.5 to 8.5 and at a constant ionic strength of 20 mM. The pH did not change during the course of the experiment, and the enzyme lost negligible activity on standing in the absence of inactivator under the conditions used. In the pH range from 5.5 to 7.0, Mes was used as a buffer and Hepes in the range from pH 7.0 to 8.5.

De-ethoxyformylation (Melchior & Farney, 1970) of modified enzyme was achieved by adding an equal volume of 200 mM hydroxylamine in 20 mM Mes or Hepes to a solution of inactivated enzyme at a given pH and following the return of activity with time in an assay mixture at pH 8, to which an aliquot of the reaction mixture had been added. A similar procedure was carried out with an unmodified enzyme solution as a control.

The extent of ethoxyformylation of histidine residues of the enzyme was determined by plotting difference absorption spectra of the enzyme at different times after the addition of diethyl pyrocarbonate to one cuvette and the same volume of ethanol to the other one. Peak values at 244 nm ($\Delta\epsilon$ = 3600 M^{-1} cm^{-1}) were used for kinetic calculations. At the same time intervals, 4-μL samples were withdrawn from the reaction mixture for the determination of the residual activity in order to relate the chemical modification reaction to that of the inactivation of the enzyme. The $\Delta\epsilon$ of 3600 M^{-1} cm^{-1}, at 244 nm, was determined at pH 6 by measuring the absorbance difference produced by solutions containing 20 μM histidine and multiples thereof in the presence of 0.8 mM diethyl pyrocarbonate. This $\Delta\epsilon$ value is larger than that usually used (Ovádi et al., 1967) owing to the fact that, as pointed out by Roosemont (1978), the $\Delta\epsilon$ value of ethoxyformylated histidine depends on whether an excess of diethyl pyrocarbonate or an excess of histidine is used in the determination. Therefore, in the modification reaction of the enzyme, an excess of diethyl pyrocarbonate was always used. When inactivation had to be related to chemical modification of the enzyme–*p*-fluorobenzoate complex, this could only be done indirectly. Diethyl pyrocarbonate (0.91 mM) was added to a solution of 33.9 μM *p*-hydroxybenzoate hydroxylase in the presence of 15.4 mM *p*-fluorobenzoate at a given pH and temperature. At given time intervals, 1-mL samples were withdrawn, added to 10 μL of 1 M imidazole, and purified by gel chromatography. After determination of the enzyme concentration and activity, the residual amount of histidines that could still be carbethoxylated in each sample was determined as described above.

Dissociation constants of different enzyme complexes were determined fluorometrically or spectrophotometrically by titration experiments. Quenching of the protein-bound FAD fluorescence emission at 525 nm (excitation at 450 nm) was monitored as a function of the concentration of NADPH or *p*-hydroxybenzoate added. For the enzyme–*p*-fluorobenzoate complex, absorption differences at 500 or 384 nm were monitored. From Benesi–Hildebrand plots (Benesi & Hildebrand, 1949), the dissociation constants of the complexes of native and modified enzyme were calculated.

The determination of the concentration of diethyl pyrocarbonate in reactions with free NADPH was carried out by the method described by Berger (1975); i.e., aliquots (50 μL) of the incubation mixture were added to 350-μL solutions of thionitrobenzoate, and the change of absorbance at 412 nm was recorded. The decrease of absorbance at 412 nm is proportional to the diethyl pyrocarbonate consumed in the reaction mixture.

The reaction of diethyl pyrocarbonate with *p*-hydroxybenzoate at a given pH value was followed by measuring the decrease of absorbance of *p*-hydroxybenzoate at 245 nm. Gel chromatography was performed by using Bio-Gel P-6DG (Bio-Rad) packed in a column (1 × 9 cm).

Results and Discussion

When ethoxyformylation of free *p*-hydroxybenzoate hydroxylase is carried out at pH 5.8 and 4 °C, the enzyme rapidly becomes inactivated (Figure 1). The inactivation reaction follows pseudo-first-order kinetics when an excess of diethyl pyrocarbonate is used, but clearly two or more reactions are involved. Extrapolation of the curves of the slower reaction to zero time yields an intercept at about 0.7 on the ordinate (Figure 1). From a secondary plot of the pseudo-first-order rate constants against the diethyl pyrocarbonate concentration, second-order rate constants of 500 and 245 M^{-1} min^{-1}, respectively, are obtained at pH 5.8 and 4 °C (Figure 1, insert).

The two-phase reaction was not observed at 20 °C. At this temperature, only the second reaction could be observed because the first reaction was too fast to be followed by conventional methods. At 20 °C, the slow reaction exhibits a second-order rate constant of 1000 M^{-1} min^{-1} at pH 6.1.

Both the fast and the slow reactions are slowed down when NADPH or 2′,5′-ADP is included in the reaction mixture

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); 2′,5′-ADP, adenosine 2′,5′-diphosphate; EDTA, ethylenediaminetetraacetate; *p*-FB, *p*-fluorobenzoate.

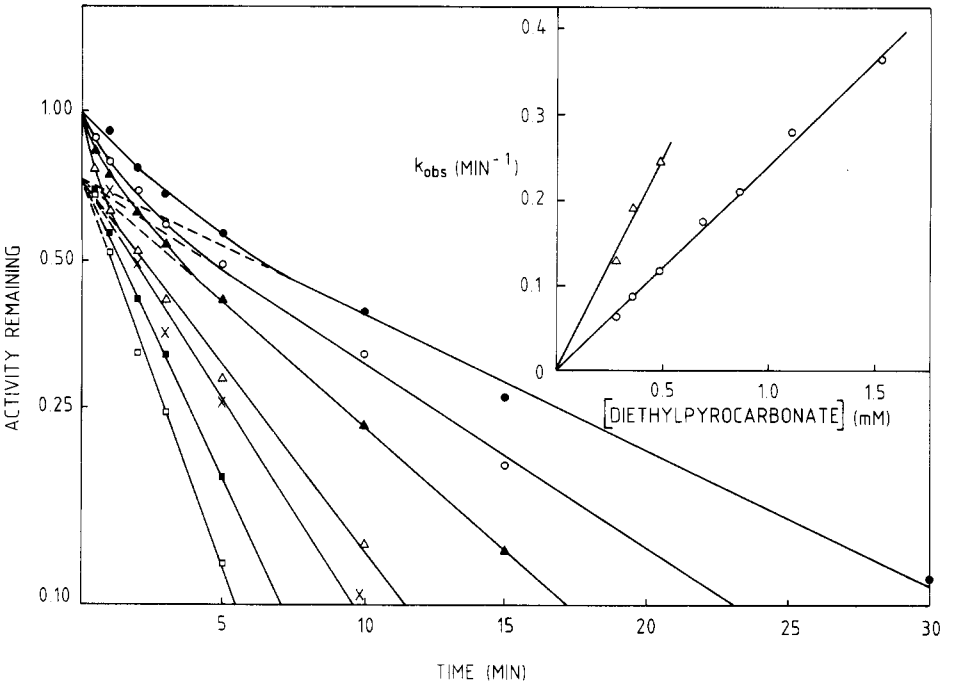


FIGURE 1: Semilogarithmic plot of the time-dependent inactivation of *p*-hydroxybenzoate hydroxylase as a function of the diethyl pyrocarbonate concentration. Each incubation mixture contained 20 μM *p*-hydroxybenzoate hydroxylase in 20 mM Mes, pH 5.8, and various concentrations of diethyl pyrocarbonate: 0.42 mM (●); 0.50 mM (○); 0.63 mM (▲); 0.83 mM (△); 1.00 mM (×); 1.25 mM (■); 1.67 mM (□). The reactions were conducted at 4 °C. Aliquots were withdrawn at intervals and assayed after dilution. The relative rate was determined by comparison with an identical enzyme sample in the absence of the inactivator. The insert shows the linear relationship between the apparent pseudo-first-order rate constants (k_{obsd}) of the two inactivation reactions and the diethyl pyrocarbonate concentration.

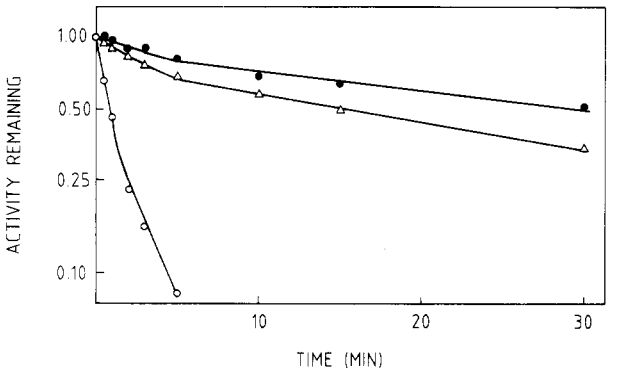


FIGURE 2: Effect of NADPH and 2′,5′-ADP on the rate of inactivation of *p*-hydroxybenzoate hydroxylase. A solution of 20 μM *p*-hydroxybenzoate hydroxylase in 20 mM Mes and at 4 °C was treated with 0.8 mM diethyl pyrocarbonate in the presence of 25 mM NADPH (△) (pH 6.2) or in the presence of 25 mM 2′,5′-ADP (●) (pH 5.9). The inactivation of the free enzyme under identical conditions is also shown (○). The pH of the solution of the free enzyme was 6.0. The data are presented as a semilogarithmic plot (cf. Figure 1).

(Figure 2). The latter compound inhibits the enzyme competitively with respect to NADPH (Santema et al., 1976). The substrate *p*-hydroxybenzoate did not influence the rates of inactivation (data not shown). Neither NADPH nor *p*-hydroxybenzoate reacts with diethyl pyrocarbonate under these conditions (about pH 6) as judged by control experiments in the absence of the enzyme. The reaction of diethyl pyrocarbonate with *p*-hydroxybenzoate actually occurs with the phenoxy form. The pK_a' value for the hydroxyl group of the substrate is 9.3 (Dawson et al., 1969), so little or no reaction is expected below pH 7, as found. Reaction between diethyl pyrocarbonate and NADPH or 2′,5′-ADP, which could be expected because diethyl pyrocarbonate reacts with adenosine (Leonard et al., 1971), was found, however, to occur at a negligible rate under the conditions used in the protection experiments. Our results therefore strongly indicate that histidine residues are involved in the binding of NADPH but not in the binding of the substrate, as claimed by Shoun et al. (1979b). The possibility of modification of other amino acid residues with unusually reactive groups, e.g., lysine or arginine, can be excluded because it has been shown (Little, 1977) that the imidazole ring of histidine is by far the preferred target for diethyl pyrocarbonate at pH 6 or lower pH values.

The experiments of Figure 2 further show that the interaction between histidine residues and NADPH most probably occurs with the 2′,5′-ADP moiety of NADPH because 2′,5′-ADP itself exhibits about the same protective effect as NADPH. It should be noted that the apparent difference in the protective effect between NADPH and 2′,5′-ADP (Figure 2) is due to a difference in the pH of the solutions.

The reaction was followed with time simultaneously both by spectrophotometric (at 244 nm) and by activity measurements (Figure 3) to see if the two different rates of inactivation could be correlated to the chemical modification reaction of histidine residues. So that the reactions could be followed as accurately as possible, the experiment was done at pH 5.6 and 4 °C, where the rate of inactivation is decreased as compared to that at higher pH values (cf. below). Both monitoring systems show two reaction rates with the point of inflection at about 3.5 min (Figure 3). According to Ray & Koshland (1961), the pseudo-first-order rate constants for the loss of activity can be calculated from the slopes of Figure 3:

$$k_1 = \frac{\text{initial slope} - k_2}{1 - F} \quad (1)$$

and

$$k_2 = \text{final slope} \quad (2)$$

where k_1 is the pseudo-first-order rate constant of the reaction of the faster modified group(s), k_2 is the pseudo-first-order rate constant of the reaction of the slower modified group(s),

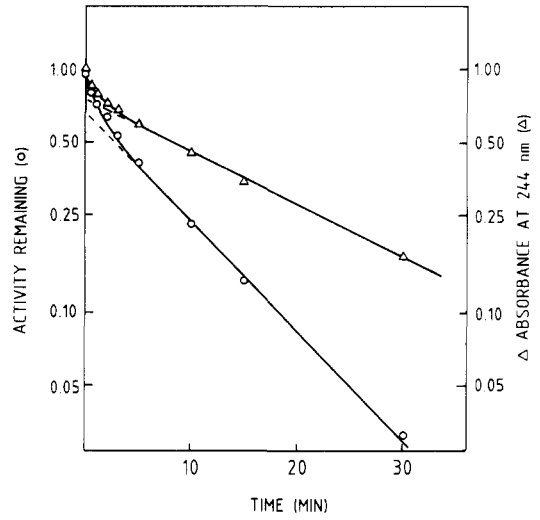


FIGURE 3: Kinetics of inactivation and chemical modification of *p*-hydroxybenzoate hydroxylase by diethyl pyrocarbonate. 20 μM enzyme in 20 mM Mes, pH 5.6, was treated with 0.81 mM diethyl pyrocarbonate at 4 °C. Aliquots of the solution were assayed at time intervals for residual activity (O) and for the increase of absorbance at 244 nm (Δ). The increase of absorbance was normalized by giving the difference of absorbance at zero time a value of 1.00 and at t = 60 min a value of 0.

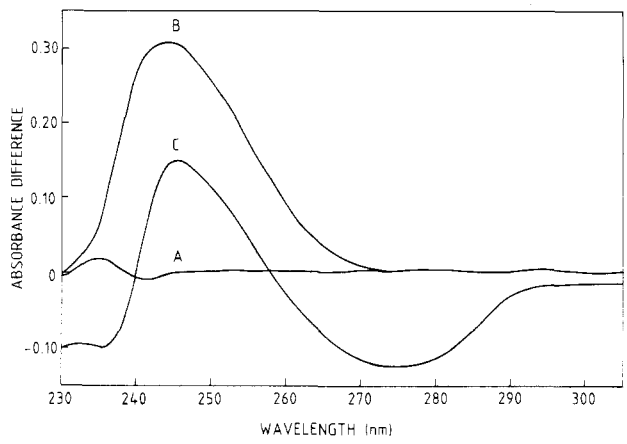


FIGURE 4: Ultraviolet difference spectrum for the inactivation of *p*-hydroxybenzoate hydroxylase by diethyl pyrocarbonate. Both cuvettes contained 0.6 mL of a 20 μM enzyme solution in 20 mM Mes, pH 5.6. Before inactivation, a base line (A) was recorded. Then 27 μL of a 18.8 mM diethyl pyrocarbonate solution in ethanol was added to the sample cuvette. An equivalent volume of ethanol was added to the reference cuvette. After 60 min at 4 °C, the difference spectrum (B) was recorded, and the enzyme solution in the sample cuvette exhibited less than 3% of the activity of the enzyme solution in the reference cuvette. The difference in absorbance corresponds to four histidine residues modified per mole of FAD bound. A similar experiment was performed in 20 mM Hepes, at pH 8 and at 20 °C. The spectrum shown (C) was recorded after 5-min incubation of the solution with diethyl pyrocarbonate. The activity of the modified enzyme solution was 0% of that in the reference cuvette.

and F is the fractional residual activity of the enzyme belonging to the two reactions. Since the fractional activity after the slow reacting groups are modified is very small, F represents the fractional residual activity of the enzyme after only the fast reacting groups have been modified. F is determined from the extrapolation of the lines of the slow reaction (cf. Figures 1 and 3). The values calculated from Figure 3 for inactivation with 0.81 mM diethyl pyrocarbonate at pH 5.6 and 4 °C are $k_1 = 0.80$ min^{-1}, $k_2 = 0.10$ min^{-1}, and $F = 0.71$. The values for the chemical modification, calculated in the same way from Figure 3, are $k_1 = 0.80$ min^{-1}, $k_2 = 0.05$ min^{-1}, and $F = 0.75$.

In the case of the chemical modification reaction, F represents the fraction of the slower modified histidine residues. According to the theory of Ray & Koshland (1961), from these kinetic results the following conclusions can be drawn. First, F indicates that there are at least two sets of histidine residues differing in reactivity toward diethyl pyrocarbonate. The ratio of the two sets of histidine residues is 1:3. The histidine residue(s) of the fast reacting set is (are) responsible for the loss of about 30% of the total activity. The equivalence of the rate constant k_1 for the inactivation and the chemical modification reaction suggests that modification of one histidine residue is responsible for the rapid loss of some activity. From this, it follows that the set of the less reactive histidine residues is composed of three residues. Of these three residues, only two are apparently essential for activity, since the rate of loss of activity is equal to the sum of the rate of the chemical modification reaction (Ray & Koshland, 1961), i.e., 0.10 min^{-1} = 2 × 0.05 min^{-1}.

The difference spectrum produced when all accessible histidine residues of the enzyme have reacted with diethyl pyrocarbonate is shown in Figure 4. Using a molar extinction coefficient of 3600 M^{-1} cm^{-1} for N-ethoxyformylated histidine residues (cf. Materials and Methods), it is calculated that four histidine residues of the enzyme are modified. The difference spectrum also clearly indicates that under the experimental conditions used, no amino acid residues other than histidines are modified (cf. also below). From the spectral data, the number of equivalents of histidine residues modified as a function of time can also be determined. When the rate of loss of activity of the enzyme is measured under conditions identical with those for the rate of the chemical modification of histidine residues, as has been done in Figure 3, the loss of activity can be correlated to the number of equivalents of histidine residues reacted. The experimental data of Figure 3 were used to obtain such a correlation (Figure 5A). Extrapolation of the line to zero activity suggests that three histidine residues per enzyme monomer (expressed as moles of His per mole of FAD) are apparently responsible for the loss of activity and that a further residue is responsible for the loss of some activity.

An alternative and more reliable approach for correlating the specific modification with the loss of activity is the statistical method developed by Tsou (1962). This method was later improved by Horiike & McCormick (1979). The relation is shown by eq 3 and 4:

$$a^{1/i} = \frac{p + s - m}{p} \qquad m > s \tag{3}$$

$$a^{1/i} = 1 \qquad m < s \tag{4}$$

where s is the number of rapidly reacting residues causing little or no loss of activity of the enzyme, p is the number of residues that react slower, but of which i residues are essential for activity, and m is the number of groups modified, which is correlated with the residual activity a. A plot of $a^{1/i}$ against m will give a straight line with a slope of $-1/p$. The value of i can be determined by plotting a, $a^{1/2}$, $a^{1/3}$, etc. against m until the best straight line fit is obtained. The value obtained in this way indicates the number of modified residues that are essential for catalysis. In our case, there is one rapidly reacting residue that is probably not essential, but which does cause about 30% inactivation. This prevented the use of the more sophisticated approach (Horiike & McCormick, 1979) which was developed for cases only where one of the kinetic constants is not involved in the inactivation of an enzyme. Therefore, the value of 70% activity was set to 100%, and the residue

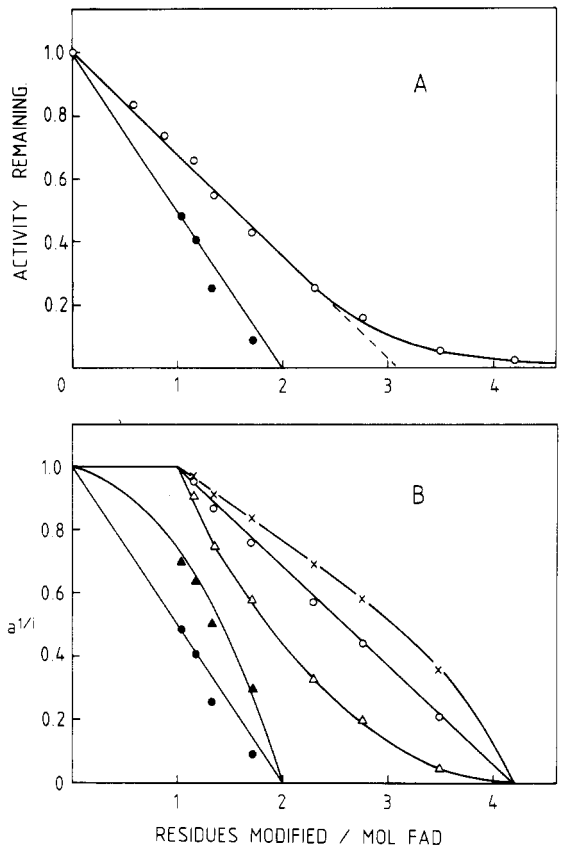


FIGURE 5: Correlation between the number of histidine residues of *p*-hydroxybenzoate hydroxylase modified by diethyl pyrocarbonate and the fractional activity remaining. (A) The open symbols represent the same data as in Figure 3 except that the change in absorbance is converted into the amount of histidines modified. The closed symbols represent the data for the enzyme–*p*-fluorobenzoate complex, obtained by the differential labeling technique (cf. Materials and Methods), in 20 mM Mes, pH 5.7 at 4 °C. The samples were withdrawn at 0, 1, 3, 10, and 30 min after the addition of diethyl pyrocarbonate. (B) The data of Figure 5A are presented in the form of a Tsou plot (see text) for $i = 1$ (△), $i = 2$ (○), and $i = 3$ (×) (free enzyme) and for $i = 1$ (●) and $i = 2$ (▲) (*p*-FB-complexed enzyme).

Table I: Comparison of the Affinity of Native and Modified Enzyme for NADPH and *p*-Hydroxybenzoate[a]

enzyme preparation	dissociation constant (mM) native enzyme	modified enzyme
enzyme–NADPH complex	0.49	5.0
enzyme–*p*-hydroxybenzoate complex	0.06	0.07

[a] The titration experiments were conducted fluorometrically at pH 6 and 20 °C. For the modification of *p*-hydroxybenzoate hydroxylase, a 20 μM solution of the enzyme was allowed to react with 0.8 mM diethyl pyrocarbonate for 45 min at 4 °C, pH 6.0. Before use in titration studies, the sample was purified by gel chromatography.

Table II: Average Number of Histidine Residues Protected in a 30 μM Solution of *p*-Hydroxybenzoate Hydroxylase in 20 mM Mes, pH 5.7[a]

conditions of differential labeling: addition	preincubation time (min)	activity remaining (%)	no. of accessible residues remaining after preincubation (mol of His/mol of FAD)[b]
none	15	12	~1.4
none	30	0	0
10 mM NADPH	15	67	~3.0
10 mM NADPH	30	51	~2.0
15 mM *p*-FB	15	14	~1.9
15 mM *p*-FB	30	3.5	~1.7

[a] The solution was preincubated in the presence of NADPH or *p*-fluorobenzoate with 0.88 mM diethyl pyrocarbonate, for a certain period of time at 4 °C. After the preincubation, an excess of a solution of imidazole was added to the reaction mixture and the preparation purified by gel chromatography. An aliquot of the purified solution, pH 5.7, was then treated again with 0.88 mM diethyl pyrocarbonate and the reaction followed at 244 nm at 13 °C. From the increase in absorbance, the number of histidine residues protected by the differential labeling technique was then determined. [b] The value given is an average of three independent measurements.

responsible for the fast loss of activity was added to *s*. The result is shown in Figure 5B. The best straight line is obtained for $i = 2$, indicating that only two of the four histidine residues modified are essential for catalysis. This conclusion would be in agreement with the results presented above if it is assumed that modification of the more reactive histidine residue exerts an indirect effect on the catalytic activity of the enzyme. That this is most likely can be seen from the following experiments.

The affinity of the modified enzyme for the substrate *p*-hydroxybenzoate and NADPH was studied with free enzyme preparations modified at pH 6 and purified by gel chromatography. The dissociation constants were determined by titration experiments monitoring the quenching of the fluorescence emission of enzyme-bound FAD. It must be noted that the dissociation constants for the enzyme–NADPH complex determined in this way are less accurate than those determined kinetically (Entsch et al., 1976). The values presented are, however, quite satisfactory for the purpose of comparison (Table I). The results clearly indicate that the affinity of the modified enzyme for NADPH is decreased, whereas the affinity for the substrate remains unaltered. Moreover, an enzyme preparation exposed to diethyl pyrocarbonate for a short period of time, resulting in the loss of about 30% of the original activity, exhibited the same affinity for NADPH as native enzyme. In addition, the light absorption spectrum in the visible region and the circular dichroism spectrum in the ultraviolet region of native and modified enzyme are identical, indicating that the conformation of the enzyme is little or not at all affected upon chemical modification of histidine residues.

Modification studies in the absence or presence of these compounds were carried out to obtain more insight into the possible involvement and interaction of the four histidine residues with the substrate *p*-hydroxybenzoate or NADPH. The enzyme was modified in the presence or absence of *p*-fluorobenzoate (see below) or NADPH for a certain period of time, the treated enzyme solution was purified, and the number of the protected histidine residues was determined by reaction with diethyl pyrocarbonate from the increase of absorbance at 244 nm. The results of the differential labeling technique are presented in Table II. The results strongly indicate that two histidine residues are essential for activity, as already concluded above from the Tsou plot (Figure 5B), and that the substrate analogue protects one to two histidine residues which are not essential for catalytic activity. Since it follows from Table I that enzyme preparations in which the four accessible histidine residues were modified retained about the same affinity for the substrate as native enzyme, it must be concluded that the histidine residues protected by *p*-fluorobenzoate are involved neither in the binding of substrate nor in the binding of NADPH. Obviously, occupation of the substrate binding site diminishes the accessibility of two his-

Table III: Time Course of the Reactivation Reaction of Modified Enzyme by Hydroxylamine[a]

time (h)	% activity
0	~2
0.5	9
1	14
1.5	75
2	86
2.5	92

[a] A 20 μM solution of enzyme was inactivated in the presence of 0.8 mM diethyl pyrocarbonate at pH 6 for 30 min at 4 °C. The solution was then purified by gel chromatography. A 10 μM solution of this preparation was incubated with hydroxylamine (116 mM) at pH 7 and the reactivation of the enzyme followed with time. An identical sample of native enzyme was also incubated with hydroxylamine and served as a reference sample.

tidine residues for diethyl pyrocarbonate. It is possible that the conformational change induced by the binding of the substrate to the enzyme (Teng et al., 1971) is responsible for the protective effect.

It is surprising that the capability of the modified enzyme to interact with NADPH is not completely abolished; however, histidine residues are obviously not solely responsible for the binding of NADPH, and other amino acid residues are also involved.

The inactivated enzyme can be reactivated by hydroxylamine. Also, in the absence of hydroxylamine, a very slow reactivation of the enzyme can be observed; allowing a completely inactivated enzyme solution to stand for 12 h at 4 °C restores about 30% of the original activity. However, hydroxylamine shows a clear catalytic effect on the reactivation reaction. As shown in Table III, the reactivation reaction is rather slow, but more than 90% of the activity can be reconstituted as compared with a control. In accordance with these observations, the affinity of the reactivated enzyme for NADPH is the same as that of the native enzyme. These data also indicate that no irreversible conformational change occurs on modification of essential histidine residues of the enzyme. This conclusion is in agreement with the spectral data (cf. above).

For determination of the pK_a' values of the essential histidine residues, the pseudo-first-order rate constant of inactivation by diethyl pyrocarbonate was measured at different pH values (Figure 6). When the free enzyme is inactivated, the pH-dependent rate constant seems to approach a maximum value at about pH 7, but it increases again at pH >7. This second increase is not observed when instead of the free enzyme the complex with *p*-fluorobenzoate is ethoxyformylated (Figure 6). It should be noted that the apparent pseudo-first-order rate constant for the inactivation of the enzyme is not affected by the presence of *p*-fluorobenzoate (Figure 6). *p*-Fluorobenzoate instead of *p*-hydroxybenzoate was used as a protecting agent because of the possible reaction of the hydroxyl group of the substrate with diethyl pyrocarbonate at pH >7. *p*-Fluorobenzoate induces the same changes in the CD spectrum of *p*-hydroxybenzoate hydroxylase upon binding as the natural substrate and also functions as an effector (Teng et al., 1971). This molecule is therefore a good substitute for the natural substrate while in addition it cannot react with the modification reagent. The observed increase in rate at higher pH values suggests that diethyl pyrocarbonate reacts with amino acid residues other than histidine in the free enzyme and that the substrate analogue protects the enzyme from this modification. Preliminary experiments at pH 8 suggest that this reaction is due to the modification of a tyrosine residue in the substrate binding site (Wijnands & Müller, 1982). In

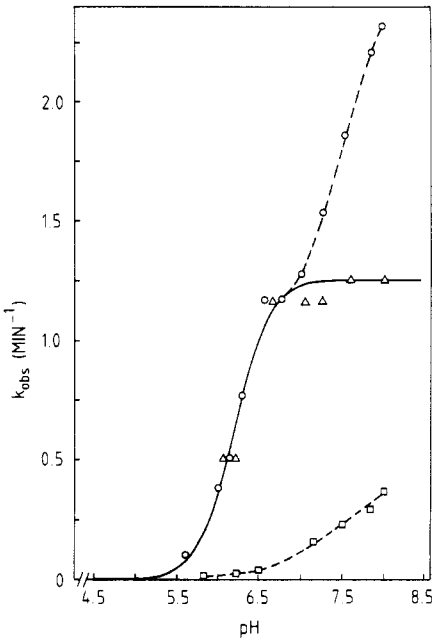


FIGURE 6: pH dependence of the rate of inactivation of *p*-hydroxybenzoate hydroxylase by diethyl pyrocarbonate in the absence or presence of NADPH and/or *p*-fluorobenzoate. The pH-dependent pseudo-first-order rate constant k_{obsd} for the inactivation of the enzyme due to the slow reacting histidine residues was calculated from the slope of curves as shown in Figures 1 and 2. The temperature was 4 °C. (○) 20 μM free enzyme; (△) 20 μM enzyme + 25 mM *p*-fluorobenzoate; (□) 20 μM enzyme + 25 mM NADPH. In all cases, 0.8 mM diethyl pyrocarbonate was added at $t = 0$. The solid line is a theoretical curve fitted to the experimental data by using eq 8 (see text). The theoretical line was calculated by using $k_{max} = 1.25$ min^{-1} and $pK_1 = 6.2$.

fact, free enzyme modified at pH 8 had lost the capability to bind *p*-hydroxybenzoate. In addition, the difference absorption spectrum of enzyme modified at pH 8 (Figure 4) shows the typical decrease of absorption at 278 nm caused by tyrosine modification (Mühlrad et al., 1967). The inactivation of free enzyme at high pH values is currently under active investigation.

Attempts failed to fit the data of the pH-dependent inactivation rate constants of the free enzyme at pH <7 and of the enzyme–*p*-fluorobenzoate complex by assuming a single, ionizing group, indicating once more that we are indeed dealing with more than one essential histidine residue. Scheme I shows the equilibrium and ethoxyformylation rate constants involved when dealing with two ionizing groups. All these microscopic values cannot be distinguished from one another because ethoxyformylation of either histidine leads to inactivation. Scheme II, on the other hand, shows the macroscopic or molecular ionization constants that are involved. According to these schemes, K_3' and the second ethoxyformylation reaction ($^1/_2k_{max}$, Scheme II) are not important because they have no influence on the inactivation; therefore, the following relationships can be deduced:

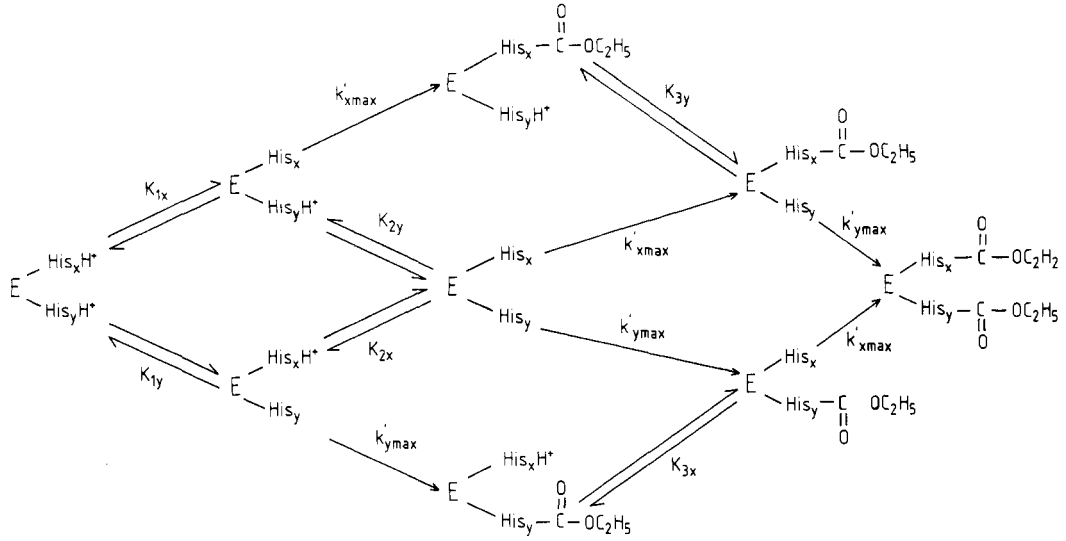
$$K_1' = K_{1x} + K_{1y} \qquad \frac{1}{K_2'} = \frac{1}{K_{2x}} + \frac{1}{K_{2y}}$$

$$k_{max} = k_{x_{max}} + k_{y_{max}}$$

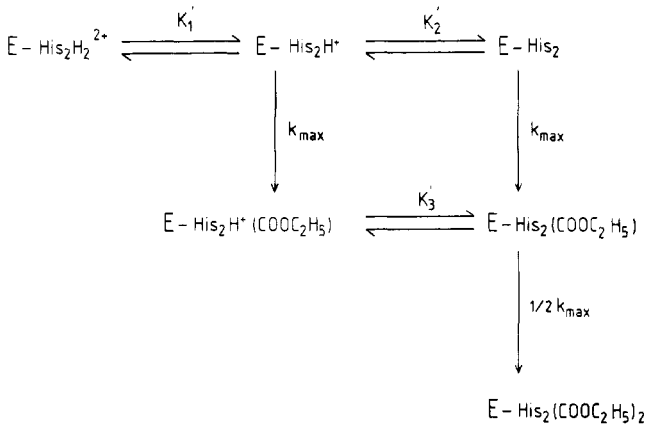
As $k_x = k_y$ (Figure 3), it can easily be shown that $K_{1x} = K_{1y}$ ($=K_1$), which means that $K_{2x} = K_{2y}$ ($=K_2$). The relations between the macroscopic and microscopic ionization constants can therefore be simplified as follows:

$$K_1' = 2K_1 \qquad K_2' = \tfrac{1}{2}K_2$$

Scheme I



Scheme II



The relation between the observed inactivation rate constant, k_{obsd}, and the pH then becomes

$$k_{obsd} = k_{max}\left[\frac{1}{2 + [H^+]/K_1 + K_2/[H^+]} + \frac{1}{1 + 2[H^+]/K_2 + [H^+]^2/(K_1K_2)}\right] \quad (5)$$

Let us now consider three extreme situations. (1) Protonation of one histidine does not influence protonation of the other, or $K_1 = K_2 = K$. Equation 5 simplifies to

$$k_{obsd} = k_{max}\left[\frac{1}{2 + [H^+]/K + K/[H^+]} + \frac{1}{(1 + [H^+]/K)^2}\right] \quad (6)$$

(2) Protonation of one histidine prevents protonation of the other, or $K_2 \ll K_1$. Equation 5 now becomes

$$k_{obsd} = \frac{k_{max}}{2 + [H^+]/K_1} \quad (7)$$

(3) Protonation of one histidine forces protonation of the other, or $K_2 \gg K_1$. This situation can be described by Hill's model for cooperativity (Hill, 1910) with two binding sites:

$$k_{obsd} = \frac{k_{max}}{1 + ([H^+]/K_1)^2} \quad (8)$$

Equation 8 implies that either both histidine residues are protonated or both are deprotonated. Our data can best be fitted by eq 8, with $k_{max} = 1.25$ min^{-1} and p$K_1 = 6.2$, as shown in Figure 6. This result is in agreement with the experimental data given above which show that *p*-fluorobenzoate indeed protects two histidine residues from modification but that these residues are not essential for activity. From Figure 6, it also follows that NADPH protects the essential histidine residues. These residues react in a cooperative manner. From this result, it is not clear whether both residues, which react in a cooperative manner, are indeed essential or only one is essential. For further clarification of this point, the differential labeling technique in the presence of *p*-fluorobenzoate was employed, and the chemical modification and the inactivation reactions were followed with time. The results are presented in Figure 5A and show the expected relation, i.e., two histidine residues responsible for the loss of activity. Application of the method of Tsou (1962) to these data, however, reveals that only one histidine residue is apparently essential for the activity of the enzyme (Figure 5B). This seems in contradiction with the results presented in Figures 5 and 6 for the free enzyme. It should be noted, however, that in cases of cooperativity the conclusions derived from Tsou plots concerning the number of essential residues may be quite erroneous (Rakitzis, 1978; Horiike & McCormick, 1979).

With the results presented at hand, one would like to indicate which histidine residues in the sequence are the essential ones. This is not yet possible because diffraction data on the enzyme–substrate complex only are available. Nevertheless, *p*-hydroxybenzoate hydroxylase from *P. fluorescens* contains nine histidine residues at positions 22, 72, 130, 162, 197, 204, 278, 289, and 351 in the sequence of the enzyme (Weijer et al., 1982). The three-dimensional model shows that not a single histidine residue is present in the substrate binding site. This seems in contradiction with our results showing that *p*-fluorobenzoate protects two histidine residues from chemical modification (Figure 5, Table II), but the model also shows that two pairs of histidine residues are located in the neighborhood of the substrate binding site. Therefore, the protection of two histidine residues from chemical modification is most probably related to the conformational change induced by the substrate (effector). The protection could also be caused by the substrate itself preventing access to the histidine residues in question. It is believed that only His-162 is involved in the binding of NADPH (J. Drenth and R. K. Wierenga, unpublished experiments). This proposal would explain our results of Figure 5 where it was concluded that only one of the two

histidine residues modified in the presence of *p*-fluorobenzoate is essential for activity. But this proposal does not explain the fact that the two histidine residues react in a cooperative manner (Figure 6). In the three-dimensional model, the closest neighboring histidine residue of His-162 is His-289. The C_α–C_α distance between His-162 and His-289 is 0.12 nm (J. Drenth and R. K. Wierenga, unpublished experiments). It might be that the reactivity of His-289 is influenced in a cooperative manner upon chemical modification of His-162. This proposal would explain our results, but a correlation between the results of chemical modification of histidine residues and their position in the sequence and the three-dimensional model cannot be offered yet. Modification of the histidine residues by chemical groups not susceptible to easy hydrolysis should make it possible to identify the essential histidine residues in the sequence of the enzyme and to elucidate their role in the catalytic mechanism. Such attempts have failed so far, but this line of research is currently further followed up.

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