Chemical Modification of Tyrosine Residues in *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*: Assignment in Sequence and Catalytic Involvement†

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ABSTRACT: *p*-Hydroxybenzoate hydroxylase was modified by diethyl pyrocarbonate at pH values >7 and by *p*-diazobenzoate. Modification of the enzyme by diethyl pyrocarbonate abolishes the affinity of the enzyme for the substrate *p*-hydroxybenzoate. Modification by *p*-diazobenzoate has the same effect on the enzyme. The enzyme is protected against these modifications by the effector *p*-fluorobenzoate. The data indicate that the modification of one tyrosine residue in the active center of the enzyme is responsible for the loss of enzyme activity. This tyrosine residue has been identified by sequence studies using radioactively labeled *p*-diazobenzoate and was found to be most probably Tyr-222. Diethyl pyrocarbonate reacts with a tyrosine residue in the active center other than Tyr-222; the former could not be identified. Sequence studies further showed that Cys-211 is also partially modified by *p*-diazobenzoate. In addition, the sequence of residues 343–345 was found to be Ser-Trp-Trp instead of the tentative assignment Ser-Tyr-Trp made earlier. The results are briefly discussed on the basis of the existing three-dimensional model of the enzyme.

The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (EC 1.14.13.2) belongs to the class of external monooxygenases and catalyzes the conversion of *p*-hydroxybenzoate into 3,4-dihydroxybenzoate. The reaction mechanism of the enzyme from *P. fluorescens* has been studied in detail (Husain & Massey, 1979). The preferred electron donor for the enzyme is reduced nicotinamide adenine dinucleotide phosphate (NADPH),[1] but reduced nicotinamide adenine dinucleotide (NADH)[1] can also serve as an electron donor, although less efficiently than NADPH (Wijnands et al., 1984; Shoun et al., 1983).

The three-dimensional structure of the enzyme at a resolution of 0.25 nm (Wierenga et al., 1979) is available, and the sequence of the protein is known (Hofsteenge et al., 1983; Weijer et al., 1983). These data form a good basis for the study of the amino acid residues involved in the binding of *p*-hydroxybenzoate and NADPH by chemical modification of the protein.

The enzyme from *P. fluorescens* contains five cysteine residues of which Cys-116 can be selectively modified by *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoate) (Müller et al., 1979; van Berkel et al., 1984). This modification does not affect the activity of the enzyme. The only other cysteine residue that can be modified fairly specifically is Cys-152, which is reactive toward a spin-label derivative of *p*-chloromercuribenzoate (van Berkel et al., 1984). Although this residue is situated relatively far from the active center of the protein, alkylation of Cys-152 abolishes the capability of the enzyme to bind the substrate. Modification of Arg-214, located in the active center and involved in the interaction with the carboxyl group of the substrate (Wierenga et al., 1979), has the same effect (Shoun et al., 1980). Histidine residues have been claimed to be involved in the binding of the substrate as well (Shoun et al., 1979). However, Wijnands and Müller (1982) have shown that histidine residues are not involved in the binding of the substrate but are involved in the binding of NADPH. In this latter study diethyl pyrocarbonate was used as a modification reagent. From a pH dependence study it was concluded that at pH values >7 other residues, probably tyrosines, are also modified by diethyl pyrocarbonate and that this modification reaction could be prevented in the presence of an effector of the enzyme (Wijnands & Müller, 1982). The three-dimensional structure shows that three tyrosine residues, i.e., Tyr-201, Tyr-222, and Tyr-385, are located in or close to the active center. This and the above-mentioned results stimulated us to investigate the possible role of tyrosine residues in the catalytic reaction of the enzyme in more detail. In this paper we show that different tyrosine residues are modified with diethyl pyrocarbonate and *p*-diazobenzoate, but that both modification reactions lead to loss of enzymic activity. The tyrosine residue modified by *p*-diazobenzoate is assigned in the sequence of the enzyme by sequencing tryptic peptides of modified enzyme.

Materials and Methods

p-Amino[^{14}C-*carboxyl*]benzoic acid (58.5 mCi/mmol) was from the Commissariat a l'Energie Atomique (Saclay, France), diethyl pyrocarbonate was from Fluka, NADPH was from Boehringer, and 4-morpholineethanesulfonic acid (Mes),[1] 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes),[1]

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[1] Abbreviations: Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; EDTA, ethylenediaminetetraacetate; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyanate; DABITH, 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiohydantoin; FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography.

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4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Hepps),[1] 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris),[1] and *p*-(chloromercuri)benzoic acid were from Sigma. Trypsin (EC 3.4.21.4), treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, was from Worthington, and proteinase from *Staphylococcus aureus* V8 (EC 3.4.21.19) was from Miles. 4-(*N*,*N*-Dimethylamino)azobenzene-4′-isothiocyanate (DABITC)[1] was from Pierce, and trifluoroacetic acid (sequencer grade) and acetonitrile [high-performance liquid chromatography (HPLC)[1] S grade] were from Rathburn. Other materials used in the isolation and identification of peptides have been described previously (Hofsteenge et al., 1983). All other chemicals were from Merck and were the best grades available.

p-Diazobenzoic acid was prepared according to the procedure of Riordan and Vallee (1972). It was prepared freshly before each experiment. Routinely, a stock solution of 20 or 40 mM of diazotized *p*-aminobenzoic acid was prepared. The pH of the solution was carefully brought to a value between 5 and 6 by addition of a solution of 5 M NaOH. For the preparation of *p*-diazo[^{14}C-*carboxyl*]benzoic acid solutions the same procedure was used. The specific activity of *p*-amino-[^{14}C-*carboxyl*]benzoic acid was decreased to 1 mCi/mmol by adding cold *p*-aminobenzoic acid. The decay of *p*-diazobenzoic acid was followed with time by the addition of 20-μL samples of a 0.2 or 2 mM solution to 1 mL of a 4 mM *p*-hydroxybenzoic acid solution in 0.1 M Tris-HCl, pH 8.8. The absorbance at 440 nm after 5 min was used directly to calculate the concentration of *p*-diazobenzoic acid in the original solution.

p-Hydroxybenzoate hydroxylase was purified from *Pseudomonas fluorescens* as described previously (Müller et al., 1979). Fresh solutions of the enzyme in the appropriate buffer were prepared each day by gel filtration over Bio-Gel P-6 DG (Bio-Rad). The enzyme concentration was determined spectrophotometrically on the basis of the flavin adenine dinucleotide (FAD)[1] content by assuming a molar absorption coefficient of 11300 M^{-1} cm^{-1} at 450 nm (Müller et al., 1979). The enzyme activity was assayed in Tris–H_2SO_4, pH 8.0, as previously reported (Müller et al., 1979).

Azo coupling of the tyrosine residues in the enzyme was performed in 40 mM Hepes, pH 7.0, and in 40 mM Hepps, pH 8.7, at 0–4 °C. At low enzyme concentrations (<20 μM) 0.2 mM *p*-diazobenzoic acid was added from a freshly prepared stock solution of 20 mM. At higher enzyme concentrations (preparative experiments) four portions of 1.25 mM *p*-diazobenzoate were added at 15-min time intervals. The reaction of *p*-diazobenzoic acid with the enzyme was stopped at desired times either by passing the mixture or an aliquot of the mixture over a Bio-Gel P-6 DG column or by dilution (100–500-fold) of an aliquot of the reaction mixture into the assay mixture.

The number of residues that were modified by *p*-diazobenzoic acid was determined spectrophotometrically as well as by incorporation of *p*-diazo[^{14}C-*carboxyl*]benzoic acid. An ϵ value of 20000 at 325 nm (pH <7) was used for monoazotyrosine (Tabachnick & Sobotka, 1959). Identification of the modified tyrosines was done by sequencing the radioactive peptides of the tryptic digest as described below.

Modification of *p*-hydroxybenzoate hydroxylase by *N*-ethylmaleimide, *p*-(chloromercuri)benzoate, or diethyl pyrocarbonate was performed as has been described earlier (van Berkel et al., 1984; Wijnands & Müller, 1982).

Dissociation constants of the binary complexes between the enzyme and NADPH or the substrate were determined fluorometrically or spectrophotometrically as published previously (Wijnands & Müller, 1982; Wijnands et al., 1984).

Spectrophotometric measurements were done on a Zeiss PMQII, a Cary 16, or an Aminco DW2A spectrophotometer and fluorometric measurements on an Aminco SPF-500 spectrofluorometer. Radioactivity measurements were carried out by using Pico-fluor 30 from United Technologies Packard and a Packard Tri-carb 2450 liquid scintillation spectrometer, or by using emulsifier scintillator 299 from Packard and a Nuclear Chicago Mark I liquid scintillation spectrometer.

Fast protein liquid chromatography (FPLC)[1] analysis was performed with Pharmacia Biotechnology Products equipment on a Mono Q HR 5/5 anion exchange column.

Identification of the essential tyrosine modified by *p*-diazobenzoate was done by analyzing, in parallel, radioactive tryptic peptides from azo-coupled enzyme samples labeled in the presence and absence of *p*-fluorobenzoate (see Table I). Tryptic digests were made as described by van Berkel et al. (1984). Prior to tryptic digestion the samples (approximately 500 nmol of each) were denatured by exhaustive dialysis against 8 M urea, followed by dialysis against 0.1 M ammonium bicarbonate, pH 8.0. Subdigests with proteinase from *S. aureus* V8 (Glu-enzyme) were made at 37 °C in 0.1 M ammonium bicarbonate, at an enzyme/substrate ratio of 1/20 (w/w) during 24 h. Peptides were purified by reversed-phase HPLC with Waters HPLC equipment (automated gradient controller Model 680, Model 441 fixed-wavelength detector, and pumps 6000 A) on a Nucleosil 10 C_{18} (Marchery-Nagel) column (300 × 4.6 mm). Sequence determinations were carried out manually by the DABITC method according to Chang et al. (1978) and Chang (1983), with identification of 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiohydantoin- (DABITH)[1] amino acids by thin-layer chromatography on polyamide sheets (2.5 × 2.5 cm). Amino acids are numbered according to the numbering for the complete primary structure of *p*-hydroxybenzoate hydroxylase (Weijer et al., 1982). All other methods used in the identification experiments have been described elsewhere (Hofsteenge et al., 1983).

RESULTS

Kinetic Studies. Chemical modification of tyrosine can be achieved either by a nucleophilic substitution at the hydroxyl group or an electrophilic substitution at the ortho position to the hydroxyl function. The most widely used nucleophilic reagent for tyrosine is *N*-acetylimidazole, and the most widely used electrophilic reagents are iodine and tetranitromethane (Means & Feeney, 1971). That tyrosine residues as well as histidine in *p*-hydroxybenzoate hydroxylase are possibly modified by diethyl pyrocarbonate at pH >7 has been shown in a preceding paper (Wijnands & Müller, 1982). This was concluded from the observation that the substrate binding capacity of the enzyme was lost by modification at high pH values and that the modification reaction could be prevented in the presence of the effector *p*-fluorobenzoic acid, while histidine modification does not affect the affinity of the enzyme for the substrate (Wijnands & Müller, 1982). Therefore, the modification reaction by diethyl pyrocarbonate at pH >7 was investigated in more detail in order to clarify the preliminary observations.

Since modification of the enzyme of diethyl pyrocarbonate at pH values >7 leads to loss of activity due to substitution of histidine residues involved in the binding of NADPH, we looked for a physical property of the enzyme that reflects the modification of the amino acid residue(s) at high pH values more specifically. We observed that modification of the enzyme by diethyl pyrocarbonate at low pH values does not influence the fluorescence properties of the enzyme, whereas

modification at high pH values leads to an increase of the fluorescence of the protein-bound FAD. The fluorescence increases by about a factor of 2 as compared to that of native enzyme. The increase of fluorescence during the modification reaction is biphasic as judged from a plot of the fluorescence increase against time. The addition of 0.9 mM diethyl pyrocarbonate to a 2 μM solution of enzyme, pH 7.5, yielded the following two pseudo-first-order rate constants at 4 °C: 0.40 min^{-1} and 0.26 min^{-1}. The rate constants increase with increasing pH value. Due to the instability of the enzyme at pH values >9 no apparent pK_a value of the amino acid residue(s) involved in the modification reaction could be determined. The modified enzyme showed no affinity for the substrate, suggesting modification of an amino acid residue in the substrate binding site. This interpretation of the data is supported by the fact that only the slow modification reaction is observed in the presence of the effector *p*-fluorobenzoate and that the affinity of the enzyme for the substrate is not affected under this condition. The fact that modification by diethyl pyrocarbonate leads to an increase of the fluorescence of the protein-bound FAD also strongly suggests that an amino acid residue located in the active center is modified. Moreover, the difference spectrum between native and modified enzyme shows a negative peak at 278 nm [see also Figure 4C in Wignands and Müller (1982)] beside a positive peak at 240 nm due to modification of histidine residues (Miles, 1977; Ovâdi et al., 1967). This negative peak also indicates that tyrosine residues are modified (Mühlrad et al., 1967; Burnstein et al., 1974).

Reactivation of the enzyme modified at pH 8 is possible by 0.1 M hydroxylamine at pH 6 and 8, but it is much slower than reactivation of the enzyme carbethoxylated at pH 6, as reported previously (Wijnands & Müller, 1982). Whereas the enzyme carbethoxylated at pH 6 was 92% reactivated after a $2^1/_2$-h incubation, the enzyme carbethoxylated at pH 8 was only 80% reactivated after 18 h. Although sulfhydryl, arginyl, α-amino acid, and ϵ-amino acid groups are also reactive toward diethyl pyrocarbonate (Mühlrad et al., 1967), only the reactions with tyrosine and histidine are known to be reversible (Melchior & Farney, 1970). Moreover, it was reported that *O*-carbethoxy-*N*-acetyltyrosine ethyl ester is 4 times less reactive with hydroxylamine than *N*-carbethoxyimidazole (Melchior & Farney, 1970). All these results strongly suggest that tyrosine residues are modified by diethyl pyrocarbonate at high pH values.

Carbethoxylated tyrosine residues are not stable under the conditions needed for protein hydrolysis in order to assign the tyrosine residue in the sequence. To achieve this goal, a reagent is needed, forming a stable bond with the tyrosine residue and being rather selective in its reaction. *p*-Aminobenzoate is an effector of the enzyme, binding at the site of the substrate. From the great similiarity between the structures of *p*-aminobenzoate and its diazonium salt it can be expected that the diazonium salt binds to the active site in a similar way as the parent compound, leading to a rather selective modification of amino acid residues in the active center.

Optimal conditions for azo coupling are a compromise between favored acidic conditions to stabilize the diazotized compound and favored alkaline conditions to facilitate the azo coupling to the phenolate moiety of tyrosine. In practice this means a pH value between 7 and 10, depending on the acidity of the phenolic group of the compound to be coupled.

When azo coupling of *p*-diazobenzoate to *p*-hydroxybenzoate hydroxylase is carried out at pH 7, the enzyme is slowly inactivated. The rate of inactivation is dependent on

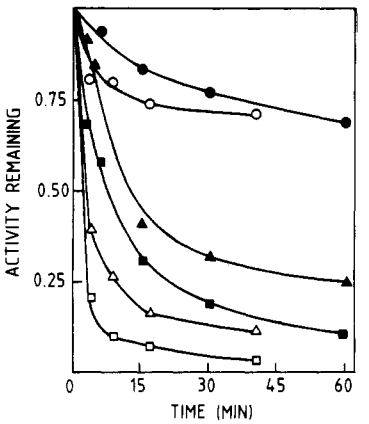

FIGURE 1: Time-dependent inactivation of *p*-hydroxybenzoate hydroxylase as a function of *p*-diazobenzoate concentration. Each incubation mixture contained 2 μM *p*-hydroxybenzoate hydroxylase in 40 mM Hepes, pH 7.0 (filled symbols), and in 40 mM Hepps, pH 8.7 (open symbols), and various concentrations of *p*-diazobenzoate: 0.018 (○, ●), 0.091 (△, ▲), and 0.18 mM (□, ■). The reactions were done at 4 °C. Aliquots were withdrawn at intervals and assayed after dilution. The relative activity was determined by comparison with an identical enzyme sample in the absence of the inactivator.

the *p*-diazobenzoate concentration and the pH (Figure 1). The reaction does not obey first-order kinetics during its whole course owing to the instability of the reagent (Means & Feeney, 1971). As judged by blank reactions, the hydrolysis of *p*-diazobenzoate is much slower than its reaction with the enzyme. The following pseudo-first-order rate constants are calculated from Figure 1 by using experimental data up to 25 min for the slower reactions and up to 10 min for the faster reactions: 0.005 min^{-1} (0.018 mM), 0.023 min^{-1} (0.091 mM), and 0.037 min^{-1} (0.18 mM) at pH 7.0; 0.014 min^{-1} (0.018 mM), 0.073 min^{-1} (0.091 mM), and 0.132 min^{-1} (0.18 mM) at pH 8.7. A plot of these observed rate constants against the concentration of diazobenzoate is linear for both pH values, and the two lines pass through the origin, suggesting that no complex is formed between the enzyme and the reagent prior to the chemical substitution reaction. The second-order rate constants are calculated to be 250 M^{-1} min^{-1} and ~800 M^{-1} min^{-1} at pH 7.0 and 8.7, respectively.

Time-dependent changes in the absorption spectrum of the enzyme upon azo coupling at pH 7 are shown in Figure 2. These difference spectra correspond well with the spectrum of monoazo(chloroacetyl)tyrosine (Tabachnik & Sobotka, 1959). Azo coupling to histidine does not seem to occur under these conditions, because identical absorption difference spectra are obtained when the enzyme is prelabeled with diethyl pyrocarbonate at pH 6 (Wijnands & Müller, 1982) (data not shown). Furthermore, not even a shoulder is found at 370 nm, the maximum wavelength of absorbance of the monoazohistidine derivative with an ϵ value of 21 100 (Tabachnik & Sobotka, 1959). As shown in the inset of Figure 2, more than 1 mol of diazobenzoate is apparently incorporated per monomeric enzyme molecule.

Prior carbethoxylation of the enzyme with diethyl pyrocarbonate at pH 8 (Wijnands & Müller, 1982) did not reveal any protection against inactivation by *p*-diazobenzoate. Decarbethoxylation of the carbethoxylated enzyme with 0.1 M hydroxylamine gave 80% reactivation within 18 h, whereas decarbethoxylation of the enzyme that had been azo coupled after modification by diethyl pyrocarbonate was not accompanied by reactivation at all. It is therefore concluded that different tyrosine residues are involved in these modifications.

FPLC analysis (anion-exchange column) of a sample of the azo-coupled enzyme revealed three peaks. One peak showed

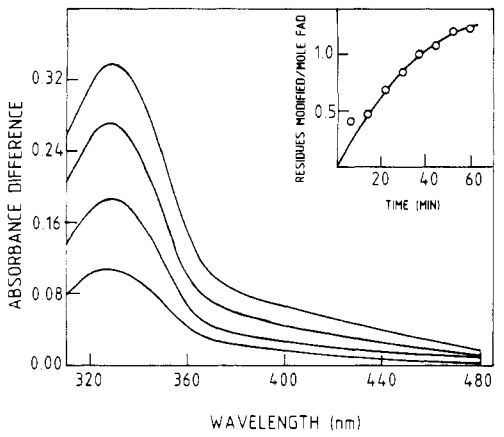

FIGURE 2: Near-ultraviolet difference spectra for the inactivation of *p*-hydroxybenzoate hydroxylase by *p*-diazobenzoate. A 100-μL sample out of 900 μL of 0.2 mM enzyme solution in 40 mM Hepes, pH 7.0, was passed over a Bio-Gel column equilibrated into the same buffer. The fraction containing the enzyme was diluted to 900 μL, and its UV spectrum was recorded. A 30-μL sample of a 40 mM *p*-diazobenzoate solution was then added to the original enzyme solution, and 100-μL samples were withdrawn at t = 7.5 min and t = 15 min. These samples were immediately treated as mentioned above, and 24 μL of the 40 mM *p*-diazobenzoate solution was added to the remaining reaction mixture, etc. All spectra were then corrected for dilution (the enzyme concentration of the t = 0 sample was 12.8 μM), and the t = 0 spectrum was subtracted. The temperature was 4 °C. The difference spectra shown were recorded at 7.5, 22.5, 37.5, and 60 min after initiation of the reaction. The inset shows the dependence of the residues modified per mole of flavin on the time. The results were calculated from the difference spectra using an ϵ value of 20 000 at 325 nm for the product (see Materials and Methods).

the same retention time as native enzyme, but the fraction exhibited low enzymic activity and an absorbance difference at 330 nm, indicating tyrosine modification by azo coupling. The other two peaks eluting at longer retention times showed the same enzymic activity and absorption spectrum as those of the former peak. These results suggest heterogeneous modification of the enzyme. Since Cys-116 in the enzyme is very reactive (Müller et al., 1979), we suspected that it becomes modified, leading to the observed FPLC pattern. In fact, when *N*-ethylmaleimide-modified enzyme (van Berkel et al., 1984) was used for the azo coupling, the two peaks eluting at longer retention times were no longer present in the FPLC chromatogram (data not shown). This clearly demonstrates that these two peaks are due to Cys-116 modification. In this context it is interesting to note that the introduction of an additional negative charge (*p*-diazobenzoate) at Cys-116, located close to the surface of the protein, is more important in the interaction with the anion-exchange material than the overall charge of the protein. The observation that azo coupling of Cys-116 leads to the appearance of two peaks in the FPLC chromatogram can be explained by the fact that the enzyme is a dimeric protein (Müller et al., 1979) and that modification of Cys-116 is not homogeneous. To avoid the "side reaction" of Cys-116, this residue was prelabeled with *N*-ethylmaleimide in the experiments to be described below.

Another fairly accessible cysteine residue in the enzyme is Cys-152, which can be modified by *p*-(chloromercuri)benzoate (van Berkel et al., 1984). Since it was still possible to modify Cys-152 in azo-coupled enzyme by *p*-(chloromercuri)benzoate, it can be concluded that no other cysteine residue than Cys-116 of the enzyme is significantly modified by *p*-diazobenzoate. Sequence studies revealed that some modification of Cys-211 takes place (see below). In a previous paper (van Berkel et al., 1984) we have shown that modification of Cys-152 by *p*-(chloromercuri)benzoate leads to pH-dependent absorbance changes at 290 nm and around 360 nm in the absorption difference spectrum. It was suggested that the apparent pK_a value of about 7.6 is due to a tyrosine residue in the active center. This proposal is now further supported by the following experiments. Modification of the enzyme by diethyl pyrocarbonate at pH 6.0 followed by *p*-(chloromercuri)benzoate modification still produces an absorption difference spectrum identical with that published previously (van Berkel et al., 1984). This result is in agreement with the fact that at this pH value only histidine residues are modified (Wijnands & Müller, 1982). In contrast, modification of the enzyme by diethyl pyrocarbonate at pH 8 followed by *p*-(chloromercuri)benzoate modification no longer produces the difference spectrum. Moreover, since diethyl pyrocarbonate modification of the enzyme at pH 8 does not prevent azo coupling of the enzyme (see above), we can safely state that the pH-dependent absorbance difference spectrum is indeed due to a tyrosine residue in the active center of the enzyme and that this tyrosine residue is not the same as the one reacting with *p*-diazobenzoate. That the tyrosine residue in question is located in the active center of the protein is strongly supported by the fact that diethyl pyrocarbonate modification of the *p*-fluorobenzoate–enzyme complex at pH 8, followed by gel filtration and *p*-(chloromercuri)benzoate modification, again produces the pH-dependent absorbance difference spectrum (van Berkel et al., 1984).

Another amino acid that is able to react with diazonium salts in general is lysine (Howard & Wild, 1957; Higgins & Harrington, 1959; Pielak et al., 1984). Like the azo coupling of sulfhydryl groups, the azo coupling of amino groups does not interfere with spectral analysis due to the low molar absorbance of the product (Pielak et al., 1984). As the degree of tyrosine modification determined spectrophotometrically coincides with the degree of modification of *N*-ethylmaleimide-modified *p*-hydroxybenzoate hydroxylase with *p*-diazo-[^{14}C-*carboxyl*]benzoate (see below), it is concluded that no lysine residues react under the conditions given.

From the above it is clear that when Cys-116 is protected by *N*-ethylmaleimide, only tyrosine residues in the enzyme react significantly with *p*-diazobenzoate at pH 7. Although it is possible that bis(azotyrosines) are formed, it has been reported that this reaction does not occur easily in proteins due to steric constraints (Pielak et al., 1984). This was confirmed for the case of *p*-hydroxybenzoate hydroxylase by the absorption spectrum of the modified enzyme at pH 12, only exhibiting an absorption band at 490 nm. Since no absorbance at 550 nm (Tabachnik & Sobotka, 1959) was observed, it can safely be concluded that no bis azo derivative is formed in the enzyme. The benzoazotyrosines in the enzyme could not be reduced to 3-aminotyrosines with dithionite or borohydride as no loss of absorbance at 325 nm was found upon the addition of either of these reagents. Although reduction is expected to take place, its failure has been reported as well (Lemke et al., 1982).

When *p*-hydroxybenzoate hydroxylase was inactivated by *p*-diazobenzoate, the diassociation constant of the modified enzyme–NADPH complex was 0.14 mM at pH 7 and I = 20 mM. As the K_d value of the native enzyme–NADPH complex is also 0.14 mM (Wijnands et al., 1984), it is concluded that the inactivation due to modification of tyrosine residues is not caused by a loss of the ability of the enzyme to bind NADPH. *p*-Hydroxybenzoate, however, did not bind to its primary binding site in the modified enzyme as only binding to the secondary binding site (Husain & Massey, 1979) could be detected after inactivation. Benzoate and *p*-fluorobenzoate

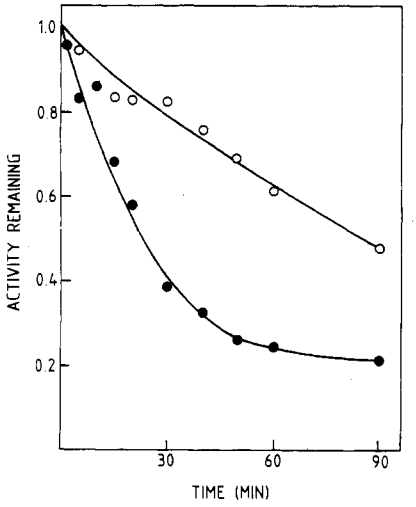

FIGURE 3: Effect of *p*-fluorobenzoate on the rate of inactivation of *p*-hydroxybenzoate hydroxylase by *p*-diazobenzoate. A solution of 2 μM enzyme in 40 mM Hepes, pH 7.0, and at 4 °C was treated with 0.2 mM *p*-diazobenzoate in the presence of 20 mM *p*-fluorobenzoate (○) or in the absence of *p*-fluorobenzoate (●).

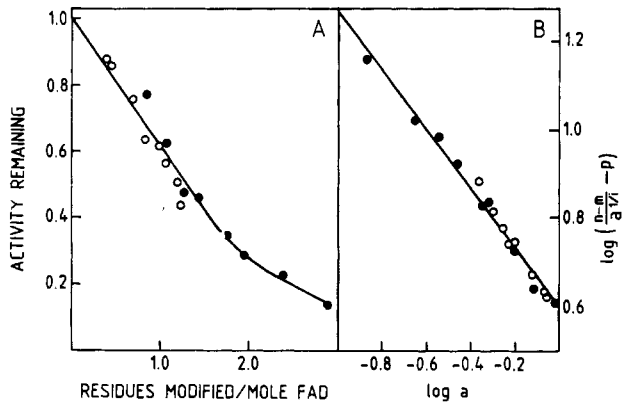

FIGURE 4: Correlation between the residual activity of *p*-hydroxybenzoate hydroxylase and the number of residues modified by *p*-diazobenzoate. The results of two separate experiments are shown. In the first experiment (○) *m* was determined spectrophotometrically (see Figure 2), and in the second (●) it was determined by incorporation of ^{14}C. Conditions of the first experiment are given in the legend of Figure 2. The enzyme concentration during the second modification experiment was 0.1 mM. A total of 4.9 nmol of *p*-hydroxybenzoate hydroxylase was used for counting. All other data are given under Materials and Methods. The results are plotted directly (A) and according to the equation given in the text (B).

were not able to bind either. Protection experiments in the presence of *p*-hydroxybenzoate are impossible due to its reactivity with *p*-diazobenzoate. We found it to be $2^1/_2$ times as reactive as *N*-acetyltyrosine ethyl ester at pH 8.8 (25 °C). This is due to the lower pK_a value of the phenolic group of *p*-hydroxybenzoate (9.3) compared to that of tyrosine (10.1) (Dawson et al., 1969). The enzyme–*p*-fluorobenzoate complex was therefore used in all protection experiments against *p*-diazobenzoate modification. Figure 3 shows that some protection against inactivation in fact occurs.

Time samples were taken during inactivation and monitored for residual activity and the extent of chemical modification to determine the number of essential tyrosine residues that are modified by *p*-diazobenzoate. In one experiment the latter was determined spectrophotometrically (Figure 2) and in another with the aid of *p*-diazo[^{14}C-*carboxyl*]benzoate. As has already been mentioned above, in the latter case, *N*-ethylmaleimide-labeled *p*-hydroxybenzoate hydroxylase was used. The correlation between the residual activity (*a*) and

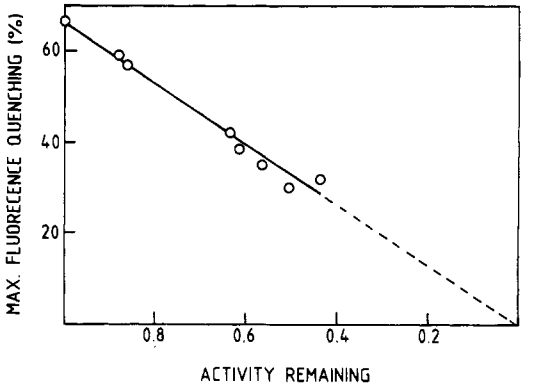

FIGURE 5: Correlation between maximal quenching of the fluorescence emission of enzyme-bound FAD by *p*-hydroxybenzoate and residual activity of the enzyme after azo coupling. The aliquots of the experiments described in the legend of Figure 2 were used for titrating with *p*-hydroxybenzoate, with the fluorescence emission being monitored at 520 nm (excitation at 450 nm). Prior to the titrations, 2-μL aliquots were withdrawn from the fractions and assayed for residual enzyme activity.

Table I: Inactivation of *p*-Hydroxybenzoate Hydroxylase by Diazo[^{14}C-*carboxyl*]benzoate and Incorporation of ^{14}C in the Absence and Presence of *p*-Fluorobenzoate[a]

p-fluorobenzoate	rel enzymic act.	Tyr residues modified/mol of FAD
−	0.25	~2.0
+	0.60	~1.2

[a] A total of 475 nmol of enzyme in the absence and presence of *p*-fluorobenzoate was first labeled with *N*-ethylmaleimide and then modified by diazo[^{14}C-*carboxyl*]benzoate. During the coupling the concentrations of enzyme and *p*-fluorobenzoate were 90 μM and 20 mM, respectively. Both reactions were stopped after 2 h. All other details are given under Materials and Methods. Only 4.6 nmol of each sample was used for quantitation and activity measurements.

the number of residues (*m*) that are modified is shown in Figure 4A. It can be seen that the number of tyrosine residues modified coincides with the amount of ^{14}C incorporated into the enzyme. It has been shown by Tsou (1962) that when *p* residues of which *i* are essential react with a rate constant *k* and *n* − *p* residues react with a rate constant αk, the following equation is valid:

$$\log [(n - m)/a^{1/i}] - p = \log (n - p) + [(\alpha - 1)/i] \log a$$

where *n* is the total number of residues that are modified. A straight line is obtained when $\log [(n - m)/a^{1/i}] - p$ is plotted against $\log a$. The data of Figure 4A are replotted in this manner in Figure 4B with $n = 5$ and $p = i = 1$, which is the best fit possible to a straight line. An α value of 0.32 is determined from the slope. Although nearly all activity is lost after the modification of 2–3 tyrosine residues (Figure 4A), a minimum number of 5 is able to explain our data. Higher values for *n* merely result in lower values for α, as α can be regarded as an average value for the reacting residues.

The samples that were analyzed spectrophotometrically were also used to determine the maximal degree of quenching of the fluorescence of the enzyme-bound FAD that could be induced by *p*-hydroxybenzoate. It can be seen in Figure 5 that the decrease in maximal quenching is correlated directly to inactivation. A difference of 0.8 tyrosine residues per mole of FAD was found on performing a quantitation experiment, comparing inactivation and ^{14}C incorporation in the absence and presence of *p*-fluorobenzoate (Table I). This is in good agreement with the results of the graphical method. It is

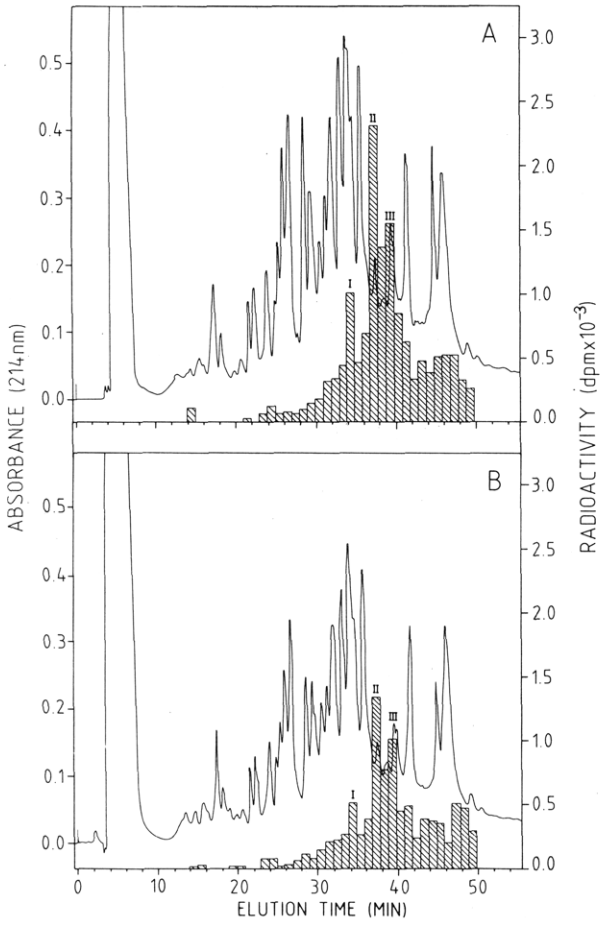

FIGURE 6: HPLC chromatograms of tryptic digests of *N*-ethylmaleimide-prelabeled *p*-hydroxybenzoate hydroxylase that had been modified by *p*-diazo[^{14}C-*carboxyl*]benzoate in the absence (A) and presence (B) of *p*-fluorobenzoate. The samples were prepared as described in Table I and under Materials and Methods. Two percent of the tryptic digest of each sample was chromatographed with a linear gradient from 0% to 67% acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.0 mL/min. Peptide elution was monitored by the absorbance at 214 nm. Vertical bars represent the total radioactivity content associated with pools of eluent collected at 1-min intervals. Preparative isolation of the radioactive peptides in pools, I, II, and III was done by five separate runs on the tryptic digest of each sample.

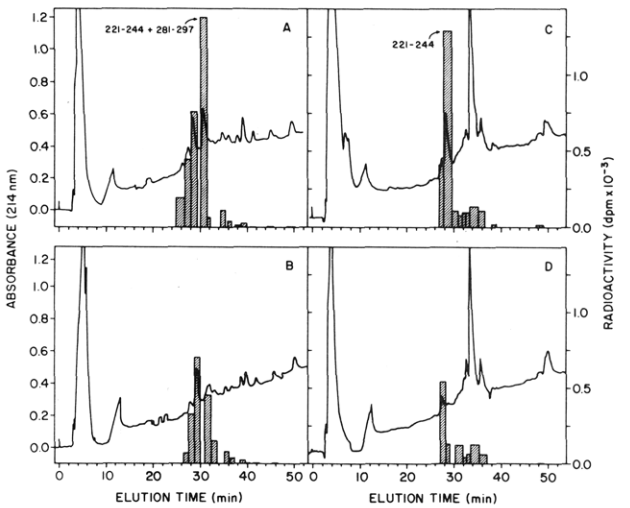

FIGURE 7: Purification of the peptide of interest of pools II and III from Figure 6 by HPLC and further digestion with the Glu-enzyme. Each 3% of pools II and III was chromatographed with a linear gradient from 0% to 67% acetonitrile in 0.1% ammonium acetate, pH 6.0. Each sample thus obtained after preparative isolation was then further digested by treatment with the Glu-enzyme. Ten percent of each digest was then chromatographed again with a linear gradient from 0% to 67% acetonitrile in 0.1% trifluoroacetic acid. Twenty percent of each of the samples thus obtained was rechromatographed with a linear gradient from 0% to 67% acetonitrile in 0.1% ammonium acetate, pH 6.0: (A, B) pool II; (C, D) pool III. Peptides that could be correlated with a known sequence in the primary structure of *p*-hydroxybenozate hydroxylase are indicated by its residue number. A flow rate of 1 mL/min was applied in all cases. Peptide elution was monitored by the absorbance at 214 nm. Vertical bars represent the total radioactivity associated with pools of eluent collected for each peptide peak. Chromatograms A and C correspond to the enzyme preparation labeled in the absence of *p*-fluorobenzoate (see Figure 6).

therefore concluded that only one of the tyrosine residues modified by *p*-diazobenzoate is essential for enzymic activity and that it can be protected from modification by the effector *p*-fluorobenzoate.

Sequence Studies. Denaturation of the labeled enzyme (see Materials and Methods) led surprisingly to the loss of most of the radioactive label (~80%). An identical observation was made in an earlier attempt to identify the tyrosine residue by using the absorbance of the azo-coupled product as a monitor. Despite this experimental uncertainty we have assumed that the distribution of the remaining label over the tryptic peptides reflects the sites modified by *p*-diazobenzoate in the enzyme in the native state.

Figure 6 shows the reversed-phase HPLC peptide mapping analyses of tryptic digests of the enzyme modified in the absence and in the presence of *p*-fluorobenzoate, together with their radioactivity profiles. Because our main interest was the identification of residues that are involved in substrate binding, we confined ourselves to the analysis of certain peptide pools of the unprotected sample. We believe that this approach is justified by the fact that the "background" radioactivity in both samples is about the same. Three pools selected in this way (pools I, II, and III in Figure 6) also happen to be the major labeled peaks in the unprotected sample. The relatively high radioactivity in pools directly adjacent to pools II and III in Figure 6 most probably originates from the presence of part of the same radioactive peptides as in pools II and III as judged by HPLC analyses (data not shown). These pools will therefore be left out of further consideration in the following. Since each of the selected pools from both digests in Figure 6 contained a complex mixture of peptides, they were further purified by HPLC using a different eluent system.

Results of amino acid analysis and three cycles of DABITC degradation of peptides of interest in Figure 7 were compatible with the presence of a peptide with N-terminal sequence Tyr-Tyr-Val-and comprising residues 221–244 in the amino acid sequence of *p*-hydroxybenzoate hydroxylase. This peptide contains an uncleaved Lys-Val bond (residues 230–231) and an Arg-Phe bond (residues 238–239). These bonds have been shown to be very resistant toward enzymic hydrolysis (Hofsteenge et al., 1983; Jekel et al., 1983). The peptide isolated from the fractionation in Figure 7C was obtained in relatively pure form, whereas that isolated from the fractionation in Figure 7A was contaminated (for 30–40% on a molar basis) with the non-tyrosine-containing peptide 281–297 (N-terminal sequence Leu-Phe-Leu-). The peptides from both panel A and panel C of Figure 7 revealed a pronounced peak of radioactivity at Tyr-222 when subjected to several cycles of DABITC degradation with measurement of the radioactivity of the derivatives cleaved off in each degradation cycle, indicating that this tyrosine residue had been modified by *p*-diazobenzoate. In the identification of DABITH-amino acids on polyamide sheets, the modified amino acid at cycle 2 eluted at the position of DABITH-Ser. Autoradiography of the

polyamide sheets also showed radioactivity of the modified tyrosine at the position of DABITH-Ser (Chang et al., 1978). Modification of one of the two tyrosines was also apparent from amino acid analysis since only 1 mol of Tyr/mol of peptide was recovered after acid hydrolysis. These results are consistent with the results in Figure 7B,D.

A peptide mixture obtained from pool I (Figure 6) was digested with proteinase from *S. aureus* V8 (Glu-enzyme) and purified by HPLC. A final peptide yielded the N-terminal sequence Gly-Phe-X-Leu-X-X. This tryptic peptide could be enriched from a mixture by removing many contaminants by an additional digestion with the Glu-enzyme. No other sequence was identified. Unsatisfying amino acid analyses were obtained because of insufficient material. Still, they were indicative for a tryptic peptide comprising residues 207–214 of *p*-hydroxybenzoate hydroxylase. Liquid scintillation counting of the DABITH derivatives of the amino acids released during each cycle of DABITC degradation only showed significant radioactivity at cycle 5, indicating that Cys-211, in addition to Tyr-222, had been modified. Tyr-222, however, seems to be the major site of attack by *p*-diazobenzoate as can be seen from the radioactivity patterns in Figure 6. Moreover, the peptide containing Tyr-222 was isolated with a yield (25 nmol after two purification steps) 25 times higher than that for the peptide containing Cys-211 (1 nmol after four purification steps). It is therefore concluded that the loss of enzymic activity upon azo coupling of *p*-diazobenzoate to *p*-hydroxybenzoate hydroxylase is caused most probably by modification of Tyr-222.

Additional Information on the Sequence of p-Hydroxybenzoate Hydroxylase. In this investigation and a previous one (van Berkel et al., 1984), reversed-phase HPLC was used for the isolation of peptides from tryptic digests of intact *p*-hydroxybenzoate hydroxylase. As an adventitious circumstance, these studies led, in a relatively easy way, to the isolation of tryptic peptides from which additional chemical evidence was obtained for the amino acid sequence of the protein as presented earlier (Weijer et al., 1982).

(a) The five CNBr fragments, CB1 through CB5, which account for the entire polypeptide chain of *p*-hydroxybenzoate hydroxylase, were aligned on the basis of the 0.25-nm electron-density map (sequential order: CB3-CB5-CB4-CB2) (Hofsteenge et al., 1980). The overlap between peptides CB5 and CB4 was proven chemically (Hofsteenge et al., 1980; Vereijken et al., 1980). Here we have isolated the overlapping peptides PT1 (residues 45–57), PT2 (residues 108–113), and PT3 (residues 270–280), which confirmed the sequential orders CB3-CB5, CB4-CB1, and CB1-CB_2, respectively. The amino acid compositions of peptides PT1, PT2, and PT3 are shown in Table II. . Peptide PT3 (residues 270–280) also confirmed the amide assignment of the residue at position 277, for which a glutamine was previously proposed from indirect chemical evidence (Hofsteenge et al., 1980, 1983). This peptide has a net charge of $0 < \epsilon < +1$ (Table II), as determined by the method of Offord (1977). Since glutamic acid is present at position 274 (Weijer et al., 1982), the residue at position 277 must be glutamine.

(b) The preliminary sequence Ser-Tyr-Trp (residues 343–345) was previously deduced from the results of amino acid analysis of a relatively impure peptide and the size and shape of residues at these positions in the electron-density map (Hofsteenge et al., 1983). In this study citraconylated *p*-hydroxybenzoate hydroxylase was digested with trypsin. After decitraconylation, the peptide PT4 (residues 342–352) was isolated in pure form by gel filtration (Sephadex G-50F, 30% acetic acid), followed by reversed-phase HPLC. The amino acid composition of peptide PT4 is shown in Table II. Sequence determination (DABITC method) yielded the N-terminal sequence Phe-Ser-Trp-Trp-Met. These results now definitively establish the sequence at residues 342–346 and confirm the overlap between peptides CB2a and CB2b (Hofsteenge et al., 1983).

Table II: Amino Acid Composition of Peptides PT1 (Residues 45–57), PT2 (Residues 108–113), and PT3 (Residues 270–280)[a]

amino acid	PT1	PT2	PT3	PT4
Asp	1.0 (1)	1.1 (1)	0.2	
Thr				0.6 (1)
Ser			0.8 (1)	1.8 (2)
Glu	2.0 (2)	1.0 (1)	2.3 (2)	
Pro			0.9 (1)	
Gly	1.9 (2)		1.2 (1)	
Ala	1.0 (1)	0.9 (1)		
Cys				
Val	1.5 (2)		1.5 (2)	1.1 (1)
Met	1.0 (1)	0.7 (1)	0.5 (1)	1.0 (1)
Ile				
Leu	2.7 (3)	1.0 (1)		1.0 (1)
Tyr				
Phe			0.9 (1)	1.0 (1)
Lys				
His			0.9 (1)	1.0 (1)
Arg	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Trp	nd	nd	nd	1.4 (2)
total residues	13	6	11	11
N-terminus	Ala	Asx	Ser	Phe
charge at pH 6.5	nd	nd	$0 < \epsilon < 1$	nd

[a] The values in parentheses indicate the number of the amino acid found in the sequence of the peptide. Quantities <0.2 mol/mol have been omitted. Hydrolysis was performed with 6 M HCl during 24 h (PT1–PT3) or with 3 M mercaptoethanesulfonic acid during 96 h (PT4). nd = not determined.

Discussion

Chemical modification studies are often used to evaluate the role of certain amino acid residues in the catalysis of enzymes. Although this approach can yield valuable information, the method has some obvious shortcomings. For instance, the substitution of an amino acid residue in the active center by a bulky group may destroy the catalytic action of an enzyme merely by virtue of steric constraints. We have concluded above from the kinetic results that the tyrosine residue modified by *p*-diazobenzoate is essential for the catalytic action of the enzyme, because the affinity of the enzyme for the substrate is abolished upon modification. Although this interpretation may be valid, it cannot be excluded that the catalytic loss of the enzyme is due to the inaccessibility of the active center for the substrate.

Diazonium salts have been shown to be useful reagents in structure–function relationship studies (Lembke et al., 1982; Mäkinen et al., 1982). Although diazonium compounds were shown to couple with α-acetylarginine and -proline, as well as tryptophan (Howard & Wild, 1957; Higgins & Harrington, 1959), a more recent study has revealed that protein reactivity toward azo coupling can mainly be attributed to tyrosine, histidine, cysteine, and lysine residues (Pielak et al., 1984). In the case of *p*-hydroxybenzoate hydroxylase, azo coupling occurs with Cys-116 and partially with Cys-211 and tyrosine residues. The reaction with Cys-116 can, however, easily be suppressed by alkylation of the residue prior to the reaction of the enzyme with the diazonium salt. Sequence studies on the modified enzyme revealed that only one tyrosine active center of the enzyme is modified, i.e., Tyr-222. This result is in agreement with the data obtained by chemical modifi-

cation of the enzyme. The presently existing three-dimensional model [Weijer et al. (1983); cf. Figure 10] of the substrate *p*-hydroxybenzoate hydroxylase complex shows that Tyr-201, Tyr-222, and Tyr-385 are located in the active center of the protein. The hydroxyl group of the substrate is located close to the hydroxyl groups of Tyr-201 and Tyr-385. The hydroxyl group of Tyr-222 is in close proximity to the carboxyl group of the substrate and the guanidinium group of Arg-214, which is involved in the binding of the carboxyl group of the substrate. The fact that Tyr-222 is modified is somewhat surprising. From the great structural similiarity between the diazonium salt and its parent compound (effector of the enzyme) we had expected that either Tyr-201 or Tyr-385 or both would become modified. The fact that Tyr-222 is modified by *p*-diazobenzoate strongly suggests that either the reagent binds, as compared to the substrate, in the reversed configuration to the enzyme as has been proposed for the binding of the substrate at high concentrations leading to inhibition of the enzyme (Husain & Massey, 1979) or it reacts directly with Tyr-222. Both proposals are in accord with the kinetic data, because if a complex were to be formed prior to reaction it would be a very weak complex. It is interesting to note that diethyl pyrocarbonate reacts with either Tyr-201 or Tyr-385. That diethyl pyrocarbonate reacts with one or both of these active center tyrosine residues is supported by the fact that the fluorescence of the enzyme-bound FAD increases on modification of the enzyme at high pH values. It is known that aromatic amino acid residues located in the flavin binding site of flavoproteins are involved in the dynamic and/or static quenching of the flavin fluorescence. It is proposed that substitution of the hydroxyl group of the tyrosine residue(s) decreases the quenching effect on the flavin fluorescence, although a slight conformational change or a small change of the microenvironment in the active center of the enzyme may have a similar effect on the flavin fluorescence. The present results seem also to support our previous proposal (van Berkel et al., 1984) that a tyrosine residue in the active center of the enzyme is ionized ($pK_a \sim 7.6$) rather than the substrate as proposed by Shoun et al. (1979). Our present results suggest that this tyrosine residue is either Tyr-201 or Tyr-385. Such an ionized tyrosine residue could facilitate hydroxylation of the substrate by formation of a hydrogen bond with the hydroxyl group of the substrate.

Finally, it should be mentioned that this study yielded some further support for the published sequence of the enzyme (Weijer et al., 1983) and provided final evidence that the residues at positions 344 and 345 are Trp.

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Registry No. EC 1.14.13.2, 9059-23-8; L-Tyr, 60-18-4; $^{+}N_2C_6H_4CO_2^{-}$-*p*, 1837-05-4; *p*-$FC_6H_4CO_2H$, 456-22-4; diethyl pyrocarbonate, 1609-47-8.

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