Structure and function of *para*-hydroxybenzoate hydroxylase

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Structure and function of *para*-hydroxybenzoate hydroxylase

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Cover: lightobject in stainless steel by Frank van der Bolt refer to the structure of *para*-hydroxybenzoate hydroxylase
Key words: aromatic hydroxylation / dehalogenation / flavoprotein / monooxygenase / *para*-hydroxybenzoate / site-specific mutagenesis

Stellingen

1 Flavine mobiliteit in *p*-hydroxybenzoate hydroxylase is essentieel voor katalyse (dit proefschrift).

2 Niet alleen de structuur van het actieve centrum, maar ook het type substraat bepaalt de regiospecifiteit van hydroxylering door *p*-hydroxybenzoaat hydroxylase (dit proefschrift).

3 De controverse tussen het experimentele reactiemechanisme van cytochroom P450 en het theoretisch-berekende mechanisme wordt door de auteurs niet besproken (Oxygen exchange with water in heme-oxo intermediates during H_2O_2 driven oxygen incorporation in aromatic hydrocarbons catalyzed by microperoxidase-8. V. Dorovska-Taran, M.A. Poshumus, S. Boeren, M.G. Boersma, C.J.Teunis, I.M.C.M. Rietjens and C. Veeger (1998), Eur. J. Biochem. *253*, 659-668; Molecular orbital study of porphyrin-substrate interactions in cytochrome P450 catalysed aromatic hydroxylation of substituted anilines. O. Zakharieva, M. Grodzicki, A.X. Trautwein, C. Veeger and I.M.C.M. Rietjens (1998), Biophys. Chem. *73*, 189-203; Molucular orbital study of the hydroxylation of benzene an monofluorobenzene catalysed by iron-oxo porphyrin π cation radical complexes. O. Zakharieva, M. Grodzicki, A.X. Trautwein, C. Veeger and I.M.C.M. Rietjens, Biol. inorg. chem. 1 (1996) 192-204.

4 De Bijlmer-enquete heeft bewezen dat de vraag om een onderzoek niet automatisch leidt tot een onderzoeksvraag.

5 De stellingname van het Brits koninklijk huis in de discussie over genetische manipulatie, manipuleert in eerste instantie de positie van het koninklijk huis.

6 Als in het onderzoek alles onder controle is gaat de vooruitgang niet snel genoeg.

7 Geen tijd hebben betekent ergens prioriteit aan geven.

8 In de meubelmakerij mag de spijker niet voor de tweede maal worden uitgevonden.

9 Het verkrijgen van inzicht in een bepaald vakgebied kan leiden tot een onverwacht uitzicht.

Stellingen behorend bij het proefschrift van Frank van der Bolt
"Structure and Function of *para*-Hydroxybenzoate hydroxylase"
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Chapter 1

Scope and outline of the thesis

1.1 General introduction

Enzymes which are biocatalysts can accelerate a reaction compared to the rate observed in solution, because they are able to lower the activation barrier of a reaction.

Enzymes can be classified on the basis of the type of reaction catalysed. This leads to a division into classes representing oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. However, this classification does not give any information about the way in which the reaction is catalysed, that is, the mechanisms and/or principles used by the enzymes to actually lower the activation barrier for a reaction.

A division, based on the type of reaction mechanism used by the enzymes, can be made on the basis of the cofactor involved or absent in the actual chemistry catalysed. Cofactors can be, for example, a flavin, a heme, a metal cluster etc. Based on the type of cofactor involved, cofactor-dependent enzymes can be divided into the following subclasses, indicative for the type and mechanism(s) of catalysis:

- flavin-dependent catalysis
- heme-dependent catalysis
- metal cluster-dependent catalysis
- pyridoxal phosphate-dependent catalysis
- quinoid cofactor-dependent catalysis
- nicotinamide-dependent catalysis
- biotin-dependent catalysis
- thiamine pyrophosphate-dependent catalysis

In the next chapter, several of the most important reaction mechanisms and catalytic principles of flavin-dependent enzymes will be outlined. This is done since the objective of the present study is related to the understanding of the catalytic mechanism of the flavoprotein *para*-hydroxybenzoate hydroxylase (PHBH).

1.2 Aim of the thesis

The aim of the present thesis was to obtain detailed insight in the role of substrate and flavin cofactor orientation and mobility and of specific amino acid residues, in the catalytic mechanism of PHBH (EC 1.14.13.2). PHBH is a flavoprotein involved in the aerobic degradation of aromatic compounds. It catalyses the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoate using NADPH and molecular oxygen (Fig. 1). This is the first step of the β-ketoadipate pathway by which certain soil micro-organisms are able to degrade and utilise hydroxylated aromatic compounds as their energy and carbon source (Ornston & Stanier, 1964; Stanier et al., 1973; Anderson & Dagley 1980; van Berkel et al., 1994; Harwood & Parales, 1996). Aromatic compounds degraded this way may originate from environmental pollution (Dagley et al., 1964; Gibson, 1968; Neujahr & Gaal, 1973; Beadle and Smith, 1982; Higson & Focht, 1989; van der Meer et al., 1992; Xun et al., 1992; van der Meer, 1997), but also from natural sources, since they are liberated during the biodegradation of lignin, one of the principle components of wood (Neujahr, 1991; van Berkel and Müller, 1991).

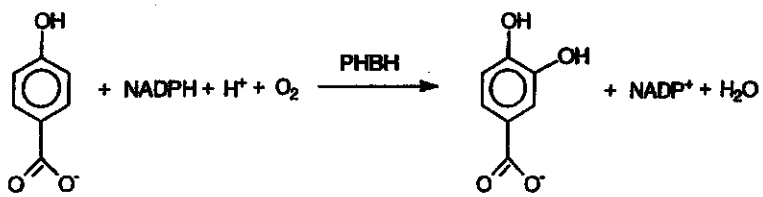

Figure 1. Reaction catalysed by PHBH

PHBH is a member of the class of flavoprotein aromatic hydroxylases. These enzymes have many catalytic properties in common and their substrate specificity is consistent with an electrophilic aromatic substitution mechanism (Massey, 1994). As can be seen from Table 1, microorganisms use this catalytic competence in an ingenious way by inducing either *ortho*- or *para*-hydroxylating enzymes (van Berkel and Müller, 1991; van Berkel et al., 1997). As a result of the renewed interest for the microbial degradation of environmental pollutants, several flavoprotein aromatic hydroxylase genes were sequenced during the last few years (Eppink et al., 1997).

Table 1. Primary substrates of flavoprotein aromatic hydroxylases

Substrate	Hydroxylation	Substrate	Hydroxylation
salicylate	ortho */para	phenol	ortho *
3-hydroxybenzoate	ortho/para	resorcinol	ortho
4-hydroxybenzoate	ortho */para	hydroquinone	ortho
2-hydroxyphenylacetate	ortho/para	orcinol	ortho
3-hydroxyphenylacetate	ortho/para	cresol	ortho *
4-hydroxyphenylacetate	ortho */para	2-nitrophenol	ortho
2-aminobenzoate	ortho	4-nitrophenol	ortho
4-aminobenzoate	para	2-hydroxybiphenyl	ortho *
melilotate	ortho	2,4-dichlorophenol	ortho *
protocatechuate	para	2,4,5-trichlorophenol	para
vanillate	para	2,4,6-trichlorophenol	para *
l-kynurenine	ortho	pentachlorophenol	para *
4-hydroxyisophtalate	ortho		
2-aminobenzoyl CoA	para *	2,6-dihydroxypyridine	ortho
3-hydroxybenzoyl CoA	para		

Enzymes with known sequence are indicated with an asterika. From van Berkel et al., 1997.

PHBH has become the model enzyme to study catalysis by flavoprotein aromatic hydroxylases, since its crystal structure is known in atomic detail (Wierenga et al., 1979, Schreuder et al., 1989; Schreuder et al., 1991). This and the cloning of the *pobA* gene enabled a detailed analysis of the active site and the creation of mutant enzymes to study the role of particular amino acid residues (Eschrich et al., 1990, Entsch et al. 1991, van Berkel et al., 1992).

The objective of the present thesis is to actually obtain insight in the role of particular amino acid residues in PHBH from *Pseudomonas fluorescens*. Special emphasis is given on the substrate and effector specificity, and on the orientation and mobility of the substrate and the flavin cofactor during catalysis.

1.3 Outline of the thesis

In 1990, the *pobA* gene encoding PHBH from *Pseudomonas fluorescens* was cloned in *Escherichia coli*. As described in **Chapter 1**, this paved the way to study the structure and function of PHBH in further detail.

Chapter 2 presents an introduction on the enzyme and the present state-of-the-art knowledge on the catalytic mechanism and active site of PHBH as well as the role of certain amino acid residues in catalysis. This chapter also describes in what way these residues may affect the mobility and chemical reactivity of the substrate and flavin cofactor.

PHBH is a homodimer in which each monomer functions catalytically independent. Each PHBH subunit contains five cysteine residues. In **Chapter 3**, the role of these sulfhydryl groups in catalysis is addressed by a combined site-directed mutagenesis and chemical modification approach.

The crystal structure of the enzyme-substrate complex of PHBH shows that the hydroxyl group of 4-hydroxybenzoate interacts with the side chain of Tyr201, which is in close contact with the side chain of Tyr385. **Chapter 4** describes the role of Tyr201 and Tyr385 in substrate activation and in the fine tuning of the substrate and effector specificity.

In **Chapter 5** structural data of wild-type PHBH and mutant Tyr222Ala complexed with substrate analogs are presented. These studies revealed a new binding mode of the flavin ring out of the active site and uncovered a proton channel, presumably involved in substrate activation. The newly discovered displacement of the flavin ring might provide an entrance for the substrate to enter the active site and an exit for the product to leave.

In the final chapters, the role of flavin mobility and substrate orientation during PHBH catalysis is investigated in more detail. In **Chapter 6**, the role of flavin motion is addressed on the basis of studies with mutant Tyr222Ala. Biochemical support is given to the idea that the equilibrium of flavin conformers is dependent on the enzyme redox state.

Chapter 7 reports on the regiospecificity of hydroxylation of tetrafluoro-4-hydroxybenzoate by wild-type PHBH and mutant Tyr385Phe. Evidence is provided for the first time that C2 hydroxylation of PHBH substrates is feasible. The implications of these results for substrate and flavin mobility and reactivity are discussed.

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Chapter 2

Introduction

2.1 Flavoenzymes

Flavoenzymes are redox proteins that contain a flavin cofactor (in most cases FAD or FMN). Flavoenzymes are spread widely in nature and catalyse diverse reactions, ranging from redox catalysis and light emission to DNA repair. In most flavoenzymes, the flavin cofactor is non-covalently bound to the protein moiety (Müller and van Berkel, 1991). Occasionally, the flavin is covalently linked to either a histidine, tyrosine or cysteine of the polypeptide chain (Mewies et al., 1998; Fraaije et al., 1998).

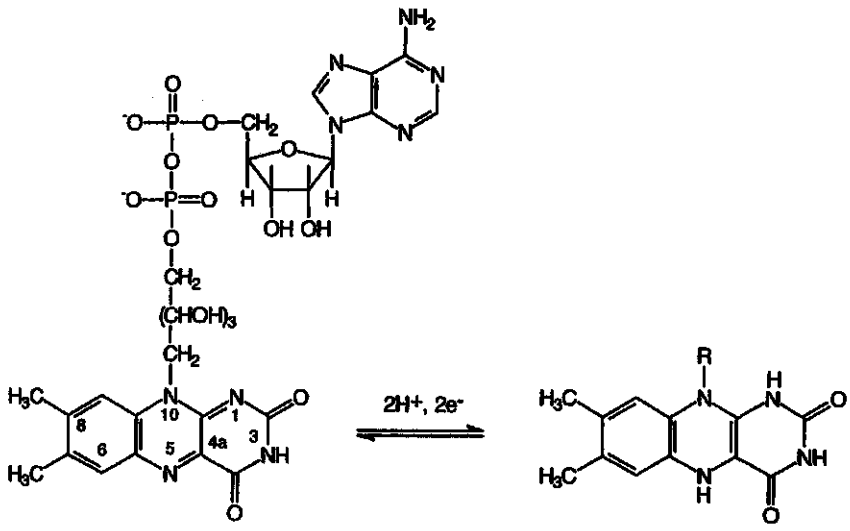


Figure 1. Structure of FAD in oxidized and fully reduced state

So far, several hundred different flavoenzymes have been purified and characterised from various organisms. Based on their catalytic mechanism and function these enzymes are divided into several classes as summarized in Table 2.1.

Table 2.1 Classification of flavoenzymes

Category	Examples	Characteristics
Oxidases	D-amino acid oxidase glucose oxidase vanillyl-alcohol oxidase	the electron acceptor is O_2 by $2e^-$ reduced to H_2O_2
Dehydrogenases	acetyl-CoA dehydrogenase	oxidation of fatty acid acyl-CoA substrates
Monooxygenases	*p*-hydroxybenzoate hydroxylase bacterial luciferase cyclohexanone monooxygenase	the acceptor is O_2: one oxygen atom in H_2O, other oxygen atom in OH of the product
Electron transferases	ferredoxin-$NADP^+$ reductase NADPH-cytP450 reductase	$1e^-$ transfer, 'flavin-shuttle' between semiquinone- and hydroquinone forms
Disulfide oxidoreductases	lipoamide dehydrogenase glutathione reductase NADH peroxidase	contains redox-active disulfide, besides NADPH often disulfide/ dithiol as substrate
Heme-containing flavoenzymes	flavocytochrome b2 cytochrome P450 BM-3 NO synthase *p*-cresol methylhydroxylase	contains heme as an extra redox group
Metal containing flavoenzymes	xanthine oxidase phtalate dioxygenase	bound transition state metal ions (Fe^{2+}, Fe^{3+}, Mo^{6+}) cofactors

From Table 2.1 it immediately follows that the classification of flavoenzymes is not very strict. There are flavoenzymes which contain one or even more heme cofactors and/or metal ions in their active site and use these additional cofactors in their catalytic mechanism. Thus, the same cofactor can be involved in different types of biocatalysis. It will be clear that the protein structure, which influences for example the surroundings and the accessibility of the cofactor, strongly influences and determines the type of catalysis performed by the flavin cofactor. In the next paragraphs, the flavin-mediated reaction catalysed by PHBH will be discussed in more detail.

2.2 *para*-Hydroxybenzoate hydroxylase

2.2.1 Overall reaction

PHBH is a flavoprotein monooxygenases. The enzyme is a dimer of identical subunits, each about 45 kDa and each containing one FAD molecule (Müller et al., 1979). PHBH catalyses the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoate, a common intermediate step in the degradation of aromatic compounds in soil bacteria (Fig. 2). The enzyme from *Pseudomonas fluorescens* which is the subject of the present study is strictly NADPH dependent (Howell et al., 1972). Some PHBH enzymes (less well studied) can also use NADH as electron donor (Seibold et al., 1996).

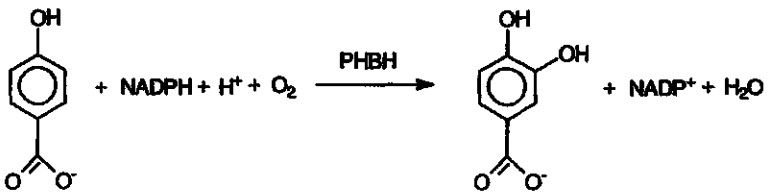


Figure 2. Reaction catalyzed by PHBH

2.2.2 Protein structure

PHBH is one of the first flavoenzymes with known three-dimensional structure (Wierenga et al., 1979). Figure 3 presents a ribbon structure of the peptide backbone of the PHBH subunit, as refined to 1.9 Å resolution (Schreuder et al., 1989). The folding topology of PHBH is shared by several other flavoenzymes, including cholesterol oxidase (Vrielink et al., 1991), glucose oxidase (Hecht et al., 1993), D-amino acid oxidase (Mattevi et al., 1996) and phenol hydroxylase (Enroth et al., 1998) but also by the GDP-dissociation inhibitor of Rab GTPases (Schalk et al., 1996). This highlights the versatile nature of the PHBH fold, which is suited for diverse biological functions (Mattevi, 1998).

The enzyme-substrate complex of PHBH crystallizes as a dimer (Wierenga et al., 1979). In the absence of substrate or in the presence of NADPH, PHBH has rather poor diffraction properties (Schreuder et al., 1991; van der Laan et al., 1989; Eppink et al. 1998b). Unlike most NAD(P)H-dependent enzymes, PHBH lacks a βαβ-fold for binding the pyridine nucleotide (Eppink et al., 1997).

Figure 3. Ribbon structure of PHBH

Anaerobic reduction of substrate complexed enzyme crystals have led to a 2.3 Å structure of the reduced enzyme-substrate complex (Schreuder et al., 1992). This structure resembles the structure of the oxidized enzyme-substrate complex. Crystal structures have also been obtained of wild-type enzyme in complex with substrate analogues and of a number of mutant enzymes (Schreuder et al., 1994; Gatti et al., 1994; Lah et al., 1994; van Berkel et al., 1994; Eppink et al. 1995; 1998a; 1998b; 1999). The PHBH structure can be divided in three domains (Schreuder et al., 1989; Fig.3):

- Residues 1-175: the FAD binding domain with the N-terminal Rossmann fold for binding the ADP moiety of FAD (Wierenga et al. 1983; Wierenga et al. 1985).
- Residues 176-290: the substrate binding domain.
- Residues 291-394: the interface domain.

However, in recent structural classifications, PHBH is taken as a two-domain protein (Mattevi, 1998).

2.2.3 Structure and function

The substrate binding site of PHBH is deeply buried in the protein and built up of all three domains (Schreuder et al., 1989). Crystallographic studies suggest that the flavin ring swings out of the active site to allow substrate binding (Schreuder et al., 1994; Gatti et al., 1994). Fig. 4 presents a close up of the substrate binding site. Arg214 forms a indispensable ionic interaction with the carboxyl group of the substrate (van Berkel et al., 1992). Ser212 and Tyr222 are also involved in binding the carboxylic moiety. Hydroxylation of the aromatic substrate is facilitated by deprotonation of the phenolic moiety (Shoun et al., 1979; van Berkel and Müller, 1989; Vervoort et al., 1992; Ridder, 1998). The hydroxyl group of the substrate is at hydrogen bond distance of Tyr201 which contacts Tyr385. Selective Phe replacements of these tyrosine residues strongly hamper substrate deprotonation and flavin reduction (Entsch et al. 1991, Eschrich et al. 1993). Pro293 is part of the active site loop and presumably of structural importance (Schreuder et al., 1989). Substitution of Asn300 by Asp introduces a negative charge in the vicinity of the flavin. This causes an apparent completely suppression of substrate phenolate formation. Nevertheless, the Asn300Asp variant slowly converts the substrate without any uncoupling of hydroxylation (Palfey et al., 1994).

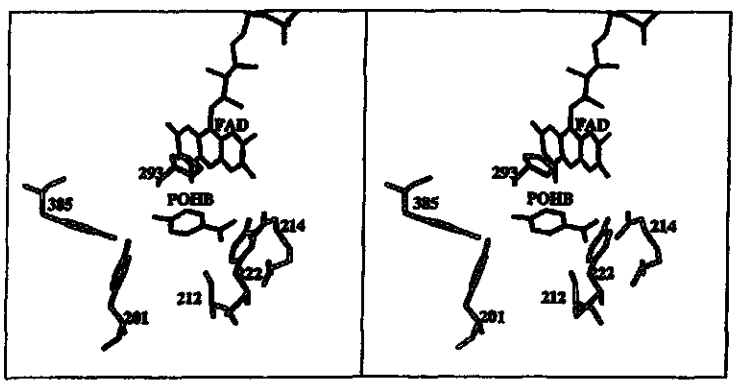


Figure 4. Stereoview of the substrate binding site of PHBH

2.2.4 Catalytic mechanism

The catalytic mechanism of PHBH has been studied by stopped-flow, absorbance and fluorescence spectroscopy (Entsch et al., 1976; Husain et al., 1979; Entsch and Ballou, 1989). The overall reaction can be divided into two half reactions, the reductive half reaction and the oxidative half reaction, each consisting of several reaction steps.

Reductive half reaction

The reductive half reaction involves ternary complex formation and leads to efficient reduction of the flavin cofactor. Two binding steps can be discriminated, although it should be emphasized that the schedule presented and discussed here, and also often encountered in the literature, includes sequential binding of substrate and NADPH. However, the actual order of binding of these two substrates may be random (Husain et al., 1979). The first step in the catalytic cycle involves the binding of the aromatic substrate:

$$EFl_{ox} + S \rightarrow EFl_{ox}S \quad (1)$$

Only in the presence of substrate fast reduction of the FAD cofactor by NADPH is observed (Nakamura et al. 1970; Howell et al., 1972). In the absence of substrate, a binary complex between NADPH and the enzyme results in a reduction rate that is 10^5 times slower. Thus, the substrate is an essential component in the reduction reaction because it acts as an effector highly stimulating the rate of flavin reduction, without being converted itself:

$$EFl_{ox}S + NADPH \rightarrow EFl_{red}H^{-}\text{-}S + NADP^{+} \quad (2)$$

NMR studies have demonstrated that the reduced flavin is in the anionic state, i.e with a deprotonated N1 (Vervoort et al., 1991). Only a few substrate analogs can stimulate the rate of flavin reduction and therefore the enzyme has a relatively narrow substrate specificity. For instance the enzyme is able to discriminate between 4-hydroxybenzoate, the parent substrate, and 4-aminobenzoate. Also some compounds are known that bind to the enzyme and elicit its fast reduction but are not converted, and are therefore called effectors. Although 4-aminobenzoate can actually bind to the enzyme, the reduction reaction in the presence of 4-aminobenzoate is very slow (Entsch et al. 1976), indicating a fine-tuning of the mechanism by which the substrate exerts its effector action. Recent studies have suggested a linkage between substrate deprotonation and flavin movement (Palfey et.al., 1999). However, the actual way in which the substrates and/or effectors increase the rate of reduction of the flavin by NADPH remains to be elucidated.

The fast release of $NADP^+$ from the reduced ternary complex is well established (Entsch et al. 1976; Husain et al., 1979; Eppink et al., 1995). Furthermore, it has been demonstrated that the rate of dissociation of the substrate from the reduced enzyme-substrate complex is 5000 times more slower than from the binary complex with the flavin in its oxidized state (Entsch et al.,1976). In addition to the increased reduction rate induced by substrate binding, the slow rate of substrate dissociation from the reduced enzyme is a second mechanism by which the enzyme controls the optimal use of valuable reducing equivalents.

Oxidative half-reaction

The oxidative half reaction includes the various reaction steps that lead to the hydroxylation of the substrate. The oxidative half reaction can be studied by reacting the anaerobic complex between the reduced enzyme and 4-hydroxybenzoate with oxygenated buffer. For this purpose, the enzyme is artificially reduced by dithionite or by photoreduction. In order to monitor the transient appearance of oxygenated flavin intermediates, the oxidative reaction is studied at low temperature, pH 6.5 either in the absence or in the presence of monovalent anions by the stopped flow technique (Entsch and Ballou, 1989). These studies revealed the existence of several oxygenated flavin intermediates, called Intermediate I, II and III (Entsch et al., 1976; Massey, 1994).

$$EFl_{red}H\text{-}S \quad + \quad O_2 \quad \rightarrow \quad EFlHOO\text{-}S \qquad (3)$$

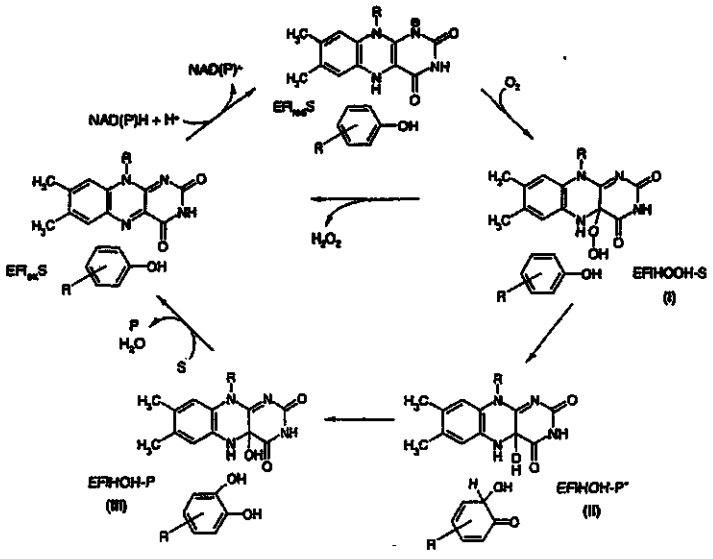
The first step in the oxidative half reaction is the binding of molecular oxygen to the $EFl_{red}H^-$-S complex. Upon reaction of the reduced flavin with molecular oxygen eventually the so-called flavin-peroxide intermediate ($EFlHOO^-$) is formed. Several authors have discussed the actual way in which this interaction can occur, since in principle the reaction between the singlet reduced flavin cofactor and the triplet molecular oxygen molecule is spin forbidden. Thus, it has been suggested that a one electron reduction of molecular oxygen by the flavin cofactor preceeds the actual formation of the $EFlHOO^-$-S complex. This implies that the $EFlHOO^-$-S complex results from the binding of a superoxide anion radical to the flavin semiquinone form of the cofactor (Müller, 1987). For PHBH the actual existence of the $EFlHOO^-$-S complex has at present not been unambiguously demonstrated. Its formation and inclusion in the catalytic cycle can be inferred from rapid kinetic experiments with an analogous enzyme, phenol hydroxylase (Maeda Yorita and Massey, 1993). Therefore, the actual formation of the C(4a)hydroperoxide intermediate (EFlHOOH-S) from its deprotonated flavinperoxy form ($EFlHOO^-$-S) might be considered as a separate step in the reaction cycle:

$$EFlHOO^-\text{-}S \quad + \quad H^+ \quad \rightarrow \quad EFlHOOH\text{-}S \qquad (4)$$

The EFlHOOH-S species is generally referred to as intermediate I and has been demonstrated in rapid kinetic and spectoroscopic studies with PHBH to have a typical absorption spectrum (Entsch et al 1976, Entsch et. al 1989). Due to the protonation of the distal oxygen of the peroxide moiety in the flavin peroxide cofactor the actual electrophilic reactivity of this cofactor can be expected to increase (Vervoort et al., 1997). Increased electrophilic reactivity of the flavin peroxide will facilitate its subsequent attack on the nucleophilic carbon centre of the substrate:

$$\text{EFlHOOH-S} \rightarrow \text{EFlHOH-P} \qquad (5)$$

This reaction step describes the step in which the activated, protonated C(4a)-hydroperoxy flavin form of the cofactor reacts with 4-hydroxybenzoate to form the product 3,4-dihydroxybenzoate and the flavin C(4a)-hydroxide (intermediate III). Initially the product is formed in its keto isomeric form (intermediate II; Schopfer et al., 1991; Maeda Yorita and Massey, 1993), which isomerizes to give the energetically favoured dihydroxy isomer, i.e. the 3,4-dihydroxybenzoate product. Evidence for the initial formation of the keto product comes from experiments with 2,4-dihydroxybenzoate as the substrate (Entsch et al.,1976; Wessiak et al., 1984; Entsch and Ballou, 1989). Several models for the structure of intermediate II have been postulated (Massey and Hemmerich, 1975; Wessiak et al, 1984; Anderson et al., 1987), but all the evidence presently available suggests that the highly absorbing intermediate II is a complex between the flavin C(4a)-hydroxide and the quinoid form of the aromatic product (Schopfer et al., 1991).



Scheme 1. Catalytic cycle of flavoprotein aromatic hydroxylases.

To increase the electron density on a certain carbon centre of the aromatic ring, the substrate needs to be activated by an already present *para* or *ortho* hydroxyl or amino group (van Berkel and Müller, 1991). In PHBH, the electron density on the C3 reaction centre of the substrate is further increased by deprotonation of the phenol. This idea is supported by spectroscopic binding studies (Shoun et al., 1979; van Berkel and Müller, 1989), by molecular orbital calculations on the reactivity of the substrate (Vervoort et al., 1992), and by reaction pathway calculations (Ridder et al., 1998). Moreover, the role of Tyr 201 and Tyr385 in substrate activation is well established (Entsch and van Berkel, 1995).

Substrate analogues which do not possess an activating electron donating group at the *para* position are not converted by PHBH (Spector and Massey, 1972). In the presence of such effector molecules, the flavin C(4a)-hydroperoxide decays to oxidized enzyme with release of hydrogen peroxide. The extent to which this uncoupling occurs is not only dependent on the nature and possibilities for activation of the substrate but is also influenced by the microenvironment of the active site. Other flavin dependent monooxygenases as well as mutant PHBH enzymes may vary in the efficiency of substrate hydroxylation.

$$EFlHOH\text{-}P \rightarrow EFl_{ox} + P + H_2O \qquad (6)$$

The final phase in the catalytic cycle is the elimination of water from the flavin C(4a)-hydroxide and the release of product from the enzyme. Some experimental evidence suggests that under certain conditions (pH 6.5 and presence of monovalent anions) or in the presence of particular substrate analogues, this step is rate limiting in catalysis. Studies with phenol hydroxylase have shown that in this step a dead-end complex can be formed between the flavin C(4a)-hydroxide form of the enzyme and the substrate leading to substrate inhibition (Maeda Yorita and Massey, 1993).

2.2.5 Reactivity with haloaromatic compounds

Halogenated organic compounds are one of the largest group of environmental pollutants, as a result of their widespread use as e.g. herbicides, insecticides, fungicides and intermediates for chemical synthesis (Gibson, 1968). Because of their toxicity, bioconcentration and persistence, halogenated compounds possibly cause negative effects on the quality of life. Research of the last decades reveal the identification of many halogenated natural products (Key et al.,1997). These natural halogenated products might well be important in the adaptation of micro-organisms to utilise xenobiotics.

A general rule for the adaptation of micro-organisms to transform or even to mineralise such compounds, is related to the number, type and position of the halogenated substituents (Fetzner et al. 1994); polyhalogenated compounds are more difficult degradable than the corresponding monohalogenated compounds (McAllister et al., 1996). The study of the biochemistry and genetics of microbial dehalogenases may help us to understand and evaluate the potential for degradation of xenobiotics in micro-organisms and in microcosm (Janssen et al., 1994). In the future, optimised or even newly designed enzymes might be used for the mineralisation of recalcitrant environmental pollutants, or even for the synthesis of novel environmentally friendly products.

PHBH can serve as a model enzyme to study the redesign of a flavoprotein aromatic hydroxylase into an efficient dehalogenase. Preliminary studies with fluorinated substrates have suggested that the actual type of reaction catalysed by PHBH appears to vary with the substituent at the centre to be hydroxylated (Husain et al., 1980). When the nucleophilic attack of the flavin C(4a)-hydroperoxide occurs at a halogenated centre, oxidative dehalogenation takes place. Due to the elimination of the halogen substituent as a halogen anion and the electron balance of the reaction the primary product formed can be characterised as a quinone product intermediate, which, only upon subsequent reduction with two additional electrons becomes chemically reduced to give the dihydroxy product. As a result, the oxidative dehalogenation of haloaromatic substrates by PHBH is expected to proceed with unusual reaction stoichiometry (Husain et al., 1980).

Several flavoprotein monooxygenases catalyse similar fortuitous dehalogenation reactions. Salicilate hydroxylase from *Pseudomonas putida* (Suzuki et al., 1991) and phenol hydroxylase from *Trichosporon cutaneum* (Peelen et al., 1995) catalyse the oxidative dehalogenation of 2-halophenols to the corresponding catechols. For the latter enzyme it was clearly demonstrated that the rate of substrate conversion decreases with increasing number of halogen substituents (Peelen et al., 1995). Recently, several microbial flavoprotein aromatic hydroxylases have been described which use a polyhalogenated aromatic compound as their physiological substrate (van Berkel et al., 1997). These enzymes, like e.g. pentachlorophenol hydroxylase (Xun et al. 1992), catalyse a *para*-hydroxylation reaction. However, their substrate specificity is far more restricted than that of heme-dependent cytochrome P450 enzymes which act as biological detoxificants in the liver (Porter and Coon, 1991; Guengerich, 1991; den Besten et al., 1993; Poulos, 1995; Rietjens et al., 1996; Oprea et al., 1997).

This thesis describes studies on the structure and function of PHBH. Special emphasis is given to the substrate and effector specificity and to the mechanism of oxidative dehalogenation of haloaromatic substrates.

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Chapter 3

Selective cysteine → serine replacements in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* allow the unambiguous assignment of Cys211 as the site of modification by spin-labeled *p*-chloromercuribenzoate

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Selective cysteine → serine replacements in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* allow the unambiguous assignment of Cys211 as the site of modification by spin-labeled *p*-chloromercuribenzoate

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***p*-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* contains five sulfhydryl groups per subunit. Cysteine → serine replacements show that the thiols are not essential for catalysis. The increased dissociation constant for FAD in mutant Cys158Ser suggests that Cys158 is important for the solvation of the pyrophosphate moiety of the prosthetic group. Wild-type *p*-hydroxybenzoate hydroxylase is rapidly inactivated by mercurial compounds. Inactivation by a spin-labeled derivative of *p*-chloromercuribenzoate is fully abolished in mutant Cys211Ser. Incorporation of the spin label in the other Cys → Ser mutants strongly impairs substrate binding without affecting the catalytic properties of the FAD. The results are discussed with respect to previous tentative assignments from chemical modification studies and in light of the 3-D structure of the enzyme–substrate complex.**

Key words: *p*-hydroxybenzoate hydroxylase/*Pseudomonas fluorescens*/site-specific mutagenesis/spin-labeling/thiol reactivity

Introduction

Since the pioneering work of Boyer (1954), *p*-chloromercuribenzoate and organomercurial analogs have been widely used as probes to study the reactivity and accessibility of protein sulfhydryl groups (Jocelyn, 1987). The high affinity and specificity of organomercurials for thiols also allows us to address the involvement of cysteine residues in enzyme catalysis. Such studies are of special relevance when the alterations observed are fully reversible upon reduction of the mercaptide bonds (van Berkel *et al.*, 1984). Chemical modification studies using bulky organomercurials have one major drawback. The loss of activity does not necessarily mean that the target cysteine residues are essential for catalysis.

For *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*, chemical modification studies have provided much insight into the dynamic properties of the free enzyme and enzyme–ligand complexes (van Berkel and Müller, 1991). *p*-Hydroxybenzoate hydroxylase is a member of the class of flavoprotein monooxygenases (van Berkel and Müller, 1991). The enzyme catalyzes the conversion of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil bacteria (Stanier and Ornston, 1973). *p*-Hydroxybenzoate hydroxylase from *P.fluorescens* contains five cysteines per subunit (Müller *et al.*, 1979; Weijer *et al.*, 1982). The 3-D structure of the enzyme–substrate complex shows that the cysteines are not directly involved in substrate hydroxylation (Weijer *et al.*, 1983; Schreuder *et al.*, 1989). Except for Cys116, all thiols are conserved in the other *p*-hydroxybenzoate hydroxylases sequenced so far (Weijer *et al.*, 1982; Entsch *et al.*, 1988; DiMarco *et al.*, 1993; Shuman and Dix, 1993). Covalent modification of Cys116 by *N*-ethylmaleimide (van Berkel *et al.*, 1984) or replacement of Cys116 by serine (Eschrich *et al.*, 1990) yields fully active enzymes resistant towards oxidation artefacts (van Berkel and Müller, 1987; van der Laan *et al.*, 1989; Eschrich *et al.*, 1990). The oxidation of Cys116 by air may explain why crystals of the enzyme–substrate complex soaked with *p*-chloromercuribenzoate show no well-defined electron density near this position (Weijer *et al.*, 1983).

p-Hydroxybenzoate hydroxylase is rapidly inactivated by mercurial compounds (van Berkel *et al.*, 1984). The inactivation shows saturation kinetics indicating that a complex is formed prior to the covalent modification of the enzyme (van Berkel *et al.*, 1984). The inactivation reaction is inhibited in the presence of the aromatic substrate and at high concentrations of the mercurial compound (van Berkel *et al.*, 1984). These data suggest a lesser accessibility of the target cysteine residue in the enzyme–substrate complex as compared with the free enzyme. The differences in reactivity are in accordance with the substrate-induced conformational changes observed by spectroscopic (Howell *et al.*, 1972; van Berkel and Müller, 1989) and crystallographic studies (Wierenga *et al.*, 1979; Schreuder *et al.*, 1991). These conformational changes may also explain why crystals of the free enzyme show poor resolution (Schreuder *et al.*, 1991).

From sequencing radioactive labeled tryptic peptides, Cys152 was tentatively assigned to react fairly specifically with a spin-labeled derivative of *p*-chloromercuribenzoate (van Berkel *et al.*, 1984). Reaction of Cys158 or Cys211, however, could not be excluded. The crystal structure shows that Cys152 is located far away from the active site and not in a position expected to be crucial for catalysis (Schreuder *et al.*, 1989). Crystals of the enzyme–substrate complex bind *p*-chloromercuribenzoate at positions which presumably are near Cys158, Cys211 and Cys332 (Weijer *et al.*, 1983). As no labeling of Cys332 is found with the free enzyme (van Berkel *et al.*, 1984), this again indicates that the substrate affects the accessibility of the protein sulfhydryl groups.

We describe here the properties of selective Cys → Ser mutant proteins. It is demonstrated that in the free enzyme, spin-labeled *p*-chloromercuribenzoate reacts specifically with Cys211 and not with Cys152. Spin labeling of Cys211 drastically diminishes the affinity for the aromatic substrate without affecting the catalytic properties of protein-bound FAD.

Materials and methods

Site-specific mutagenesis

Site-specific mutagenesis of the gene encoding *p*-hydroxybenzoate hydroxylase from *P.fluorescens* was performed according to the method of Kunkel *et al.* (1987), essentially as described elsewhere (van Berkel *et al.*, 1992). The oligonucleotides 5′-GCCTGGATTCCGACTACATC-3′ (Cys152Ser), 5′-CGCCGGCTCCGATGGCTTC-3′ (Cys158Ser), 5′-CGCCCTGTCCAGCCAGCG-3′

(Cys211Ser) and 5'-CGGCAATCTCCCTGCGGCG-3' (Cys332Ser) were used as primers for the construction of mutants. All mutations were introduced into the *Escherichia coli* gene encoding mutant Cys116Ser (Eschrich *et al.*, 1990). The catalytic properties and the crystal structure of this mutant are identical to wild-type (Eschrich *et al.*, 1990). All mutations were confirmed by nucleotide sequencing according to Sanger *et al.* (1977).

Enzyme purification

Mutant proteins were purified from *E.coli* TG2, essentially as described (van Berkel *et al.*, 1992). Expression and yield were comparable with that of enzyme Cys116Ser (Eschrich *et al.*, 1990).

Analytical methods

Standard activity measurements were performed at 25°C in 100 mM Tris–sulfate pH 8.0, containing 150 μM NADPH, 150 μM *p*-hydroxybenzoate and 10 μM FAD. Enzyme concentrations and kinetic parameters were determined as described (Eschrich *et al.*, 1993). The hydroxylation efficiency of mutant proteins was estimated from oxygen consumption experiments (Eschrich *et al.*, 1993). Dissociation constants of complexes between enzyme and substrate were determined fluorimetrically as described previously (Müller and van Berkel, 1982). Electron spin resonance spectra were recorded on a Bruker ER 200D spectrometer. The amount of incorporated spin label was measured by treating the modified enzymes with excess dithiothreitol (van Berkel *et al.*, 1984).

Chemical modification of sulfhydryl groups

Spin-labeled *p*-chloromercuribenzoate was synthesized by condensation of *p*-chloromercuribenzoylchloride with 4-amino-2,2,6,6-tetramethylpiperidinooxy (Zantema *et al.*, 1979). Chemical modification of mutant proteins by spin-labeled *p*-chloromercuribenzoate was performed at pH 7.0, essentially as described for wild-type (van Berkel *et al.*, 1984). Excess spin label was removed by gel filtration over Biogel P-6DG.

Results

Catalytic properties of mutant proteins

Table I shows that the cysteine residues of *p*-hydroxybenzoate hydroxylase from *P.fluorescens* are not essential for enzyme catalysis. All Cys → Ser mutants efficiently hydroxylate the substrate with k_{cat} and K_m values in the same range as that of wild-type. From this it can be concluded that the rapid inactivation of the enzyme by *p*-chloromercuribenzoate derivatives (van Berkel *et al.*, 1984) is due to the bulkiness of the aromatic group introduced. The Cys → Ser mutations hardly affect substrate binding. Flavin fluorescence titration experiments show that the dissociation constants for the complexes between the substrate and the mutants are in the same range as found for wild-type (K_d = 30 ± 10 μM, pH 7.0; van Berkel *et al.*, 1984). Mutant Cys158Ser binds FAD somewhat more weakly than wild-type. For this mutant the addition of excess FAD to the assay mixture is essential to achieve optimal turnover. From activity measurements varying the amount of flavin an apparent K_m for FAD of ~0.5 μM is estimated. This is about one order of magnitude higher than the value found for wild-type (Müller and van Berkel, 1982).

Chemical modification by spin-labeled p-chloromercuribenzoate

Two cysteine residues in *p*-hydroxybenzoate hydroxylase from *P.fluorescens* react with spin-labeled *p*-chloromercuribenzoate (van Berkel *et al.*, 1984). Cys116 reacts extremely rapidly with the spin label without loss of enzyme activity. Subsequently, a second cysteine reacts more slowly resulting in ~95% loss of activity. The inactivation shows saturation kinetics and is strongly inhibited by the presence of the substrate.

When the Cys → Ser mutants are incubated with spin-labeled *p*-chloromercuribenzoate, rapid inactivation is observed with mutants Cys152Ser, Cys158Ser and Cys332Ser (Figure 1). The rate of inactivation is highly comparable with that of wild-type (van Berkel *et al.*, 1984). In contrast, inactivation by spin-labeled *p*-chloromercuribenzoate is almost fully blocked in mutant Cys211Ser (Figure 1).

Table I. Kinetic parameters of Cys → Ser mutant proteins of *p*-hydroxybenzoate hydroxylase from *P.fluorescens*

Enzyme	Product (%)	k_{cat} (s^{-1})	K_m NADPH (μM)	K_m B_2OH (μM)
Cys116Ser	98	55	50	25
Cys152Ser	95	45	56	10
Cys158Ser	93	40	31	16
Cys211Ser	93	35	25	37
Cys332Ser	95	40	29	17

Turnover numbers are maximum values extrapolated to infinite concentrations of *p*-hydroxybenzoate and NADPH. Kinetic constants have maximal error values of 10%. B_2OH, *p*-hydroxybenzoate. Product, 3,4-dihydroxybenzoate.

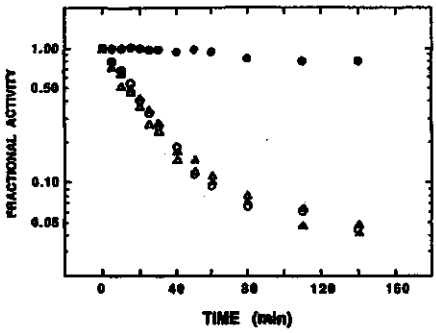

Fig. 1. Time-dependent inactivation of mutant *p*-hydroxybenzoate hydroxylase by spin-labeled *p*-chloromercuribenzoate. 1.6 μM mutant protein *in* 80 mM HEPES buffer, pH 7.0, I = 0.1 M, was incubated at 21°C in the presence of 50 μM spin-labeled *p*-chloromercuribenzoate. Mutant Cys152Ser (○); Cys158Ser (▲); Cys211Ser (●); Cys332Ser (△).

When spin-labeled mutants Cys152Ser, Cys158Ser and Cys332Ser (Figure 2) are characteristic for a moderately immobilized spin label (Zantema *et al.*, 1979). The EPR spectra of these mutants resemble that of spin-labeled *N*-ethylmaleimide-pretreated wild-type enzyme (van Berkel *et al.*, 1984). Quantitation of the amount of spin label incorporated in the mutants (Table II) confirms the fact that Cys211 is the main site of modification. With mutant Cys211Ser, the intensity of the integrated EPR signal is five times lower than for wild-type and characteristic of a relatively mobile spin label (Figure 2). This is not due to the presence of free label nor is the small fraction of mobile spin label observed in mutant Cys158Ser. Repeated gel filtration of the spin-labeled mutants does not change the shape of the EPR spectra. The small fraction of covalently bound mobile spin label in the mutants Cys211Ser and Cys158Ser, therefore, is due to modification of either Cys152 or Cys332.

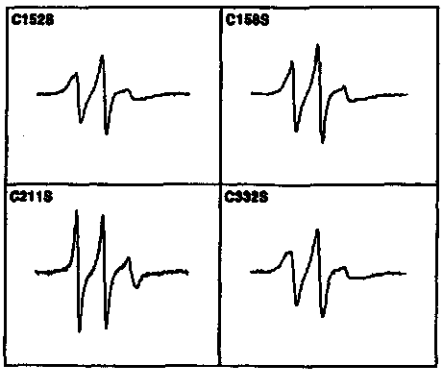

Fig. 2. EPR spectra of spin-labeled *p*-hydroxybenzoate hydroxylase mutant proteins. 50 μM mutant protein in 80 mM HEPES buffer, pH 7.0, *I* = 0.1 M, was incubated at 21°C in the presence of 150 μM spin-labeled *p*-chloromercuribenzoate for a period of 1 h. The incubation mixture was then passed over Biogel P-6DG prior to measurement. EPR conditions: microwave frequency, 9466 MHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.25 G; magnetic field, 3340–3440 G. The relative gain for the Cys211Ser spectrum is two times higher than for the other spectra.

Table II. Quantitation of incorporation of spin-labeled *p*-chloromercuribenzoate in *p*-hydroxybenzoate hydroxylase

Enzyme	Spin label incorporated (mol/mol FAD)
Cys116Ser	1.00 ± 0.05
Cys152Ser	1.01 ± 0.05
Cys158Ser	1.25 ± 0.06
Cys211Ser	0.20 ± 0.01
Cys332Ser	1.01 ± 0.05

Spin-labeled mutants (Figure 2) were reactivated with an excess of dithiothreitol. The amount of spin label released was then determined by comparison of doubly integrated EPR spectra with that of a known solution of the free spin label.

Table III. Kinetic parameters of Cys → Ser mutant proteins of *p*-hydroxybenzoate hydroxylase as treated with spin-labeled *p*-chloromercuribenzoate

Enzyme	Product (%)	k_{cat} (s^{-1})	K_m NADPH (μM)	K_m B_2OH (μM)
Cys116Ser	96	55	76	1800
Cys152Ser	96	48	107	1800
Cys158Ser	96	43	89	1500
Cys211Ser	95	35	86	30
Cys332Ser	95	45	87	1500

For experimental details see footnote to Table I.

Properties of spin-labeled mutant proteins

Modification of wild-type *p*-hydroxybenzoate hydroxylase by spin-labeled *p*-chloromercuribenzoate strongly impairs substrate binding (van Berkel *et al.*, 1984). The spin-labeled mutants Cys152Ser, Cys158Ser and Cys332Ser also show a weak affinity for the substrate as judged by fluorescence titration experiments (K_d > 1 mM, pH 7.0). The affinity of the substrate for mutant Cys211 → Ser is not changed by the spin labeling procedure (K_d = 30 μM, pH 7.0). Binding of the substrate to this modified enzyme results in a 75% decrease of flavin fluorescence. This percentage of quenching is in the same range as found for the non-spin label-treated Cys → Ser mutants (not shown).

The spin-labeled mutants Cys152Ser, Cys158Ser and Cys332Ser show ~5% of residual activity in the standard assay (Figure 1). The activity of the spin-labeled mutants strongly increases upon raising the concentration of *p*-hydroxybenzoate. Table III shows that the k_{cat} values for the spin-labeled mutants are in the same range as those found for the untreated mutants (cf. Table I). The high K_m for *p*-hydroxybenzoate for the spin-labeled mutants Cys152Ser, Cys158Ser and Cys332Ser (Table III) is in agreement with the fluorescence binding studies. This confirms that substrate binding is strongly affected by spin labeling of Cys211. In a previous study (van Berkel *et al.*, 1984), the residual activity of spin-labeled wild-type enzyme was tentatively assigned to the presence of a small fraction of native enzyme. The kinetic parameters recorded in Table III, however, show that the low activity of the spin-labeled mutant proteins in the standard assay (Figure 1) results from impaired substrate binding. Covalent binding of the spin label only slightly affects the K_m for NADPH (Table III). The formation of >90% of product (Table III) shows that reduction of the spin-labeled mutant proteins by NADPH is fully coupled to substrate hydroxylation. This is in accordance with an earlier conclusion (van Berkel *et al.*, 1984) that the covalently bound spin label points with its side chain away from the active site. Studies with other mutants have shown that perturbation of the active site strongly influences catalysis (Entsch *et al.*, 1991; van Berkel *et al.*, 1992; Eschrich *et al.*, 1993).

Discussion

The kinetic parameters of Cys → Ser mutants as presented in this paper show that the cysteine residues of *p*-hydroxybenzoate hydroxylase from *P. fluorescens* are not essential for enzyme catalysis. Mutant Cys158Ser shows a decreased affinity for FAD. This weaker interaction can be rationalized by inspection of the 3-D model of the enzyme–substrate complex (Schreuder *et al.*, 1989). The high resolution crystal structure shows that the carbonyl oxygen of Cys158 interacts with two buried water molecules located in hydrogen bond distance of the pyrophosphate moiety of the FAD. Replacement of Cys158 by Ser may disturb the configuration of this hydrogen bond network, thereby influencing the solvation of the pyrophosphate group.

Modification of one cysteine residue by spin-labeled *p*-chloromercuribenzoate is responsible for the almost complete loss of enzyme activity (van Berkel *et al.*, 1984). The target cysteine, however, could not be unambiguously assigned, most probably due to reshuffling of mercaptide bonds upon unfolding of the modified enzyme (van Berkel *et al.*, 1984). By protein engineering it has now been demonstrated clearly that Cys211 is the main site of modification. Inactivation by spin-labeled *p*-chloromercuribenzoate is fully blocked in mutant Cys211Ser and only 0.2 mol of spin label is incorporated per mol enzyme.

The kinetic parameters of the spin-labeled mutants Cys152Ser, Cys158Ser and Cys332Ser and the fluorescence binding studies show that inactivation of these enzymes results from impaired substrate binding. The crystal structure of the enzyme–substrate complex shows that Cys211 is located in strand B5 of the substrate binding domain (Schreuder *et al.*, 1989). This strand also comprises residues Ser212 and Arg214 directly involved in binding the carboxylic moiety of the substrate. The side chain of Cys211 points away from the active site at the opposite side of the β-sheet and is not involved in substrate binding. The

properties of mutant Cys211Ser show that Cys211 is not critical for providing the correct interactions between the substrate and strand B5. Based on chemical modification data alone, one might have argued that Cys211 would assist substrate binding. Introduction of the bulky 1.4 nm-long spin-labeled benzoate derivative may disturb the active site geometry by pushing strand B5 towards the flavin or a flip of Cys211 may cause the spin label to occupy the active site. Another more likely explanation is that labeling of Cys211 inhibits the access of the substrate to the active site. As noted before (van Berkel *et al.*, 1984), the covalently bound spin label is located presumably in a cleft or close to the protein surface and far away from the active site. This is in accordance with the present observation that the spin-labeled mutants are fully capable of coupling flavin reduction to substrate hydroxylation. Entrance to the substrate-binding pocket seems to be reached via a cleft between the FAD-binding domain and the substrate-binding domain (Weijer *et al.*, 1983). Entrance to the NADPH-binding pocket is far more unclear (Weijer *et al.*, 1983; van Berkel *et al.*, 1988). The location of the spin label may therefore help in the understanding of how the substrate and NADPH enter the active site. Unfortunately, no good quality crystals of spin-labeled enzyme have been obtained so far. A possible reason is the weak binding of the substrate, preventing the change in domain interactions (Wierenga *et al.*, 1979; Weijer *et al.*, 1983).

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Chapter 4

Role of Tyr201 and Tyr385 in substrate activation by *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

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Role of Tyr201 and Tyr385 in substrate activation by *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

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The crystal structure of the enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* shows that the hydroxyl group of 4-hydroxybenzoate interacts with the side chain of Tyr201, which is in close contact with the side chain of Tyr385. The role of this hydrogen bonding network in substrate activation was studied by kinetic and spectral analysis of Tyr→Phe mutant enzymes.

The catalytic properties of the enzymes with Tyr201 or Tyr385 replaced by Phe (Tyr201→Phe and Tyr385→Phe) with the physiological substrate are comparable with those of the corresponding mutant proteins of *p*-hydroxybenzoate hydroxylase from *P. aeruginosa* [Entsch, B., Palfey, B. A., Ballou, D. P. & Massey, V. (1991) *J. Biol. Chem.* 266, 17341–17349]. Enzyme Tyr201→Phe has a high K_m for NADPH and produces only 5% of 3,4-dihydroxybenzoate/catalytic cycle. Unlike the wild-type enzyme, the Tyr201→Phe mutant does not stabilize the phenolate form of 4-hydroxybenzoate. With enzyme Tyr385→Phe, flavin reduction is rate-limiting and the turnover rate is only 2% of wild type. Despite rather efficient hydroxylation, and deviating from the description of the corresponding *P. aeruginosa* enzyme, mutant Tyr385→Phe prefers the binding of the phenolic form of 4-hydroxybenzoate.

Studies with substrate analogs show that both tyrosines are important for the fine tuning of the effector specificity. Binding of 4-fluorobenzoate differentially stimulates the stabilization of the 4*a*-hydroperoxyflavin intermediate. Unlike wild type, both Tyr mutants produce 3,4,5-trihydroxybenzoate from 3,4-dihydroxybenzoate. The affinity of enzyme Tyr201→Phe for the dianionic substrate 2,3,5,6-tetrafluoro-4-hydroxybenzoate is very low, probably because of repulsion of the substrate phenolate in a more nonpolar microenvironment.

In contrast to data reported for *p*-hydroxybenzoate hydroxylase from *P. aeruginosa*, binding of the inhibitor 4-hydroxycinnamate to wild-type and mutant proteins is not simply described by binary complex formation. A binding model is presented, including secondary binding of the inhibitor. Enzyme Tyr201→Phe does not stabilize the phenolate form of the inhibitor. In enzyme Tyr385→Phe, the phenolic pK_a of bound 4-hydroxycinnamate is increased with respect to wild type. It is proposed that Tyr385 is involved in substrate activation by facilitating the deprotonation of Tyr201.

p-Hydroxybenzoate hydroxylase is the paradigm of the class of flavoprotein aromatic hydroxylases (Van Berkel and Müller, 1991). The enzyme catalyzes the conversion of 4-hydroxybenzoate to 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil bacteria (Stanier and Ornston, 1973). For *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*, high-resolution crystal structures of the enzyme-substrate, enzyme-product and reduced enzyme-substrate complex are available (Schreuder et al., 1991). The highly similar genes encoding *p*-hydroxybenzoate hydroxylase from *P. aeruginosa* (Entsch et al., 1988) and *P. fluorescens* (Van Berkel et al., 1992) have been cloned and expressed in *Escherichia coli* and the properties of a set of mutated enzymes are known (Eschrich et al., 1990; Entsch et al., 1991a, b; Van Berkel et al., 1992).

Like most other flavoprotein aromatic hydroxylases, *p*-hydroxybenzoate hydroxylase shows a rather narrow substrate specificity (Husain et al., 1980). Scheme 1 shows the reaction cycle, as deduced from stopped-flow techniques (Entsch et al., 1976; Entsch and Ballou, 1989). The substrate acts also as an effector, strongly stimulating the rate of flavin reduction by NADPH (Hosokawa and Stanier, 1966). After $NADP^+$ release, the reduced enzyme-substrate complex reacts with molecular oxygen forming a labile covalent 4*a*-hydroperoxyflavin adduct. This intermediate then attacks the substrate under formation of 4*a*-hydroxyflavin and product. Finally, the 4*a*-hydroxyflavin is rapidly dehydrated and product is released. The exact mechanism of hydroxylation remains to be elucidated (Merényi et al., 1991; Entsch et al.,

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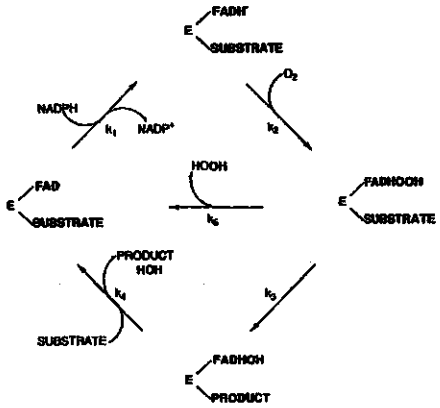
Enzymes. p-Hydroxybenzoate hydroxylase (EC 1.14.13.2); catalase, hydrogen-peroxide oxidoreductase (EC 1.11.1.6); glucose oxidase, β-D-glucose:oxygen 1-oxidoreductase (EC 1.1.3.4); glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP 1-oxidoreductase (EC 1.1.1.49).



Scheme 1. Reaction cycle of *p*-hydroxybenzoate hydroxylase. $FADH^-$ = reduced flavin (adenine dinucleotide). FADHOOH = reduced 4*a*-hydroperoxyflavin. FADHOH = reduced 4*a*-hydroxyflavin.

1991a). In the presence of a non-hydroxylatable effector like 6-hydroxynicotinate (Howell et al., 1972), the presumed 4*a*-hydroperoxyflavin intermediate decays to oxidized enzyme with the production of stoichiometric amounts of hydrogen peroxide (reaction k_6) (Entsch et al., 1976).

Crystallographic data provide a detailed insight into the conformation of the substrate binding site. The carboxylic moiety of the substrate forms a salt bridge with the side chain of Arg214 (Weijer et al., 1983). Studies of Arg214-mutated enzymes have shown that this salt bridge is essential for tight binding and correct orientation of the substrate, thereby increasing the stabilization of the transient 4*a*-hydroperoxyflavin (Van Berkel et al., 1992). The side chains of Tyr201 and Tyr385 form a hydrogen bonding network with the 4-hydroxy moiety of the substrate (Schreuder et al., 1990). Chemical modification of either one or both of these tyrosine residues abolishes the affinity of the enzyme for 4-hydroxybenzoate (Wijnands et al., 1986). Rapid reaction studies on Tyr→Phe mutants from *P. aeruginosa* (Entsch et al., 1991b) have confirmed an earlier proposal (Van Berkel and Müller, 1989) that Tyr201 activates the substrate for efficient hydroxylation. Furthermore, it was shown that both mutations decrease the rate of enzyme reduction. We here describe the catalytic and binding properties of Tyr201→Phe and Tyr385→Phe mutated *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. As expected from the strongly conserved amino acid sequences (Entsch et al., 1988), the catalytic properties of these mutants are highly comparable with those of the *P. aeruginosa* Tyr→Phe mutant proteins. From binding studies, we present here a refined working hypothesis for the role of Tyr201 and Tyr385 in substrate activation. Some preliminary results have been reported elsewhere (Westphal et al., 1991).

MATERIALS AND METHODS

General

Restriction endonucleases, DNA polymerase I (Klenow fragment) and T4-DNA ligase were from Bethesda Research Laboratories. Universal M13 sequencing primer, dNTPs, 2',3'-dideoxy-nucleoside 5'-triphosphates (ddNTP), calf intestinal phosphatase, glucose oxidase (grade II), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, glucose 6-phosphate, NADPH and dithiothreitol were all products of Boehringer. [α-^{32}P]dATP (3000 Ci/mol) was obtained from New England Nuclear. Oligonucleotides for mutagenesis were prepared using an Applied Biosystems DNA synthesizer. Mes, Hepes, Hepps and Cibacron-blue-3GA–agarose (type 3000-CL) were from Sigma. QAE-Sepharose fast-flow was from Pharmacia/LKB.

4-Hydroxybenzoate and substrate analogues were purchased from Aldrich. 4-Hydroxycinnamic acid (predominantly *trans*) was from Janssen Chimica. 2,3,5,6,-Tetrafluoro-4-hydroxybenzoate was prepared from pentafluorobenzoate as decribed (Husain et al., 1980).

Mutagenesis

E. coli TG2 (pAW45), containing the gene encoding *p*-hydroxybenzoate hydroxylase from *P. fluorescens*, has been described elsewhere (Van Berkel et al., 1992). Site-directed mutagenesis, using *E. coli* RZ1032 for generation of uracil-containing single-stranded DNA, was performed in the bacteriophage M13mp18 according to the method of Kunkel et al. (1987).

The oligonucleotides 5'-CGAACTGATCTTCGCCAACCATC (Tyr201→Phe) and 5'-CCGAGAACTTGTCGGCCTG (Tyr385→Phe) were used for the construction of mutants. Both mutations were introduced into the *E. coli* gene encoding the microheterogeneity-resistant Cys116→Ser mutant enzyme (Eschrich et al., 1990; Van Berkel et al., 1992). The mutations were confirmed by nucleotide sequencing using the M13 dideoxy-chain-termination method of Sanger et al. (1977).

Mutated *p*-hydroxybenzoate hydroxylase genes were expressed in transformed *E. coli* TG2 grown in 6-l batches of tryptone/yeast medium containing 75 μg/ml ampicillin at 37°C with vigorous aeration (Westphal and de Kok, 1988).

Mutated enzymes were purified from *E. coli* TG2, essentially as described (Van Berkel et al., 1992). The expression and yield of the enzymes Tyr201→Phe and Tyr385→Phe is comparable to wild-type and enzyme Cys116→Ser. For convenience, and in view of identical catalytic properties (Eschrich et al., 1990), enzyme Cys116→Ser will be referred to as wild-type enzyme.

Analytical methods

HPLC analysis was done on a Hewlett Packard 1081 B high-pressure liquid chromatography system using a Zorbax ODS C-18 (100×3 mm) column. The liquid phase consisted of a mixture of 20% methanol and 1% acetic acid. Detection varied over 260–300 nm using a Pye Unicam LC-UV detector with 8-mm light path.

^{1}H-NMR experiments were performed on a Bruker AMX 500-MHz spectrometer. Samples were dissolved in deuterated dimethylsulfoxide.

4-Hydroxycinnamic acid was purified before use by preparative HPLC using a Lichrosorb 10 RP-18 (250×9 mm) column. In each run, 10 mg 4-hydroxycinnamic acid was purified isocratically using a mixture of 25% methanol and 1% acetic acid. The flow rate was 2 ml/min and 5-ml fractions were collected. Purified 4-hydroxycinnamic acid eluted on analytical HPLC as a single symmetrical peak and con-

tained more than 98% of the *trans*-isomer as evidenced by ^{1}H-NMR.

Absorption spectra were recorded at 25°C on an automated Aminco DW-2000 spectrophotometer. Enzyme concentrations were determined spectrophotometrically using a molar absorption coefficient $\varepsilon_{450} = 10.2\ mM^{-1}\ cm^{-1}$ for protein-bound FAD (Van Berkel et al., 1992).

p-Hydroxybenzoate hydroxylase activity was routinely assayed spectrophotometrically at 340 nm by measuring the oxidation of NADPH in 100 mM Tris/sulfate pH 8.0 and 25°C (Müller et al., 1979). Steady-state kinetics were performed at 25°C in air-saturated 100 mM Tris/sulfate pH 8.0 or 50 mM potassium phosphate, pH 7.0, both containing 0.5 mM EDTA and 10 μM FAD. The concentration of 4-hydroxybenzoate or NADPH was varied using 0.2 mM of the fixed substrate. The hydroxylation efficiency of the mutated enzymes in the above-mentioned mixtures was estimated from oxygen consumption experiments using 0.2 mM NADPH and 1 mM of aromatic substrate (s), performed either in the absence or presence of 150 units catalase (Entsch et al., 1976). Product analysis, using a NADPH-regenerating system, was done by either analytical HPLC (Entsch et al., 1991b) or recording absorption spectra (Husain et al., 1980).

Rapid-reaction kinetics were carried out using a temperature-controlled High-Tech Scientific SF-51 spectrophotometer (dead time, 1.3 ms). The instrument was interfaced to an IBM microcomputer for data acquisition and analysis, using software from High-Tech Scientific Inc. All experiments were performed anaerobically at 15°C in 50 mM potassium phosphate pH 7.0, containing 0.5 mM EDTA, essentially as described (Entsch et al., 1976; Husain and Massey, 1979). In flavin reduction experiments, 25 units glucose oxidase and 20 mM glucose were added in order to achieve optimal anaerobiosis. Flavin reduction was monitored at 450 nm, either in the absence or presence of saturating concentrations of substrate/effector and varying concentrations of NADPH. Second-order rate constants and dissociation constants were determined from nonlinear least-square analysis (Van Berkel et al., 1992). In flavin reoxidation experiments, protein-bound FAD was anaerobically reduced by a small excess of sodium dithionite (Entsch et al., 1976).

Fluorescence-emission spectra were recorded on an automated Aminco SPF-500C spectrofluorimeter. Dissociation constants of enzyme/ligand complexes were determined fluorimetrically at pH 7.0, essentially as reported earlier (Van Berkel and Müller, 1989). Binding studies as a function of pH were performed in 40 mM Mes (pH 5–7), 40 mM Hepes (pH 7–8) and 40 mM Hepps (pH 8–9). Buffers were adjusted to 50 mM ionic strength with 0.5 M sodium sulfate as described before (Wijnands et al., 1984).

The ionization state of enzyme-bound 4-hydroxybenzoate was measured by recording absorption spectra as a function of pH (Shoun et al., 1979). The length of the two-compartment cell used was 0.875 cm. The ionization state of enzyme-bound 4-hydroxycinnamate was measured at pH 8.0 and 25°C by recording absorption spectra of 20 μM enzyme in the absence or presence of 4-hydroxycinnamate (Entsch et al., 1991b). Both sample and reference cell were titrated with known concentrations of the reagent, diluted in pH 8.0. Deprotonation of enzyme-bound 4-hydroxycinnamate was monitored either at 353 nm (enzyme Cys116→Ser) or 370 nm (enzyme Tyr385→Phe) using an LKB Ultrospec III spectrophotometer. All measurements were corrected for the increase in absorption of samples titrated in the absence of enzyme and for dilution. All data from binding studies were evaluated by a singular-value-decomposition-based analysis algorithm using the FORTRAN *Numerical Recipes* subroutine package (Press et al., 1986) of KALEIDAGRAPH (Adelbeck Software).

Table 1. Dissociation constants of some complexes between mutant *p*-hydroxybenzoate hydroxylase and benzoate ligands. Dissociation constants were determined at 25°C in 50 mM potassium phosphate pH 7.0 by fluorimetric titration experiments, essentially as described (Van Berkel et al., 1992). The fluorescence quantum yield (Q) is expressed relative to that of free enzyme (100%). Q was determined relative to free FAD.

Ligand	Dissociation constant (fluorescence quantum yield) of the complex		
	Cys116→Ser (Q = 0.6)	Tyr201→Phe (Q = 0.73)	Tyr385→Phe (Q = 0.72)
	μM (%)		
Benzoate	156 (33)	20 (19)	34 (60)
4-Hydroxybenzoate	30 (20)	33 (32)	11 (20)
4-Aminobenzoate	16 (9)	36 (76)	35 (14)
4-Fluorobenzoate	120 (39)	28 (18)	44 (52)
2,4-Dihydroxybenzoate	89 (11)	78 (13)	28 (12)
3,4-Dihydroxybenzoate	284 (10)	845 (18)	150 (12)
2,3,5,6-Tetrafluoro-4-hydroxybenzoate	300 (42)	2550 (14)	250 (42)

RESULTS

General properties of mutant proteins

The flavin visible absorption spectra of the free and 4-hydroxybenzoate complexed enzymes Tyr201→Phe and Tyr385→Phe closely resemble that of wild type (Van Berkel and Müller, 1989), indicating that the mutations cause no major structural changes. For enzyme Tyr385→Phe from *P. aeruginosa* it was reported that the crystal structure of the enzyme-substrate complex is the same as for wild type (Entsch et al., 1991b).

In the oxidized state, binding of benzoate analogues results in quenching of the fluorescence of protein-bound FAD. This property was used for the estimation of the dissociation constants of the enzyme-benzoate ligand complexes. Table 1 shows that the affinity for 4-hydroxybenzoate is not strongly influenced by the mutations. Most benzoate analogues tested interact somewhat more strongly with enzyme Tyr385→Phe than with wild type (Table 1). This may be caused by the more nonpolar microenvironment. Enzyme Tyr201→Phe shows a more divergent binding behaviour. The more apolar analogues benzoate and 4-fluorobenzoate strongly interact with this mutant whereas the affinity of the more polar analogues 3,4-dihydroxybenzoate and 2,3,5,6-tetrafluoro-4-hydroxybenzoate is considerably decreased with respect to wild type. The results obtained with the tetrafluoro compound (phenolic pK_a free in solution = 5.3) indicate that the interaction of the phenolate form of substrates with the introduced phenylalanine side chain of enzyme Tyr201→Phe is unfavored energetically.

Binding of NADPH to the free enzyme is hardly affected by the mutations. At pH 7.0 (I = 50 mM), both the dissociation constants (about 250 μM) and the fluorescence

Table 2. Kinetic parameters and hydroxylation efficiency of mutant *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. All experiments were performed at 25°C in air-saturated 50 mM potassium phosphate pH 7.0 or 100 mM Tris/sulfate pH 8.0, containing 0.5 mM EDTA, 10 μM FAD. Turnover numbers (k_{cat}) are maximum values extrapolated to infinite concentrations of 4-hydroxybenzoate and NADPH. For other details see Materials and methods. BzOH, 4-hydroxybenzoate. Product, 3,4-dihydroxybenzoate.

Enzyme	pH	Product	k_{cat}	K_m	
				NADPH	BzOH
		%	s^{-1}	μM	
Cys116→Ser	7.0	100	37	73	20
	8.0	100	55	50	25
Tyr201→Phe	7.0	5	2.5	390	30
	8.0	5	6.5	1300	55
Tyr385→Phe	7.0	75	0.6	18	8
	8.0	78	0.9	15	15

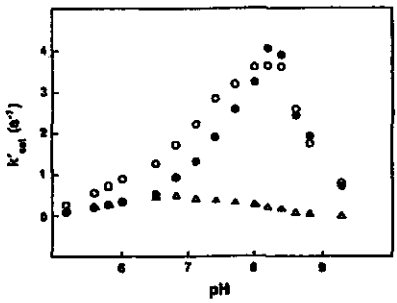


Fig. 1. pH-dependent activity of mutant *p*-hydroxybenzoate hydroxylase from *P. fluorescens* with 3,4-dihydroxybenzoate. All experiments were performed at 25°C in air-saturated buffers containing 0.3 mM NADPH and 1 mM 3,4-dihydroxybenzoate. For buffers used see Materials and methods. Relative activity of enzyme Cys116→Ser (○), enzyme Tyr201→Phe (△) and enzyme Tyr385→Phe (●).

quantum yield of the enzyme-NADPH complexes are in the same range as found for wild type (Wijnands et al., 1984). This is not unexpected because the adenosine 2',5'-bisphosphate moiety of the coenzyme is believed to be located far away from the substrate binding site (Van Berkel et al., 1988).

Catalytic properties

The catalytic properties of the mutated enzymes differ from wild type. At pH 8.0, the optimum pH for turnover of the wild-type enzyme (Van Berkel and Müller, 1989), the turnover rate of enzyme Tyr201→Phe is about 10% of that of wild-type and the K_m for NADPH is relatively high (Table 2). The turnover rate of enzyme Tyr201→Phe is much higher than the value reported for the corresponding enzyme from *P. aeruginosa*. For the Tyr→Phe mutants from *P. aeruginosa*, however, no maximal rates and K_m values were estimated (Entsch et al., 1991b). Table 2 also shows that K_m for NADPH for enzyme Tyr201→Phe is more pH dependent than for wild type (see also below). Replacement of Tyr201 with Phe decreases the efficiency of hydroxylation. With this mutant, 4-hydroxybenzoate mainly acts as an effector and only 5% of the product 3,4-dihydroxybenzoate is formed/catalytic cycle (Table 2).

The turnover rate of enzyme Tyr385→Phe is only 2% of wild type (Table 2). In contrast to enzyme Tyr201→Phe, for this mutant K_m for NADPH is somewhat decreased as compared to wild type. With enzyme Tyr385→Phe, relatively efficient hydroxylation occurs and about 75% of 3,4-dihydroxybenzoate is produced/catalytic cycle. The values for hydroxylation are in good agreement with those reported for the corresponding enzymes from *P. aeruginosa* (Entsch et al., 1991b).

Under NADPH-regenerating conditions, enzyme Tyr 385→Phe from *P. aeruginosa* catalyzes the conversion of the product of the physiological reaction, 3,4-dihydroxybenzoate to the cytotoxic 3,4,5-trihydroxybenzoate (Entsch et al., 1991b). We have studied the reaction of the Tyr mutants from *P. fluorescens* with 3,4-dihydroxybenzoate in somewhat more detail. Especially at high pH values, 3,4-dihydroxybenzoate is a relatively good effector for enzyme Tyr385→Phe (Fig. 1). Under standard assay conditions, the apparent turnover rate is comparable to wild type (Fig. 1). In contrast to wild type, enzyme Tyr385→Phe partially hydroxylates 3,4-dihydroxybenzoate, yielding 3,4,5-trihydroxybenzoate as a product. The efficiency of hydroxylation is relatively low and only slightly dependent on pH. Oxygen-consumption experiments, performed either in the absence or presence of catalase, showed that about 20% of 3,4,5-trihydroxybenzoate is produced/catalytic cycle. This value is in full agreement with that reported for the corresponding *P. aeruginosa* enzyme. Hydroxylation of 3,4-dihydroxybenzoate is also catalyzed by enzyme Tyr201→Phe. Under standard assay conditions, both HPLC and ultraviolet spectral analysis showed the exclusive formation of 3,4,5-trihydroxybenzoate as a product. With enzyme Tyr201→Phe, inefficient catalysis occurs (Fig. 1), partly as a result of the high dissociation constants for 3,4-dihydroxybenzoate and NADPH (cf. Table 1 and Table 3). The percentage of hydroxylation is rather low and varies between 5% and 15%. These values, however, are significantly

Table 3. Effector specificity of mutant *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. All experiments were performed at 15°C in 50 mM potassium phosphate, pH 7.0. Flavin reduction was monitored at 450 nm by conventional or stopped-flow absorption spectroscopy, either in the absence or presence of saturating concentrations of effector and varying concentrations of NADPH. Rate constants and dissociation constants were determined from non-linear least-square analysis as described (Van Berkel et al., 1992).

Effector	Reduction rate (K_s NADPH) of the complex		
	Cys116→Ser	Tyr201→Phe	Tyr385→Phe
	s^{-1} (mM)		
None	0.003 (0.26)	0.01 (0.62)	0.01 (0.81)
4-Hydroxybenzoate	122 (0.25)	3.4 (0.87)	0.53 (0.16)
2,4-Dihydroxybenzoate	0.61 (0.06)	0.03 (0.42)	0.12 (0.15)
3,4-Dihydroxybenzoate	3.3 (0.75)	1.1 (1.70)	0.33 (0.03)
4-Aminobenzoate	0.03 (0.40)	1.0 (4.0)	0.02 (2.0)
4-Fluorobenzoate	0.50 (1.50)	0.61 (0.21)	0.06 (0.46)

higher than observed for wild type (<1%). This implies that with both Tyr mutants, and in contrast to wild type (Entsch et al., 1976), binding of 3,4-dihydroxybenzoate induces the stabilization of the 4*a*-hydroperoxyflavin intermediate. The above results also show that 3,4-dihydroxybenzoate can become orientated in both Tyr mutants in such a way that hydroxylation at the 5′-position is feasible. The X-ray structure of the enzyme-product complex indicates that in the wild-type enzyme the orientational freedom of the product is restricted by hydrogen bonding of the 3′-hydroxyl group of the product with the carbonyl oxygen of Pro293 (Schreuder et al., 1989).

p-Hydroxybenzoate hydroxylase shows a narrow effector specificity (Spector and Massey, 1972). Except for the physiological substrate, only fluorinated 4-hydroxybenzoate derivatives strongly stimulate the rate of enzyme reduction (Husain et al., 1980). The potential substrate *p*-aminobenzoate is very slowly hydroxylated by the wild-type enzyme because flavin reduction is rate-limiting (Entsch et al., 1976). This points to a direct role of Tyr201 and/or Tyr385 in the regulation of the effector specificity. We have compared the effector specificity properties of wild type and Tyr mutants by performing flavin reduction experiments in the absence and presence of some substrate analogs. Binding of 4-hydroxybenzoate stimulates the rate of reduction of enzymes Tyr201→Phe and Tyr385→Phe by NADPH to a much smaller degree than observed with wild type (Table 3) and in accordance with data reported for the corresponding enzymes from *P. aeruginosa* (Entsch et al., 1991b). Reduction of enzyme Tyr385→Phe is rate-limiting in overall catalysis (cf. Table 2). The rate of reduction of enzyme Tyr201→Phe in complex with 4-hydroxybenzoate is stimulated 300-fold when compared to the free enzyme (Table 3). Under the same conditions, reduction of wild-type enzyme is still 40 times faster. The apparent K_d for NADPH for the ternary complex of enzyme Tyr201→Phe is relatively high and in accordance with the high K_m for NADPH obtained from steady-state kinetics. It should be noted here that the apparent K_d for NADPH reflects the ratio of k_{-m} and k_{red} for NADPH in the enzyme-substrate complex. As noted above, fluorescence quenching studies indicate that the affinity for NADPH is not changed in mutant Tyr201→Phe. In the presence of substrate analogs no stimulation of enzyme reduction is observed (Table 3). For enzyme Tyr201→Phe in complex with 4-aminobenzoate, the rate of enzyme reduction is increased. The efficiency of reduction of this complex is, however, low in view of the high apparent K_d for NADPH.

Stabilization of the 4*a*-hydroperoxyflavin intermediate is essential for the efficient hydroxylation of substrates. This stabilization is strongly influenced by the type of substrate/effector present in the active site (Entsch et al., 1976; Entsch and Ballou, 1989). In order to probe the effect of the mutations on the stabilization of the 4*a*-hydroperoxyflavin, the reaction of oxygen with the reduced enzymes was studied in the presence of the non-hydroxylatable effector 4-fluorobenzoate. NMR studies with [^{13}C]FAD-enriched apoprotein have shown that this ligand induces electronic and structural perturbations in the wild-type enzyme which are comparable to 4-hydroxybenzoate (Vervoort et al., 1991). In the absence of the effector, the reaction of oxygen with the reduced enzymes follows pseudo-first-order kinetics and no stabilization of the 4*a*-hydroperoxyflavin is observed. Table 4 shows that oxidized flavin is formed with rates comparable to wild type. In the presence of 4-fluorobenzoate, a rapid increase of absorption at 390 nm is followed by a much slower monophasic increase at 480 nm and indicative for the formation and subsequent breakdown of the 4*a*-hydroperoxyflavin (Entsch et al., 1976; Entsch and Ballou, 1989). Table 4 shows that, with enzyme Tyr201→Phe, the transient oxygenated flavin is stabilized considerably. For enzyme Tyr385→Phe, the stabilization of the 4*a*-hydroperoxyflavin is comparable to wild type. These results are in line with data reported for the 4-hydroxybenzoate complexed Tyr-mutated enzymes from *P. aeruginosa* (Entsch et al., 1991b) and indicate that local structural perturbations around the *para*-position of the substrate do not strongly influence the micropolarity near the flavin ring. The different lifetimes of the 4*a*-hydroperoxyflavin intermediate in the 4-fluorobenzoate-complexed enzymes suggest, however, that small variations in the electronic structure of the flavin are induced.

Table 4. 4-Fluorobenzoate-induced stabilization of 4*a*-hydroperoxyflavin in mutant *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Dithionite-reduced enzymes were mixed with oxygenated buffer at 15°C, pH 7.0, either in the absence or presence of 1 mM 4-fluorobenzoate. For further experimental details see Materials and Methods. For explanation of symbols see Scheme 1.

Enzyme	k_2	k_3
	$M^{-1}\,s^{-1}$	s^{-1}
Cys116→Ser	2.6×10^5	–
Complex	1.5×10^5	22
Tyr201→Phe	2.4×10^5	–
Complex	2.5×10^5	1.4
Tyr385→Phe	2.3×10^5	–
Complex	2.0×10^5	13

Binding studies

For *p*-hydroxybenzoate hydroxylase from *P. desmolytica* (Shoun et al., 1979) and *P. aeruginosa* (Entsch et al., 1991b) it was reported that, at pH 8.0, 4-hydroxybenzoate is predominantly bound in the phenolate form. From a chemical point of view, the dianionic state of substrate is expected to facilitate the electrophilic attack of the 4*a*-hydroperoxyflavin (Husain et al., 1980; Vervoort et al., 1992). For the *P. fluorescens* enzyme, Tyr201 was postulated to possess a lowered pK_a, thereby facilitating the activation of the substrate (Van Berkel and Müller, 1989). Binding of 4-hydroxybenzoate to *p*-hydroxybenzoate hydroxylase from *P. fluorescens* also stimulates the ionization of the hydroxyl moiety of the substrate. Fig. 2 shows the difference absorption spectrum of wild-type enzyme as a function of pH. Because the dissociation constant of the wild-type enzyme-substrate complex is hardly pH-dependent in the pH range 5.5–8.5 (Van Berkel and Müller, 1989) and assuming a maximum value of 16 $mM^{-1}\,cm^{-1}$ for the molar difference absorption coefficient of the phenolate form of bound substrate at 290 nm (Shoun et al., 1979), a pK_a value of about 7.2 can be estimated. Despite tight binding (Table 1), titration of the Tyr-mutated enzymes with 4-hydroxybenzoate does not result in marked changes in the absorbance around 290 nm (inset Fig. 2). Comparable results were reported for enzyme Tyr201→Phe from *P. aeruginosa* (Entsch et al., 1991b). For enzyme Tyr385→Phe from *P. aeruginosa* preferential binding of the phenolate form of substrate was reported but no data were presented (Entsch et al., 1991b). The present results indicate that both tyrosines are important for the optimal

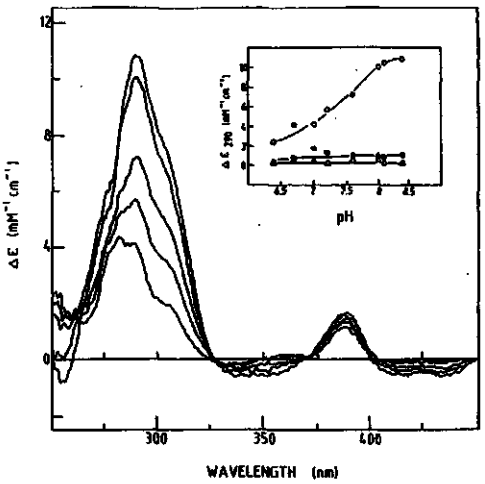


Fig. 2. Absorption difference spectra observed upon binding of 4-hydroxybenzoate to mutant *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Both cuvettes contained 1.0 ml 20 μM enzyme in one compartment and 1.0 ml 200 μM 4-hydroxybenzoate in the same buffer in the other compartment. The temperature was 25°C. For buffers used see Materials and methods. Before mixing the solutions in the two compartments of the sample cell, a baseline was recorded. From bottom to top: difference spectrum of enzyme Cys-116→Ser at pH 6.8, pH 7.2, pH 7.6, pH 8.0 and pH 8.4, respectively. The inset shows the molar absorption difference at 290 nm as a function of pH: enzyme Cys116→Ser (○); enzyme Tyr201→Phe (△); enzyme Tyr385→Phe (●).

activation of the substrate and in accordance with the relatively slow rate of hydroxylation of enzyme Tyr385→Phe (Entsch et al., 1991b).

Additional support for the activating role of Tyr201 in *p*-hydroxybenzoate hydroxylase from *P. aeruginosa* was obtained from binding studies with the inhibitor 4-hydroxycinnamate (Entsch et al., 1991b). It was reported that at pH values above pH 7.5, wild type and enzyme Tyr385→Phe predominantly bind the phenolate form of the inhibitor whereas enzyme Tyr201→Phe only binds the phenolic form. For all three enzymes, binding of 4-hydroxycinnamate was described by simple binary complex formation, yielding dissociation constants ranging over 100−200 μM. For the *P. fluorescens* enzymes, binding of 4-hydroxycinnamate does not follow simple 1:1 binding. Fig. 3A shows that at pH values below 7, titration of wild-type enzyme initially results in an increase of flavin fluorescence. At higher concentrations of the inhibitor, the fluorescence decreases again, indicating binding of the inhibitor at a second site. Such a binding behaviour was recently also reported for enzyme Arg214→Lys *in complex with 4-hydroxybenzoate* (Van Berkel et al., 1992). The fluorescence properties of wild-type enzyme in complex with 4-hydroxycinnamate are independent of the enzyme concentration (using 2−10 μM enzyme) excluding possible FAD dissociation. Fig. 3A also shows that, in contrast to the free enzyme (Van Berkel and Müller, 1989), the fluorescence quantum yield of the enzyme-inhibitor complex is strongly dependent on the pH of the solution. At pH values around 7, initially the fluorescence hardly changes. This is followed by a fluorescence decrease at higher concentrations of the inhibitor. At pH values above 7, addition of low concentrations of the inhibitor immediately results in a strong decrease of flavin fluorescence. The binding data of wild-type enzyme fit well, assuming binding of 4-hydroxycinnamate at two independent sites:

$$F_{obs} = F_0 + \frac{\Delta F_A [I] + (\Delta F_B [I]^2/K_{dB})}{K_{dA} + [I] + ([I]^2/K_{dB})} \qquad (1)$$

where F_{obs} is the experimental fluorescence observed, [I] the concentration of the inhibitor and ΔF_A, ΔF_B, K_{dA} and K_{dB} represent the fluorescence intensity changes and dissociation constants of the high-affinity and low-affinity sites, respectively (Van Berkel et al., 1992). The dissociation constants obtained from the optimal fits are summarized in Fig. 3B. It should be noted here that, above pH 7.2, data of the wild-type enzyme can also be fitted assuming simple binary complex formation. The much higher variation in dissociation constants and correlation coefficients, however, strongly suggests that above pH 7.2 secondary binding also occurs. This conclusion is supported by fluorescence binding data of the Tyr-mutated enzymes. For both mutants, the fluorescence quantum yield of the free enzyme remains nearly constant over the experimental pH range. Titration with 4-hydroxycinnamate results in an initial increase of flavin fluorescence, followed by a decrease at higher concentrations of the inhibitor (Fig. 3C, E). In contrast to wild type, however, no initial decrease of fluorescence is observed at high pH values. When the data of Fig. 3C and 3E are treated according to Eqn (1), values of about 50 μM and 700 μM are obtained for the dissociation constants of the high-affinity and low-affinity sites, respectively (Fig. 3D, F). For both mutants, the dissociation constant for the high-affinity site is only slightly pH dependent.

Over the entire pH interval and for all three enzymes, secondary binding of the inhibitor results in quenching of flavin fluorescence. Fitting the fluorescence data of Fig. 3 according to Eqn (1) yields values of F_B ranging over 5−10% for the fluorescence quantum yield of the ternary enzyme-inhibitor complexes. For the high-affinity site, however, replacement of Tyr201 or Tyr385 for Phe leads to changes in the fluorescence quantum yield of the enzyme-inhibitor complex. For enzyme Tyr201→Phe, the fluorescence quantum yield of the complex is nearly pH independent (Fig. 4). In contrast, the fluorescence quantum yield of the wild-type−4-hydroxycinnamate complex decreases with increasing pH (Fig. 4). The data of the wild-type enzyme fit well according to the expression (Van Berkel and Müller, 1989):

$$F_{obs} = \frac{F_{max}}{(K_a/[H^+]) + 1} + \frac{F_{min}}{([H^+]/K_a) + 1} \qquad (2)$$

yielding an apparent pK_a value of 6.5 for the optimal fit (R = 0.998) where F_{max} = 298% and F_{min} = 27% of the flavin fluorescence of the free enzyme. For enzyme Tyr385→Phe (Fig. 4), the fluorescence quantum yield of the complex only gradually decreases with increasing pH. When treating the data of enzyme Tyr385→Phe according to Eqn (2), a less optimal fit (R = 0.992) is obtained, yielding an apparent pK_a value of 6.9 with F_{max} = 212% and F_{min} = 100%, respectively. The above results suggest that for the high-affinity sites, the flavin fluorescence quantum yield of the enzyme−4-hydroxycinnamate complexes is related to the ionization state of the inhibitor bound (see also below). The fitted fluo-

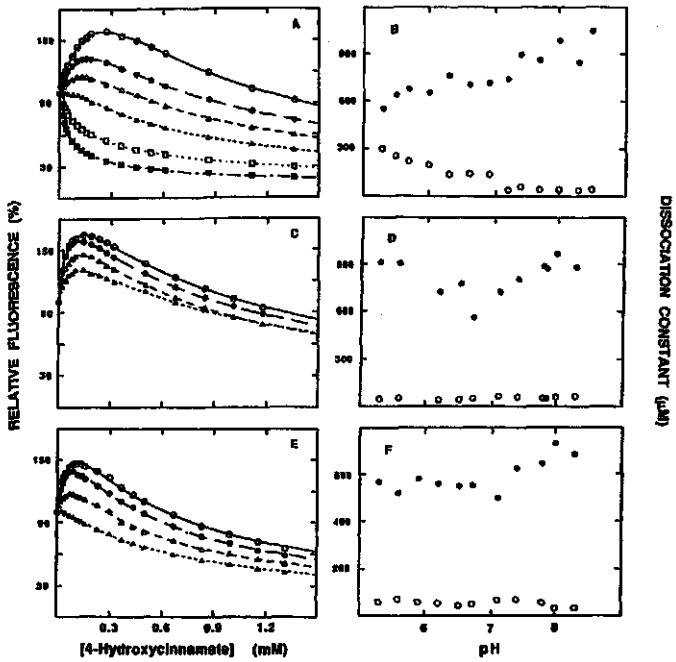


Fig. 3. pH-dependent fluorescence properties of mutant *p*-hydroxybenzoate hydroxylase upon titration with 4-hydroxycinnamate. 2 μM enzyme was titrated with 4-hydroxycinnamate at 25°C. Flavin fluorescence was monitored at 525 nm with excitation set at 450 nm. The fluorescence quantum yield of the free enzymes is given a value of 100%. The dissociation constants for the high-affinity and low-affinity sites, as calculated according to Eqn (1), are plotted as a function of pH. For buffers used and other experimental details see Materials and methods. (A) Fluorescence titration of enzyme Cys116→Ser at pH 5.3 (○), pH 6.3 (●), pH 6.6 (△), pH 6.9 (▲), pH 7.4 (□) and pH 8.0 (■). (B) pH dependence of the dissociation constant of the complex between enzyme Cys116→Ser and 4-hydroxycinnamate: high-affinity site (○) and low-affinity site (●). (C) Fluorescence titration of enzyme Tyr201→Phe at pH 5.6 (○), pH 6.5 (●), pH 7.1 (△) and pH 8.0 (▲). (D) pH Dependence of the dissociation constant of the complex between enzyme Tyr201→Phe and 4-hydroxycinnamate: high-affinity site (○) and low-affinity site (●). (E) Fluorescence titration of enzyme Tyr385→Phe at pH 5.6 (○), pH 6.5 (●), pH 7.1 (△) and pH 8.0 (▲). (F) pH dependence of the dissociation constant of the complex between enzyme Tyr385→Phe and 4-hydroxycinnamate: high-affinity site (○) and low-affinity site (●).

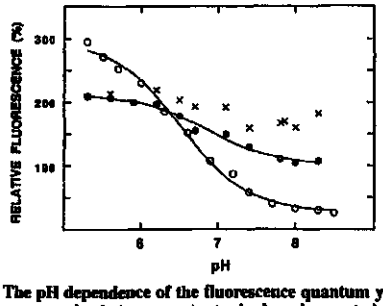


Fig. 4. The pH dependence of the fluorescence quantum yield of the binary complex between mutant *p*-hydroxybenzoate hydroxylase and 4-hydroxycinnamate. The fluorescence quantum yield of the binary complexes between enzyme and 4-hydroxycinnamate, using the data from Fig. 3 and as treated according to Eqn (1). The fluorescence quantum yield of the free enzymes is given a value of 100%. Enzyme Cys116→Ser (○), enzyme Tyr201→Phe (×) and enzyme Tyr385→Phe (●).

rescence quantum yield values of the mutant enzyme-inhibitor complexes strongly differ from the values reported for the corresponding complexes of the *P. aeruginosa* enzymes (Entsch et al., 1991b). As already noted above, for the *P. aeruginosa* enzymes, however, secondary binding of the inhibitor was not taken into account.

The ionization state of bound 4-hydroxycinnamate was studied in more detail by recording absorption spectra as a function of pH. Fig. 5A shows that titration of wild-type enzyme with 4-hydroxycinnamate results in large pH-dependent absorption changes around 350 nm. Over the entire pH interval, the data of Fig. 5A fit well, assuming simple binary complex formation. By treating the data in this way, the dissociation constant of the wild-type enzyme−4-hydroxycinnamate complex is only slighly pH-dependent and varies from 156 μM at pH 6.4 to 80 μM at pH 8.0. The maximum difference absorption coefficient at 353 nm is about 23 mM^{-1} cm^{-1} and an apparent pK_a value of 6.9 for the ionization of bound 4-hydroxycinnamate is estimated (Fig. 5C). The above results are in close agreement with the corresponding values reported for the *P. aeruginosa* wild-type en-

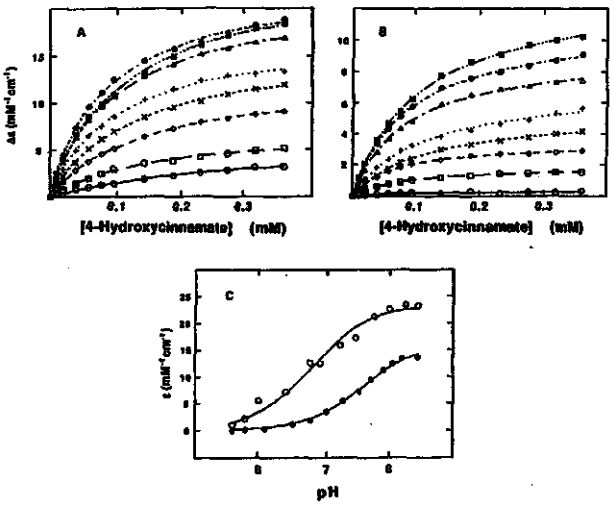


Fig. 5. pH-dependent absorption properties of mutant *p*-hydroxybenzoate hydroxylase upon titration with 4-hydroxycinnamate. 20 μM enzyme was titrated with 4-hydroxycinnamate at 25°C. The increase in absorption was monitored at 353 nm (enzyme Cys116→Ser) or 370 nm (enzyme Tyr385→Phe). For buffers used and other experimental details see Materials and methods. (A) Titration of enzyme Cys116→Ser at pH 6.0 (○), pH 6.4 (□), pH 6.9 (◇), pH 7.2 (×), pH 7.45 (+), pH 7.75 (△), pH 8.0 (●) and pH 8.25 (■). (B) Titration of enzyme Tyr385→Phe at pH 6.1 (○), pH 6.75 (□), pH 7.0 (◇), pH 7.25 (×), pH 7.5 (+), pH 7.7 (△), pH 7.9 (●) and pH 8.2 (■). (C) The molar absorption differences obtained from A and B were extrapolated to infinite 4-hydroxycinnamate concentration and plotted against pH: enzyme Cys116→Ser (○), enzyme Tyr385→Phe (●).

zyme (Entsch et al., 1991b). In contrast to the fluorescence titration experiments, the absorption data do not yield information about secondary binding of the inhibitor. This might explain the variation in the pK_a values and dissociation constants as derived from both techniques.

Despite tight binding (cf. Fig. 3D), over the entire experimental pH interval, titration of enzyme Tyr201→Phe with 4-hydroxycinnamate does not result in significant absorption changes around 350 nm. This is in full accord with data reported for the corresponding enzyme from *P. aeruginosa* (Entsch et al., 1991b), and clearly shows that enzyme Tyr201→Phe only binds the phenolic form of the inhibitor.

For enzyme Tyr385→Phe from *P. aeruginosa* (Entsch et al., 1991b) it was reported that binding of the phenolate form of 4-hydroxycinnamate is similar to wild type, except that at high pH values binding is accompanied with much lower absorption changes. When enzyme Tyr385→Phe from *P. fluorescens* is titrated with 4-hydroxycinnamate, over the entire experimental pH interval the maximum difference in absorption is centered around 370 nm, a clear red shift with respect to the wild-type enzyme. This shows that mutation of Tyr385 can have a direct effect on the electronic properties of the aromatic ligand bound. Fig. 5B shows the absorption changes at 370 nm as a function of pH. Again the binding data fit well, assuming simple binary complex formation, and yield dissociation constants varying between 47 μM (pH 6.1) and 105 μM (pH 8.2). Interestingly, the derived maximum absorption coefficients (Fig. 5C) are much more pH-dependent when compared to the corresponding values reported for the *P. aeruginosa* enzyme (Entsch et al., 1991b). An apparent pK_a value of 7.6 is calculated from the experimental data with a maximum difference absorption coefficient of $\Delta\varepsilon_{max}$ = 16.8 mM^{-1} cm^{-1} at 370 nm. The above results show that the electronic properties of bound 4-hydroxycinnamate are influenced by the polarity of the microenvironment around the 4-hydroxyl group.

DISCUSSION

Hydroxylation of substituted aromatic compounds by the flavin-dependent aromatic hydroxylases is a highly sophisticated process. Efficient hydroxylation of substrates is dependent on (a) the molecular orbital characteristics of bound substrate, (b) the electronic properties of the reduced flavin, determining the stabilization of the 4*a*-hydroperoxyflavin intermediate and (c) the mutual orientation of the 4*a*-hydroperoxyflavin and the hydroxylation site of bound substrate. With most flavin-dependent aromatic hydroxylases, the requirements for efficient hydroxylation are achieved by independently acting subunits (Van Berkel and Müller, 1991). In this respect, it is interesting to note that, very recently, a *P. putida* 4-hydroxyphenylacetate 3-hydroxylase was described which needs a second non-redox protein, crucial for coupling the oxidation of NADH to substrate hydroxylation (Arunachalam et al., 1992).

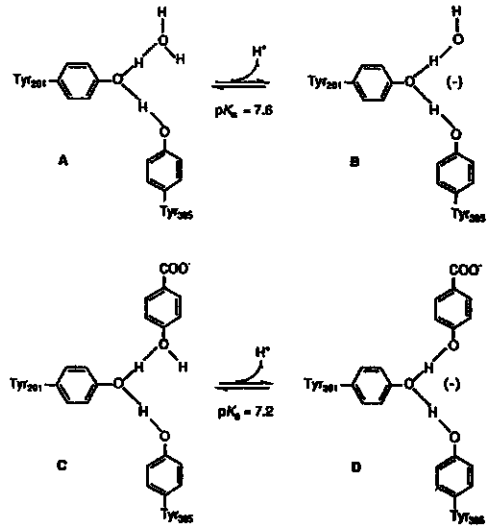
A further high level of control over the substrate specificity is introduced by the effector specificity. The extremely slow reduction of 4-aminobenzoate-complexed wild-type enzyme is indicative of the fine tuning of the effector specificity of *p*-hydroxybenzoate hydroxylase (Entsch et al., 1976). The stimulatory effect of substrate binding on the rate of enzyme reduction is most probably due to alterations in the mutual orientation of the flavin and the pyridine ring of

NADPH (van Berkel and Müller, 1991). The positioning of the nicotinamide ring is presumably directed by the conformation of the active-site loop, comprising residues 291–298 (Schreuder et al., 1991). This active-site loop is sharply bent and in close contact with the hydrogen bonding network about the 4-hydroxyl group of the substrate (Schreuder et al., 1989). The results presented in this paper show that any perturbation of this hydrogen bonding network influences the effector specificity. In this respect it is interesting to note that the amino acid sequence of the active-site loop and its connected secondary structure elements (β-strand D3 and α-helix H10) is conserved to a large extent in other FAD-dependent external aromatic hydroxylases, sequenced so far (Kälin et al., 1992). From this we conclude that the topology of the nicotinamide binding site of salicylate hydroxylase and phenol hydroxylase is comparable to *p*-hydroxybenzoate hydroxylase. This conclusion is supported by NMR experiments which show that, in the reduced state, both *p*-hydroxybenzoate hydroxylase and salicylate hydroxylase carry a negative charge at flavin N1 (Vervoort et al., 1991). For *p*-hydroxybenzoate hydroxylase it was suggested that the interaction between flavin N1 and the microdipole of α-helix H10 is probably also essential for efficient reduction of the enzyme-substrate complex (Müller et al., 1983).

In contrast with published data (Entsch et al., 1991b), both Tyr→Phe mutants are capable of hydroxylation of the product 3,4-dihydroxybenzoate. This suggests more rotational freedom for the product in these enzymes than in wild type. This points to a weaker interaction of the 3OH group of the product with the carbonyl oxygen of Pro293 (Schreuder et al., 1989), possibly via some change in conformation of the active-site loop. The formation of 3,4,5-trihydroxybenzoate implies that, in contrast to wild-type (Entsch et al., 1976), binding of 3,4-dihydroxybenzoate to the Tyr mutants induces some stabilization of the 4a-hydroperoxyflavin intermediate. NMR studies suggest that such a stabilization is dictated by the electronegativity of the C (4a) atom in the intermediate which again is dependent on the micropolarity and/or geometry of the active site (Vervoort et al., 1991).

From a chemical point of view, hydroxylation of 4-hydroxybenzoate is favored when the substrate is in the dianionic state (Husain et al., 1980; Vervoort et al., 1992). We agree with Entsch et al. (1991b) that Tyr201 activates the substrate (Van Berkel and Müller, 1989) by facilitating the deprotonation of the 4′-hydroxyl group. From the observed stabilization of the 4a-hydroperoxyflavin in enzyme Tyr 201→Phe (Entsch et al., 1991b) it might be expected that dianionic substrates are potential substrates for this mutant. The present binding studies with 2,3,5,6-tetrafluoro-4-hydroxybenzoate however indicate that introduction of a more hydrophobic residue at position 201 highly disfavors the interaction with the phenolate form of substrates. Enzyme Tyr-385→Phe rather slowly hydroxylates the substrate (Entsch et al., 1991b). In binding studies no deprotonation of the substrate is observed. From this we propose that in the free enzyme, Tyr385 is essential for lowering the pK_a of the 4′-OH group of Tyr201 (Van Berkel and Müller, 1989), thereby stimulating the ionization of the substrate upon binding (Scheme 2). In the free enzyme, the 4′-OH proton of Tyr201 presumably is taken up by a water molecule, located at the substrate binding site.

Although providing indirect evidence, the important role of Tyr385 in the activation of the substrate is supported by the binding studies with the inhibitor 4-hydroxycinnamate.



Scheme 2. Hypothetical scheme for the (de)protonation state of the active site of *p*-hydroxybenzoate hydroxylase. (A, B) Free enzyme; (C, D) enzyme 4-hydroxybenzoate complex.

With enzyme Tyr385→Phe, ionization of the inhibitor is clearly more difficult than with wild type. Tight binding of 4-hydroxycinnamate to *p*-hydroxybenzoate hydroxylase is quite surprising in view of the weak binding of the related compound 4-hydroxyphenylacetate (Van Berkel et al., 1992). Moreover, from crystallographic data (Schreuder et al., 1991), tight binding of the inhibitor is not expected because of steric constraints. Wild-type enzyme preferentially binds the phenolate form of 4-hydroxycinnamate. This indicates that in the enzyme-inhibitor complex, the hydrogen bonding network formed by both tyrosines is highly conserved. We therefore conclude that the side chain of Arg214, making a salt bridge with the carboxylic moiety of the substrate (Schreuder et al., 1989) must change its position in order to accomodate the extra introduced ethylene bond. Modeling studies (H. Schreuder, personal communication) suggest that the guanidinium group of Arg214 should move about 0.15 nm for proper binding of the inhibitor.

Binding of 4-hydroxycinnamate to wild-type and mutant enzymes is accompanied by variable pH-dependent changes in flavin fluorescence. These changes are most simply explained by binding of the inhibitor at two independent sites. For mutant Arg214→Lys in complex with 4-hydroxybenzoate, it was suggested that secondary binding of the substrate might occur at the NADPH binding site (Van Berkel et al., 1992). On the other hand, inhibition of wild-type enzyme by excess 2-fluoro-4-hydroxybenzoate was reported to be due to an effect in the oxidative half-reaction (Husain et al., 1980). Additional information from other techniques, e.g. isothermal titration calorimetry (Freire et al., 1990), might shed some more light on this poorly understood phenomenon.

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Chapter 5

Crystal structures of wild-type *p*-hydroxybenzoate hydroxylase complexed with 4-aminobenzoate , 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate and of the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate. Evidence for a proton channel and a new binding mode of the flavin ring.

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Crystal Structures of Wild-Type *p*-Hydroxybenzoate Hydroxylase Complexed with 4-Aminobenzoate, 2,4-Dihydroxybenzoate, and 2-Hydroxy-4-aminobenzoate and of the Tyr222Ala Mutant Complexed with 2-Hydroxy-4-aminobenzoate. Evidence for a Proton Channel and a New Binding Mode of the Flavin Ring†

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ABSTRACT: The crystal structures of wild-type *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*, complexed with the substrate analogues 4-aminobenzoate, 2,4-dihydroxybenzoate, and 2-hydroxy-4-aminobenzoate have been determined at 2.3-, 2.5-, and 2.8-Å resolution, respectively. In addition, the crystal structure of a Tyr222Ala mutant, complexed with 2-hydroxy-4-aminobenzoate, has been determined at 2.7-Å resolution. The structures have been refined to *R* factors between 14.5% and 15.8% for data between 8.0 Å and the high-resolution limit. The differences between these complexes and the wild-type enzyme–substrate complex are all concentrated in the active site region. Binding of substrate analogues bearing a 4-amino group (4-aminobenzoate and 2-hydroxy-4-aminobenzoate) leads to binding of a water molecule next to the active site Tyr385. As a result, a continuous hydrogen-bonding network is present between the 4-amino group of the substrate analogue and the side chain of His72. It is likely that this hydrogen-bonding network is transiently present during normal catalysis, where it may or may not function as a proton channel assisting the deprotonation of the 4-hydroxyl group of the normal substrate upon binding to the active site. Binding of substrate analogues bearing a hydroxyl group at the 2-position (2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate) leads to displacement of the flavin ring from the active site. The flavin is no longer in the active site (the "in" conformation) but is in the cleft leading to the active site instead (the "out" conformation). It is proposed that movement of the FAD out of the active site may provide an entrance for the substrate to enter the active site and an exit for the product to leave.

The flavoprotein *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) catalyzes the conversion of the substrate *p*-hydroxybenzoate (4-hydroxybenzoate) into the product 3,4-dihydroxybenzoate with help of NADPH and molecular oxygen. It is first step of the β-keto adipic acid pathway by which certain soil bacteria are able to degrade and utilize hydroxylated aromatic compounds such as *p*-hydroxybenzoate. These compounds are liberated during the biodegradation of lignin, one of the principle components of wood.

The reaction mechanism of *p*-hydroxybenzoate hydroxylase, as deduced by Entsch and co-workers using stopped-flow spectroscopic techniques (Entsch et al., 1976), is depicted in Figure 1. It is a multistep reaction involving three substrates, 4-hydroxybenzoate, NADPH, and molecular oxygen, and three products, 3,4-dihydroxybenzoate, $NADP^+$, and water.

It is remarkable that a single polypeptide of 43 kDa (*p*-hydroxybenzoate hydroxylase occurs in solution as a homodimer, but its active sites are independent) is able to perform three different reactions: (i) the reduction of FAD by NADPH to form $FADH^-$, (ii) the reaction of $FADH^-$ with molecular oxygen to form the flavin 4a-hydroperoxide, and (iii) the hydroxylation of the substrate by the flavin 4a-hydroperoxide to form the product 3,4-dihydroxybenzoate. In addition, the activity of this enzyme is tightly regulated. Reduction of the enzyme in the absence of substrate leads to the production of potentially harmful hydrogen peroxide (Spector & Massey, 1972) and the waste of NADPH equivalents. These events do not occur because, under normal conditions, the enzyme is only reduced by NADPH when the substrate is present (Husain & Massey, 1979).

More than 20 years of research on *p*-hydroxybenzoate hydroxylase have yielded detailed spectroscopic information on every step of the reaction (Entsch et al., 1976) and have revealed many other aspects of the reaction such as the stereochemistry of the reduction reaction (Manstein et al., 1986) and the fact that the substrate binds to the active site as a phenolate anion (Shoun et al., 1979; Entsch et al., 1991; Eschrich et al., 1993). Crystallographic studies of reduced and oxidized forms of the enzyme–substrate complex and of the enzyme–product complex (Wierenga et al., 1979; Schreuder et al., 1988a, 1989, 1992) have revealed detailed structural information on some key intermediates of the reaction cycle and allowed molecular modeling of the flavin 4a-hydroperoxide

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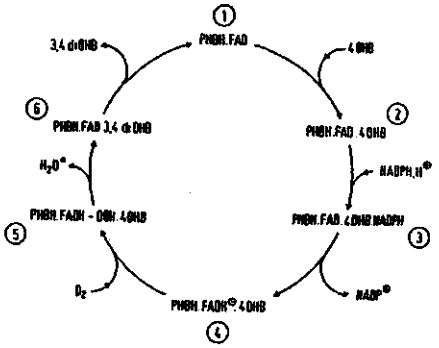

FIGURE 1: Catalytic cycle of *p*-hydroxybenzoate hydroxylase based on work by Entsch et al. (1976) and many others. PHBH, *p*-hydroxybenzoate hydroxylase; 4OHB, the substrate 4-hydroxybenzoate; $FADH^-$, reduced flavin bearing a negative charge at the N1 (Vervoort et al., 1991); FADH-OOH, flavin 4a-hydroperoxide intermediate; 3,4-diOHB, the reaction product 3,4-dihydroxybenzoate. The reaction starts at (1) with the binding of the substrate 4-hydroxybenzoate and NADPH in random order (2, 3), followed by reduction of the flavin ring to form $FADH^-$ (4). Subsequently, molecular oxygen reacts with the reduced flavin to form a flavin 4a-hydroperoxide intermediate (5). This latter intermediate hydroxylates the substrate 4-hydroxybenzoate to form the reaction product 3,4-dihydroxybenzoate and a flavin 4a-hydroxide intermediate. The flavin 4a-hydroxide breaks down into a water molecule and oxidized flavin (6). After the product has left the active site, the enzyme is ready for the next reaction cycle.

intermediate and the hydroxylation reaction (Schreuder et al., 1988b, 1990). In addition, van der Laan et al. (1989a) reported a crystal structure of *p*-hydroxybenzoate hydroxylase in which the FAD molecule has been replaced by an ADPR molecule. The latter complex was obtained by crystallizing native *p*-hydroxybenzoate hydroxylase in the presence of ADPR. The apparently easy replacement of bound FAD by ADPR indicated that the flavin ring itself does not contribute strongly to the binding of the FAD molecule.

Despite a plethora of spectroscopic, biochemical, and structural data, many questions are still unanswered. These questions include the interaction of NADPH with *p*-hydroxybenzoate hydroxylase [see van Berkel et al. (1988)], the catalysis of the reduction reaction, and the mechanism causing the 10^5-fold increase in reduction rate after binding of substrate and effector molecules (Husain & Massey, 1979). Other questions involve the precise way by which the protein influences the reactivity of the flavin and the various reactants in order to achieve optimal overall catalytic efficiency.

The cloning of the *pobA* gene (encoding *p*-hydroxybenzoate hydroxylase) of *Pseudomonas aeruginosa* (Entsch et al., 1988), followed by the cloning of this gene from *Pseudomonas fluorescens* (van Berkel et al., 1992), and recently from *Acinetobacter calcoaceticus* (DiMarco et al., 1993), opened the way to address these questions by site-directed mutagenesis. Mutations of Tyr201 and Tyr385 showed their role in the activation of the substrate 4-hydroxybenzoate toward hydroxylation (Entsch et al., 1991; Eschrich et al., 1993; Lah et al., 1994). The result of the mutation of Asn300 (the only side chain which directly interacts with the flavin ring) into Asp was less clear since the mutated side chain moved away from the flavin ring, causing a number of local structural changes in the protein (Lah et al., 1994). Mutations of residues that are involved in binding the carboxylate group of the substrate such as Arg214 (van Berkel et al., 1992) and Tyr222 (van Berkel et al., 1993) showed that these residues are essential for efficient hydroxylation, presumably because they limit the rotational freedom of the substrate.

Here we describe the crystal structures of the Tyr222Ala mutant complexed with the substrate analogue 2-hydroxy-4-aminobenzoate and, in addition, crystal structures of wild-type enzyme complexed with 4-aminobenzoate, 2,4-dihydroxybenzoate, and 2-hydroxy-4-aminobenzoate. Removal of the bulky side chain of Tyr222 from the active site in the Tyr222Ala mutant led to surprisingly small changes in the crystal structure, while introduction of a single hydroxyl group at the 2-position in substrate analogues led unexpectedly to an alternative position of the flavin ring which is completely different from its normal position in the active site of the enzyme. These results suggest a weak binding of the flavin ring which is in line with the easy replacement of FAD by ADPR as observed by van der Laan et al. (1989b). Changing the 4-hydroxyl group of the substrate to a 4-amino group in substrate analogues led to binding of an extra water molecule next to Tyr385, resulting in a continuous hydrogen-bonding network between the 4-amino group of the substrate analogue and His72.

EXPERIMENTAL PROCEDURES

Preparation of the Enzymes. The Cys116Ser mutant (Eschrich et al., 1990) was used as "wild-type", in order to prevent problems with the crystallization ascribed to the oxidation of the cysteine at this position (van der Laan et al., 1989b; van Berkel & Müller, 1987). The position of residue 116 is far away from the active site, and its mutation does not influence any of the catalytic properties of the enzyme. Site-specific mutagenesis was performed according to the method of Kunkel et al., (1987), essentially as described elsewhere (van Berkel et al., 1992). The oligonucleotide GCCGC-TACGCCGTACAGGTGC was used for the construction of the mutant Tyr222Ala. The Tyr222Ala mutation, made in the gene bearing the Cys116Ser mutation, was confirmed by nucleotide sequencing using the M13 dideoxy-chain-termination method (Sanger et al., 1977). Mutant proteins were expressed in transformed *Escherichia coli* TG2 and purified at pH 8 (van Berkel et al., 1992). The expression and yield of mutant Tyr222Ala is in the same range as found for wild-type (Eschrich et al., 1990; van Berkel et al., 1992).

Crystallization. Crystals of "wild-type" enzyme with 2,4-dihydroxybenzoate and 4-aminobenzoate were obtained by soaking crystals of the enzyme–4-hydroxybenzoate complex, grown by the free interface liquid–liquid diffusion technique (Drenth et al., 1975; van der Laan et al., 1989b), for 4 days in mother liquor containing 38% saturated ammonium sulfate, 0.1 M potassium phosphate buffer (pH 7.5), 0.04 mM FAD, 0.3 mM EDTA, and 20 mM 2,4-dihydroxybenzoate or 4-aminobenzoate instead of the normal substrate 4-hydroxybenzoate.

Crystals of "wild-type" enzyme and the Tyr222Ala mutant, both complexed with the substrate analogue 2-hydroxy-4-aminobenzoate, were obtained using the hanging drop method. The protein solution contained 10 mg/mL enzyme in 10 mM potassium phosphate buffer (pH 7.5). The reservoir solution contained 50% saturated ammonium sulfate, 0.04 mM FAD, 0.30 mM EDTA, 2 mM substrate analogue, 20 mM sodium sulfite- and 0.1 M potassium phosphate buffer (pH 7.5). Drops of 2 μL of protein solution and 2 μL of reservoir solution were allowed to equilibrate at 4 °C against 1 mL of reservoir solution. Crystals with dimensions of up to 0.2 × 0.3 × 0.4 mm^3 grew within 7 days. It was not possible to obtain crystals

Table 1: Data Collection and Refinement Statistics

complex	WT[a] + 4-amino-benzoate	WT[a] + 2,4-dihydroxy-benzoate	WT[a] + 2-hydroxy-4-amino-benzoate	Y222A + 2-hydroxy-4-amino-benzoate
code	4AB	24DOB	2O4AB	Y222A
cell dimensions (Å)				
a	72.0	72.3	72.2	72.2
b	146.6	146.2	146.3	146.5
c	88.7	89.0	88.8	88.4
unique reflections	14 434[b]	15 242	11 552	11 648
resolution (Å)	2.3	2.5	2.8	2.7
R_{sym} (%)	4.7	3.9	9.8	8.6
completeness (%)	67.8	91.3	96.8	87.8
starting model	POHB[c]	2O4AB	POHB[c]	2O4AB
initial *R* factor	19.2	22.4	20.5	19.7
final *R* factor (%)	15.6	15.8	14.5	14.8
water molecules	267	203	208	231
rms bond lengths (Å)	0.009	0.009	0.009	0.009
rms bond angles (deg)	1.50	1.53	1.57	1.56
average *B* factors (Å^2)				
protein	27.4	25.0	21.5	22.6
flavin ring	20.1	16.8	15.3	18.2
substrate analogue	14.9	12.1	9.3	15.7

[a] Cys116Ser "wild-type". [b] It should be noted that the 4AB data set, although it extends to higher resolution, contains less reflections than the 24DOB data set, owing to its lower completeness. The 4AB data set is 90% complete to 3.3 Å, but completeness drops gradually toward higher resolution. In the resolution shell between 2.5 and 2.3 Å, 40.0% of the reflections have been observed, and the shell between 2.36 and 2.30 Å is 31.4% complete. [c] *p*-Hydroxybenzoate–substrate complex (Schreuder et al., 1989).

of the Tyr222Ala mutant in the presence of the normal substrate. This may be due to the weak affinity (K_d = 1300 μM; van Berkel et al., 1993) of this mutant for the normal substrate 4-hydroxybenzoate. The 2-hydroxy-4-aminobenzoate molecule binds much tighter (K_d = 20 μM), and its complex could be crystallized.

Data Collection. Data of the crystals of wild-type with 2,4-dihydroxybenzoate and 4-aminobenzoate were collected with a FAST television area detector (Enraf Nonius, Delft, The Netherlands) mounted on an Elliot rotating anode generator, operating at 45 kV and 75 mA equipped with a graphite monochromator. Madness software (Messerschmidt & Pflugrath, 1987) was used to run the detector, to index the data, and to produce a "xrec.xds" type raw data file which was fed into the XDS software (Kabsch, 1988) to obtain integrated intensities by means of three-dimensional profile fitting. Data of the crystals of wild-type enzyme and the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate were collected using a multiwire area detector (Siemens, Analytical Instruments, Inc., Madison, WI) mounted on a Siemens rotating anode generator, operating at 45 kV and 100 mA, equipped with a graphite monochromator. Data were processed with the XDS package (Kabsch, 1988). Data collection statistics are given in Table 1. The crystals obtained by liquid–liquid diffusion were of better quality (larger and with a more regular shape) than the crystals obtained by the hanging drop method. As a result the diffraction of the crystals of the 4-aminobenzoate and 2,4-dihydroxybenzoate complexes extends to higher resolution than the diffraction of the crystals of complexes of 2-hydroxy-4-aminobenzoate with "wild-type" and the Tyr222-Ala mutant.

Refinement. Refinement was carried out using the program XPLOR (Brünger, 1992). The parameter set as determined by Engh and Huber (1991) was used for the protein part of the structure, while the parameters for the FAD molecule were obtained from Dr. A. Vrielink as were used for the refinement of cholesterol oxidase (Vrielink et al., 1991). The latter parameters, however, led to unacceptable deviations from planarity for the flavin ring. This effect may be due to the lower resolution we used (2.8 Å versus 1.8 Å for the refinement of cholesterol oxidase), or it may be due to the combination of original XPLOR (CHARMM) parameters for the FAD with the much stricter Engh and Huber parameters for the protein part of the structure. We did not investigate this effect further but added empirically improper angle restraints to the FAD topology definition to maintain acceptable planarity of the flavin ring while still allowing some twisting or bending of the flavin ring.

The cell dimensions of the crystals listed in Table 1 deviate less than 1% from each other and from the wild-type crystals (cell dimensions a = 71.5 Å, b = 145.8 Å, c = 88.2 Å; Schreuder et al., 1989). We did not use rigid-body refinement to adapt the starting model to a different crystal form but used an alternative method instead. The starting coordinates were converted to fractional coordinates and subsequently converted back to orthogonal coordinates using the cell dimensions of the new crystal form. This method works as least as well as rigid-body refinement, as evidenced by the low starting *R* factors listed in Table 1. The error in protein geometry introduced by this method is of the same order as the rms deviation of the bond lengths and is quickly corrected during the first cycle of subsequent refinement.

The structures of "wild-type" enzyme complexed with 4-aminobenzoate and 2-hydroxy-4-aminobenzoate were refined starting from the coordinates of the wild type–4-hydroxybenzoate complex (Schreuder et al., 1989) after application of the correction for the slightly different cell dimensions as mentioned above. Refinement was started with manual inspection of unweighted $2F_o - F_c$ and $F_o - F_c$ maps based on the corrected starting model in order to obtain a clear view of the differences between the new crystal form and the starting model, not biased by any coordinate refinement. These starting maps indicated only few corrections, except for some very clear and significant differences in the active site region. Refinement consisted of three macrocycles of map inspection and rebuilding using FRODO (Jones, 1985) with subsequent energy minimization and temperature factor refinement using XPLOR (Brünger, 1992). The structures of "wild-type" with 2,4-dihydroxybenzoate and the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate were refined using two macrocycles of map inspection and refinement. Here the structure of "wild-type" complexed with 2-hydroxy-4-aminobenzoate was used as the starting model. The last three structures mentioned all have an alternative binding mode for the flavin ring. The final statistics are listed in Table 1.

Superpositions. Prior to analysis, all four structures discussed in this paper were superimposed onto the 1.9-Å structure of the enzyme–substrate complex (Schreuder et al., 1989) using the algorithm of Kabsch (1976). Only C$^\alpha$ atoms were used for the superposition. In order to asses the theoretical contacts of the 2-hydroxyl group of the 2,4-dihydroxybenzoate molecule with the flavin ring occupying the standard, well-known "in" position (see Results and Discussion), we measured the distance between the 2-hydroxyl group of the substrate analogue in the enzyme–2,4-dihydroxybenzoate complex and the flavin ring in the superimposed enzyme–substrate complex. We did not correct for movements of the protein to adapt to the substrate analogue, so the distances thus obtained do not represent the real situation but indicate the magnitude of rearrangements necessary to accommodate the substrate analogue.

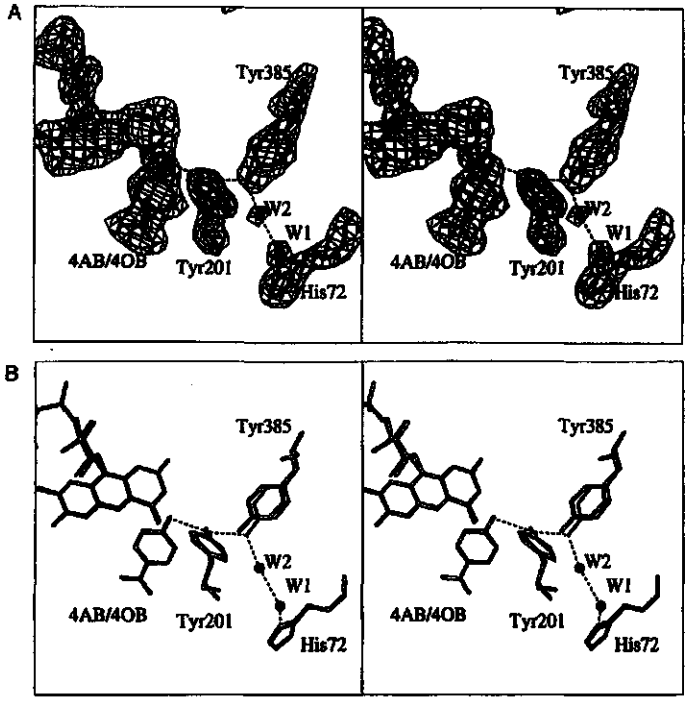

FIGURE 2: Structure of the 4-aminobenzoate complex. For clarity, only the flavin, the substrate (analogue), and the residues involved in the hydrogen-bonding network are shown. Abbreviations: 4OB, 4-hydroxybenzoate; 4AB, 4-aminobenzoate. (A) $F_o - F_c$ omit map, contoured at 4σ. The residues shown (His72, Tyr201, Tyr385, the flavin, the 4-aminobenzoate, and both water molecules) were omitted from the map calculation. (B) Superposition of the structures of the enzyme–substrate complex (Schreuder et al., 1989) and the 4-aminobenzoate complex. The enzyme–substrate complex is drawn with open bonds, the 4-aminobenzoate complex with solid bonds. Circles indicate bound water molecules. Two water molecules are shown. W1 next to His72 is present both in the 4-hydroxybenzoate and in the 4-aminobenzoate complex, but W2 next to Tyr385 is only present in the 4-aminobenzoate complex, giving rise to a continuous hydrogen-bonding network between the N4 of the substrate analogue and the ND1 of His72.

RESULTS

The highly refined 1.9-Å starting structure (Schreuder et al., 1989) resulted in well-refined structures of the *p*-hydroxybenzoate hydroxylase complexes presented here. Table 1 shows that both the geometry (rms deviations of 0.009 Å for the bond lengths and 1.5–1.6° for the bond angles) and the *R* factors (15–16%) are excellent. Only two residues (Arg44 and Asp144) have ϕ, ψ angles outside the allowed regions. Even Ala80, which deviates in the 1.9-Å native structure, is within allowed regions in the present structures.

Superposition of the various complexes with the wild-type enzyme–substrate complex shows that the overall folding did not change in any of the structures. The rms deviations for all 391 C^{α} atoms present in our structures varies from 0.15 Å for the 4-aminobenzoate complex to 0.25 Å for the Tyr222Ala mutant. The average *B* factors as listed in Table 1 do not differ significantly between the different complexes and with the 1.9-Å native structure (average *B* factors: 24.9 Å^2 for the protein, 16.6 Å^2 for the flavin ring, and 15.7 Å^2 for the substrate).

4-Aminobenzoate Complex. Analysis of the electron density maps of the "wild-type"–4-aminobenzoate complex indicated only a few but very clear differences between the 4-aminobenzoate complex and the 4-hydroxybenzoate complex. These differences involve the hydrogen-bonding network

Table 2: Lengths of the Hydrogen Bonds Involved in the Hydrogen-Bonding Network around Tyr201 and Tyr385 in Various Crystal Structures of *p*-Hydroxybenzoate Hydroxylase

hydrogen bond	length of hydrogen bond (Å) for complex[a]				
	POHB	24DOB	4AB	2O4AB	Y222A
O4/N4[b]–OH Tyr201	2.7	3.0	3.1	3.1	3.3
OH Tyr201–OH Tyr385	2.8	2.5	2.9	2.6	2.7
OH Tyr385–W2[c]			2.8	2.8	3.1
W2–W1			3.0	3.0	2.9
W1–ND1 His72	2.5	2.7	2.8	2.6	2.6

[a] See Table 1 for the codes of the different *p*-hydroxybenzoate hydroxylase complexes. [b] O4 for the 4-hydroxybenzoate and 3,4-dihydroxybenzoate molecules; N4 for the 4-aminobenzoate and 2-hydroxy-4-aminobenzoate molecules. [c] W1, water molecule next to His72; W2, water molecule next to Tyr385 (see Figure 2).

of the 4-amino and 4-hydroxyl group of the substrate (analogue) and, respectively, the active site tyrosines 201 and 385 (see Figure 2 and Table 2). The hydrogen bond between the 4-amino group and Tyr201 is 0.4 Å longer than the equivalent hydrogen bond in the enzyme–substrate complex, reflecting the difference between a NH–OH hydrogen bond and an O^-–OH hydrogen bond. It should, however, be noted that in the 2,4-dihydroxybenzoate complex a rather long hydrogen bond of 3.0 Å is present between the O4 of the substrate analogue and the OH of Tyr201. The 4-aminobenzoate molecule occupies exactly the same position as the

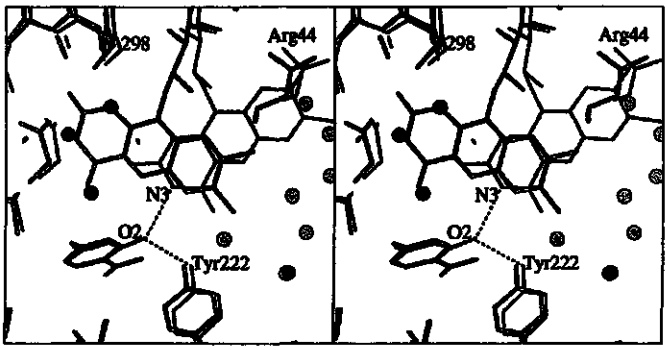

FIGURE 3: Superposition of the structures of the enzyme–substrate complex (Schreuder et al., 1989) and the 2,4-dihydroxybenzoate complex. The enzyme–substrate complex is drawn with open bonds, the 2,4-dihydroxybenzoate complex with solid bonds. Circles indicate bound water molecules. Broken lines indicate hydrogen bonds between the O2 of the 2,4-dihydroxybenzoate molecule and the N3 of the flavin (2.8 Å) and the OH of Tyr222 (3.0 Å). The flavin does not occupy its normal "in" position in the 2,4-dihydroxybenzoate complex but has rotated about 30° into the cleft leading to the active site. Three water molecules (black circles) occupy the binding sites of the N1, N3, and O4 of the flavin when it is in the "in" position. For clarity, only a thin slice of the structure is shown. As a result, the side chain of Arg44, which runs at van der Waals distance behind the flavin in the "out" conformation, is only visible from the CD onward.

Table 3: Contacts ($d < 3.5$ Å) between the Flavin Ring and the Protein or Substrate (Analogue)[a]

	protein atom in complex			
flavin atom	4AB[b]	24DOB	2O4AB	Y222A
N1	(3.4) N Gly298 (3.1) N Leu299 (3.2) Wat230	(3.3) Wat172 (3.4) Wat186	(3.3) Wat172	(3.0) Wat209
C2	(3.4) O Ala296 (3.4) N Leu299		(3.4) Wat187	(3.2) Wat209
O2	(3.1) O Ala296 (2.9) N Leu299 (3.5) CA Leu299 (3.3) CB Leu299 (3.1) N Asn300 (3.1) ND2 Asn300	(3.1) Wat172 (3.1) Wat182 (2.9) Wat186	(3.4) CB Ala45 (3.1) Wat187 (3.2) Wat202 (3.0) Wat206	(3.4) CB Ala45 (2.9) Wat209 (3.2) Wat222 (3.5) Wat224
N3	(3.0) O Val47 (3.4) O Ala296	(2.8) O2 DOB[c]	(2.9) O2 HAB[d]	(3.0) O2 HAB[d]
C4	(3.5) CB Ala45			
O4	(3.2) N Gly46 (3.2) N Val47	(3.3) OH Tyr222	(3.4) OH Tyr222	
C4A	(3.5) CA Ala45 (3.5) CB Ala45			
C5A				(3.3) CD Arg44
C6			(3.5) CD Arg44	(3.4) CD Arg44
C8			(3.4) NH1 Arg44	(3.2) Wat121
C8M	(3.3) Wat69		(3.4) Wat122	(3.4) Wat121 (3.1) Wat121
C9			(3.2) Arg44 (3.3) Wat115	(2.8) Wat121
N10	(3.3) Wat230			
C10	(3.2) Wat230			

[a] Distances (Å) are given between brackets. [b] See Table 1 for the codes of the different *p*-hydroxybenzoate hydroxylase complexes. [c] 2,4-Dihydroxybenzoate. [d] 2-Hydroxy-4-aminobenzoate.

substrate molecule in the enzyme–substrate complex, and the hydroxyls of Tyr201 and Tyr385 move 0.4 and 0.55 Å, respectively, to accommodate the longer hydrogen bond. In addition, difference maps clearly indicated the presence of an extra water molecule next to Tyr385. Because of this extra water molecule, the hydrogen-bonding network that ends at Tyr385 with the normal substrate now extends to His72 (see Figure 2). This "bridging" water molecule appears to be firmly bound since its temperature factor of 21.5 Å^2 is even lower than the overall temperature factor of the protein (27.4 Å^2; Table 1).

2,4-Dihydroxybenzoate Complex. Soaking 2,4-dihydroxybenzoate into the active site of *p*-hydroxybenzoate hydroxylase crystals causes an entirely unexpected and dramatic shift in the position of the flavin ring. As shown in Figure 3, the flavin ring has rotated over about 30° with respect to its position in the 4-hydroxybenzoate complex and is now located in the cleft leading to the active site. The pyrimidine ring of the flavin in the new, "out" position occupies the position of the dimethyl benzene ring in the 4-hydroxybenzoate complex. In contrast to the nine potential hydrogen bonds (defined by donor–acceptor distances <3.5 Å) between the protein and the flavin ring in the "in" position (e.g., the enzyme–4-aminobenzoate complex; Table 3), we did not observe any direct hydrogen bond between the protein and the flavin ring in the alternative "out" conformation. The only exception is a rather long (3.3 Å) potential hydrogen bond between the flavin O4 and the hydroxyl group of Tyr222. However, a

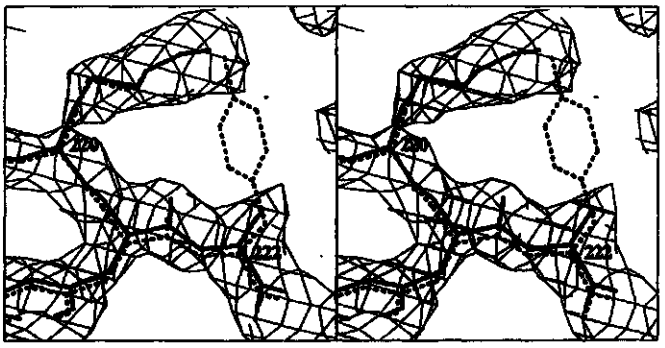

FIGURE 4: Electron density around the mutation site in the crystal structure of the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate. The structure of the Tyr222Ala mutant is drawn with solid bonds; the structure of "wild-type" enzyme with 2-hydroxy-4-aminobenzoate is drawn in broken lines. The electron density shows clearly the absence of the bulky side chain of Tyr222 in the mutant. The electron density also indicates that the side chain of Arg220 moves toward the now empty pocket of Tyr222.

strong hydrogen bond (2.8 Å) seems to be present between the 2-hydroxyl group of the substrate analogue and the N3 of the flavin ring. The O2 and O4 oxygens of the flavin ring make hydrogen bonds with fixed solvent molecules. Three water molecules were observed in the flavin binding pocket in the active site. One water molecule is present in the carbonyl–oxygen binding pocket (Schreuder et al., 1988b), which normally binds the O4 of the flavin ring. The other two water molecules occupy the positions of the N1 and N3 nitrogens. Arg44, which runs at 3.5–4.0 Å distance at the *si*-side of the flavin ring, may provide additional stabilization for the flavin ring in the "out" position due to π–π stacking interactions. The new position of the flavin ring did not change the deviating ϕ,ψ angles of Arg44. The rms difference for the flavin ring between the "in" and the "out" position is 4.7 Å. The flavin C7 methyl atom, which is farthest away from the pivot point, moves 6.4 Å. The mobility of the flavin ring, as deduced from the average temperature factors in Table 1, does not seem to be significantly different in both orientations. Finally, the orientations of the bound 4-hydroxybenzoate and 2,4-dihydroxybenzoate molecules are virtually identical, suggesting that the enzyme rigidly fixes the substrate (analogue) molecule in the active site. Superposition of the 4-hydroxybenzoate and 2,4-dihydroxybenzoate complexes shows that a short contact of 3.0 Å would be present between the O2 of the substrate analogue and the C6 carbon of the flavin ring when the ring would occupy its standard, "in" position inside the active site.

2-Hydroxy-4-aminobenzoate Complex. The crystal structure of wild-type *p*-hydroxybenzoate hydroxylase, complexed with the substrate analogue 2-hydroxy-4-aminobenzoate, shows that the effect of the 4-amino group (as is found in the 4-aminobenzoate complex) and the effect of an extra hydroxyl group in the 2-position (as is observed in the 2,4-dihydroxybenzoate complex) are independent. An extra water molecule is present next to Tyr385, creating a continuous hydrogen-bonding network between the 4-amino group and His72, just as was observed for the 4-aminobenzoate complex. The water appears to be less firmly bound than in the 4-aminobenzoate complex. The hydrogen bond with Tyr385 is long (3.4 Å), and its temperature factor is rather high (36.9 Å^2). The flavin ring occupies the "out" position, just as was found for the 2,4-dihydroxybenzoate complex. Two water molecules are observed in the flavin-binding pocket. One occupies the carbonyl-oxygen (O4) binding pocket; the other is near the position of the C2 carbon of the flavin in the "in" position. The lower resolution of this structure (2.8 Å versus 2.5 Å for the structure of the 2,4-dihydroxybenzoate complex) makes the position of these water molecules less certain than in the 2,4-dihydroxybenzoate complex. In particular, the water near the flavin C2 may represent the average of the two water molecules found near this position in the 2,4-dihydroxybenzoate complex.

Complex of the Tyr222Ala Mutant with 2-Hydroxy-4-aminobenzoate. The mutation of Tyr222 into Ala results in the removal of a large side chain from the active site (Figure 4). This side chain is involved in the binding of the carboxyl group of the substrate. Nevertheless, as is shown in Figure 5, the active site hardly changes with respect to the complex of wild-type enzyme with 2-hydroxy-4-aminobenzoate. The flavin ring occupies the new, "out" position, and the substrate analogue is bound in the same way as with the wild-type–2-hydroxy-4-aminobenzoate complex. The side chain of Arg220 moves 1.3 Å into the empty space which is occupied by Tyr222 in the wild-type enzyme. Even the two water molecules in the flavin-binding pocket and the water molecule next to Tyr385 (B = 24.6 Å^2) have positions similar to those in the wild-type–2-hydroxy-4-aminobenzoate complex.

Planarity of the Flavin Ring. Previous studies have established that the oxidized flavin ring is somewhat twisted when bound to the active site of *p*-hydroxybenzoate hydroxylase. The twisting angle between the pyrimidine ring and the dimethylbenzene ring is 10° in the 1.9-Å structure of the enzyme–substrate complex (Schreuder et al., 1989), which is the highest resolution structure of *p*-hydroxybenzoate hydroxylase determined so far. A similar twist has recently been observed by a different laboratory for a number *p*-hydroxybenzoate hydroxylase mutants of *P. aeruginosa* (Lah et al., 1994) and was also observed in the 1.8-Å structure of cholesterol oxidase (Vrielink et al., 1991). Analysis of the angle between the pyrimidine and dimethylbenzene rings in the present structures (Table 4) reveals that a twist of 12° is present in the structure of the 4-aminobenzoate complex, which has flavin in the "in" conformatio, and that the flavin ring is much more planar (angles of 2–5°) in the other three complexes which contain flavin in the "out" conformation.

DISCUSSION

The present studies show that not only site-specific mutations but also substrate analogues are powerful tools to study the intricate catalytic mechanism of *p*-hydroxybenzoate hydroxy-

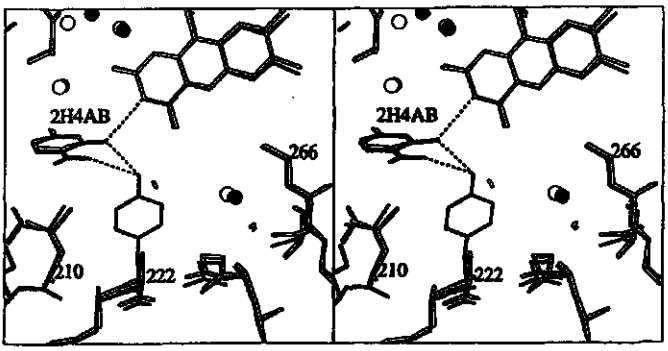

FIGURE 5: Superposition of the structures of the 2-hydroxy-4-aminobenzoate complexes of "wild-type" enzyme and of the Tyr222Ala mutant. "Wild-type" enzyme is drawn with solid lines, the Tyr222Ala mutant with open bonds. Dashed lines show hydrogen bonds between the OH of Tyr222 and the O2 and carboxyl oxygen of the substrate analogue and a hydrogen bond between the O2 of the analogue and the N3 of the flavin ring. The flavin ring has the "out" conformation in both complexes (see Figure 3). The position of the flavin ring, the substrate analogue, and almost all active site residues is virtually identical in both structures, despite the fact that a bulky side chain has been removed from the active site in the Tyr222Ala mutant.

Table 4: Angles between the Dimethylbenzene and Pyrimidine Ring in Various Crystal Structures of *p*-Hydroxybenzoate Hydroxylase

complex[a]	angle (deg)
POHB	10
4AB	12
DOB	2
2O4AB	2
Y222A	5

[a] For abbreviations see Table 1.

lase and that they can reveal unexpected properties of the enzyme. The two major findings of this study are (i) the presence of a bridging water molecule in the 4-aminobenzoate complexes and (ii) the fact that the flavin ring slides out of the active site in the 2-hydroxybenzoate complexes. We will now discuss the possible implications of these findings for the catalytic mechanism.

However, before discussing a possible role for the bridging water molecule, we will first review the evidence for its presence. The presence or absence of specific water molecules in protein crystal structures cannot always be determined with certainty (especially at low and intermediate resolutions), but the accumulated crystallographic evidence regarding the bridging water molecule in *p*-hydroxybenzoate hydroxylase is unambiguous. The water molecule is not observed in any complex with substrate or substrate analogues that bear a 4-hydroxyl group, i.e., the oxidized and reduced forms of the enzyme–substrate complex (Schreuder et al., 1989, 1990), the enzyme–product complex (Schreuder et al., 1988a), the enzyme–ADPR complex (van der Laan et al., 1988a), the complexes of 2,4-dihydroxybenzoate with *p*-hydroxybenzoate hydroxylase from *P. fluorescens* (this study) and from *P. aeruginosa* (Lah et al., 1993), and neither in the complexes of both 4-hydroxybenzoate and 2,4-dihydroxybenzoate with the Tyr385Phe and Asn300Asp mutants of the *P. aeruginosa* enzyme (Lah et al., 1994). In contrast, the extra water molecule is present in all structures with substrate analogues possessing a 4-amino group. These structures include the complex of the *P. fluorescens* enzyme with 4-aminobenzoate and 2-hydroxy-4-aminobenzoate, the complex of the Tyr222Ala mutant with 2-hydroxy-4-aminobenzoate (this study), and the complex of 4-aminobenzoate with the *P. aeruginosa* enzyme (Lah et al., 1993). In addition, the extra water molecule is present in the structure of the Tyr201Phe mutant of the *P. aeruginosa* enzyme complexed with 4-hydroxybenzoate (Lah et al., 1994). Here the mutation has disrupted the hydrogen bond between Tyr201 (now Phe) and Tyr385, allowing Tyr385 to donate a hydrogen bond to the bridging water molecule (see Figure 6).

Having established beyond reasonable doubt that a bridging water molecule is present in the 4-aminobenzoate complexes, one might ask why such a bridging water molecule is not present in the 4-hydroxybenzoate complexes. Analysis of the 4-hydroxybenzoate and 4-aminobenzoate complexes suggests that the presence of the bridging water molecule is determined by the strength of the hydrogen bonds with its two neighbors. In the 4-aminobenzoate complex, the bridging water molecule seems to make a reasonably strong hydrogen bond with Tyr385 (2.8 Å) and a somewhat weaker hydrogen bond with the water next to His72 (3.0 Å). In the 4-hydroxybenzoate complex, the tyrosines 201 and 385 are approximately 0.3 Å closer to the substrate than in the 4-aminobenzoate complex due to the longer NH–OH hydrogen bond in the latter complex. As a result, the distance between the hydroxyl group of Tyr385 and water next to His72 (W1 in Figures 2 and 6) is 0.4 Å longer in the 4-hydroxybenzoate complex. Superposition of the 4-aminobenzoate and 4-hydroxybenzoate complexes suggests that, because of this greater distance between Tyr385 and the water next to His72, the hydrogen bonds of a bridging water would become longer and presumably weaker. The result would be a water molecule situated in a restricted pocket fixed only by two weak hydrogen bonds. Such a situation is both thermodynamically and energetically unfavorable and could explain why a bridging water molecule has never been observed in the presence of substrate (analogues) bearing a 4-hydroxyl group. The bridging water molecule observed in the Tyr201Phe mutant (Lah et al., 1994) could be explained by the fact that no hydrogen bond is present between Tyr201 (now a Phe) and Tyr385, allowing Tyr385 to move closer to the bridging water molecule (see Figure 6).

Has the bridging water molecule a catalytic function? Although no firm experimental data are currently available, it is tempting to speculate that it might serve such a role. The bridging water molecule is not observed in the crystal structure of the enzyme–substrate complex, but it is unlikely that such a water molecule would not transiently also be present in the normal enzyme–substrate complex. As has been argued by Lah et al. (1993), the continuous network of hydrogen bonds

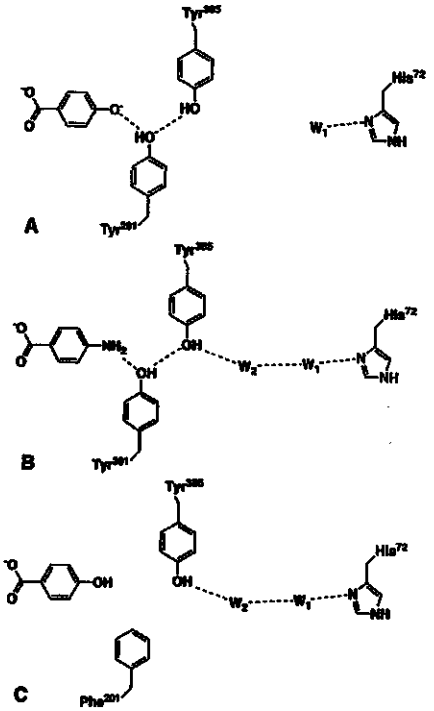

FIGURE 6: Schematic drawing of the active site hydrogen-bonding network in various structures of *p*-hydroxybenzoate hydroxylase. Protons cannot be determined by protein crystallography so their positions have been inferred. (A) 4-Hydroxybenzoate complex (Schreuder et al., 1989). Biochemical studies have established that the substrate binds as its phenolate anion, which means that the protons of Tyr201 and Tyr385 must be directed toward the substrate as drawn. The direction of the hydrogen-bonding network would not change if the substrate would be in its phenol form, because then the O4 of the substrate would most likely donate a hydrogen bond to the carbonyl oxygen of Pro293 [see Schreuder et al. (1989)]. No water is present between Tyr385 and W1. (B) 4-Aminobenzoate complex (this study). The 4-amino group will donate a hydrogen bond to Tyr201, and consequently the direction of the hydrogen bond between Tyr201 and Tyr385 will change as well. An extra water molecule (W2) is present between Tyr385 and W1, resulting in a continuous hydrogen-bonding network between the 4-amino group of the substrate analogue and the ND1 of His72. (C) Hydrogen-bonding network in the Tyr201Phe mutant of the enzyme from *P. aeruginosa* (Lah et al., 1994) complexed with the substrate. Also in this structure an extra water molecule is observed between Tyr385 and W1.

transiently present between the 4-hydroxyl group of the substrate and His72 may serve as a "proton wire", like similar proton channels observed in enzymes ranging from serine proteases to rhodopsin (Meyer, 1992), which transport protons in and out of the active site. The 4-hydroxy group of the substrate gets deprotonated when it binds to the active site (Shoun et al., 1979; Entsch et al., 1991; Eschrich et al., 1993), and the transport of the 4-hydroxyl proton out of the active site might proceed via this hydrogen-bonding network.

One might, however, argue that the long hydrogen bonds inferred from the modeling mentioned above would preclude efficient proton transport. This need not be true since the transient bridging water molecule will be dynamic, and it may oscillate from a state where it makes a strong hydrogen with Tyr385 and no hydrogen bond with W2 to a state with no hydrogen bond with Tyr385 but with a strong hydrogen bond with W2. Additional studies such as site-directed mutagenesis of His72 are required to establish whether or not the proton channel really serves a role in catalysis or whether it is merely a curiosity.

The second major finding of this study is that the flavin is able to slide out of the active site. As will be discussed below, experimental evidence exists that this is functional because it allows the substrate to enter and the product to leave the active site. The substrate is completely buried when the flavin occupies the "in" position, and we have previously argued that the substrate may enter and the product may leave the active site via a path near Arg214 and the dimethylbenzene part of the FAD, but that a movement of side chains or even a shift of domains seemed to be necessary to accomplish this (Schreuder et al., 1988b). The present results suggest that it is the flavin ring which moves away to allow entrance of the substrate and exit of the product. Analysis of the solvent-accessible surface using the program WHATIF (Vriend, 1990; Voorintholt et al., 1989) reveals that the solvent channel near the flavin N10 extends to the substrate binding pocket when the flavin is in the "out" orientation.

Experimental evidence that the flavin ring occupies the "out" position when the substrate (analogues) bind to the active site comes from the regioselectivity of the hydroxylation of the substrate analogue 2,4-dihydroxybenzoate. The 2,4-dihydroxybenzoate molecule is only hydroxylated at the 3-position, not at the 5-position (Spector & Massey, 1972). Frontier orbital calculations indicate that both positions are almost equally reactive toward hydroxylation (Vervoort et al., 1992). This means that the 2,4-dihydroxybenzoate molecule must bind in only one specific orientation in the active site, the orientation in which the 2-hydroxyl group points toward the flavin ring (the observed orientation, see Figure 7a), and not in the alternative orientation, in which the 2-hydroxyl group would point away from the flavin ring (see Figure 7b). We analyzed the theoretical contacts of the 2-hydroxyl group of the 2,4-dihydroxybenzoate molecule in each of its two possible orientations with the flavin in the "in" position (see the legend to Figure 7). The results indicate that a short contact of 3.0 Å with the flavin C6 would be present in the observed orientation and short contacts of 3.1 Å with the CG2 of Val47 and of 2.9 Å with the CD2 of Leu199 would be present in the alternative orientation. In both cases, rearrangements in the order of 0.5 Å are required to relieve the short contacts. It is difficult to envisage why a 0.5-Å readjustment would only be possible for the observed orientation and not for the alternative orientation. We therefore conclude that there are no apparent steric reasons for a preferred binding orientation of the 2,4-dihydroxybenzoate molecule in the active site when the flavin occupies the "in" position.

The situation becomes completely different when the flavin occupies the "out" position. The short contacts with the Val47 and Leu199 side chains will still be present for the alternative orientation, but the short contact with the flavin in the observed binding mode is absent because the flavin is not present in the active site (Figure 7c). On the contrary, the 2-hydroxyl group of the substrate analogue now makes a strong hydrogen bond with the N3 of the flavin. Both the absence of the short contact and the presence of the hydrogen bond with the flavin N3 would favor the observed orientation, strongly suggesting that the flavin is in the "out" position when the substrate analogue binds to the active site. The hydrogen bond between the 2-hydroxyl group of the substrate analogue and the N3 of the flavin could also explain why the 2-hydroxy-4-aminobenzoate

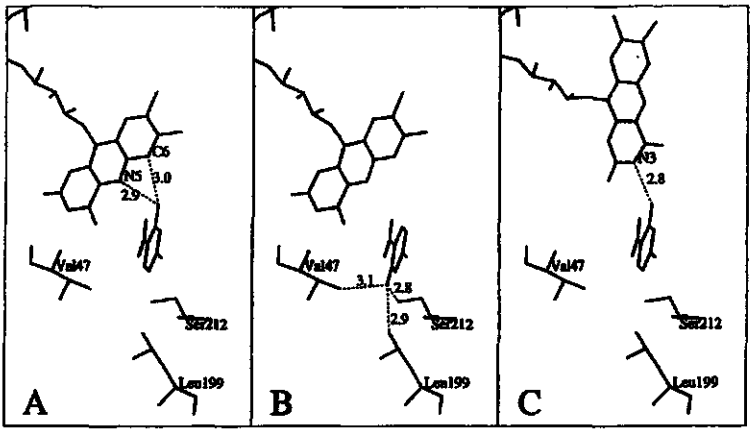

FIGURE 7: Theoretical contacts between the O2 of the 2,4-dihydroxybenzoate molecule and *p*-hydroxybenzoate hydroxylase with the flavin in the "in" position, and observed contacts with the flavin in the "out" position. The theoretical contacts of the observed orientation were obtained by superimposing the structures of the 4-hydroxybenzoate complex (Schreuder et al., 1989) and the 2,4-dihydroxybenzoate complex (this study). The contacts of the alternative orientation were obtained by rotating the 2,4-dihydroxybenzoate molecule 180° around the C1–C6 axis (see Experimental Procedures). (A) Theoretical contacts in the observed orientation; (B) theoretical contacts in the alternative binding mode; (C) observed contacts in the 2,4-dihydroxybenzoate–wild-type complex.

molecule binds more strongly to the Tyr222Ala mutant than the 4-hydroxybenzoate, which cannot make this hydrogen bond.

Is it possible that the flavin is reduced in the "out" position? Given the limited space present in the active site to fit a nicotinamide ring [see Schreuder et al. (1990)] and the multiple reactions (flavin reduction, formation of the flavin peroxide, and substrate hydroxylation) which have to be performed by the enzyme in a single active site, it is tempting to speculate that the "out" position would be the site for the reduction of the flavin by NADPH. The cleft leading to the active site is much wider than the narrow active site pocket, and the *si*-side of the flavin ring is shielded by the side chain of Arg44, which is compatible with the observed reduction at the *re*-side of the flavin ring (Manstein et al., 1986). Shifting of the flavin conformation towards the "out" position by binding of substrate or effector molecules could then explain the increase in reduction rate induced by these effector compounds.

However, available evidence argues against reduction of the flavin in the "out" position. The crystal structures show that the 2,4-dihydroxybenzoate molecule shifts the equilibrium position of the flavin ring strongly toward the "out" position due to the presence of the 2-hydroxyl group. If the flavin would be reduced in the "out" position, one would expect a considerable increase in reduction rate in the presence this substrate analogue. This is not observed. Kinetic measurements indicate that 2,4-dihydroxybenzoate is only a weak effector and that it stimulates the reduction rate much less than the normal substrate 4-hydroxybenzoate (Eschrich et al., 1993). These observations argue against a reduction of the flavin in the "out" position and suggest therefore that the reduction occurs with the flavin in another orientation, which may be the "in" orientation or another not yet observed third orientation. The less likely alternative would be that the flavin is reduced in the "out" position and that the reduction rate is governed by some unknown effects. Compounds like 2,4-dihydroxybenzoate should then somehow inhibit the flavin reduction while exposing the flavin toward NADPH.

The final question we want to address is the reason why the flavin ring is apparently able to easily slide in and out of the active site, despite multiple van der Waals contacts and hydrogen bonds with the protein in the "in" position (see Table 3). The binding energy is determined by the free energy difference between solvated enzyme and solvated ligand versus the enzyme–ligand complex. Indeed, the flavin interacts with several solvent molecules when it is in the "out" position and three solvent molecules occupy the empty flavin binding pocket, offsetting possibly to some extent the lost flavin–protein interactions. The flavin binding may also be influenced by the twisted conformation of the flavin in the "in" position (see Table 4). Modeling studies show that this twisted flavin conformation resembles the conformation of the flavin–4a-hydroperoxide reaction intermediate (Schreuder et al., 1990). The enzyme may facilitate the formation of the flavin–4a-hydroperoxide by forcing the flavin into a twisted conformation. The energy needed for this deformation has to come from the binding energy and will contribute to the observed weak binding of the flavin ring. Indeed, in agreement with this notion, the flavin is almost planar in the "out" position, especially in the most accurately determined structure of the 2,4-dihydroxybenzoate complex. Weak binding of the flavin ring also explains why an ADPR molecule could easily displace the FAD in the crystals of the *p*-hydroxybenzoate hydroxylase–ADPR complex (van der Laan et al., 1989a).

In summary, these studies and also the studies by Lah et al. (1993) on the *P. aeruginosa* enzyme have established the presence of a water channel in *p*-hydroxybenzoate hydroxylase which may or may not assist in the deprotonation of 4-hydroxyl group of the substrate when it binds to the active site. They also reveal an alternative binding mode for the flavin ring which may provide an entrance for the substrate and an exit for the product molecule.

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Chapter 6

Flavin motion in *p*-hydroxybenzoate hydroxylase
Substrate and effector specificity of the Tyr222Ala mutant

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Flavin motion in *p*-hydroxybenzoate hydroxylase
Substrate and effector specificity of the Tyr222→Ala mutant

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The side chain of Tyr222 in *p*-hydroxybenzoate hydroxylase interacts with the carboxy moiety of the substrate. Studies on the Tyr222→Phe mutant, [F222]*p*-hydroxybenzoate hydroxylase, have shown that disruption of this interaction hampers the hydroxylation of 4-hydroxybenzoate. Tyr222 is possibly involved in flavin motion, which may facilitate the exchange of substrate and product during catalysis. To elucidate the function of Tyr222 in more detail, in the present study the substrate and effector specificity of the Tyr222→Ala mutant, [A222]*p*-hydroxybenzoate hydroxylase, was investigated.

Replacement of Tyr222 by Ala impairs the binding of the physiological substrate 4-hydroxybenzoate and the substrate analog 4-aminobenzoate. With these compounds, [A222]*p*-hydroxybenzoate hydroxylase mainly acts as a NADPH oxidase. [A222]*p*-hydroxybenzoate hydroxylase tightly interacts with 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate. Crystallographic data [Schreuder, H. A., Mattevi, A., Obmolova, G., Kalk, K. H., Hol, W. G. J., van der Bolt, F. J. T. & van Berkel, W. J. H (1994) *Biochemistry 33*, 10161–10170] suggest that this is due to motion of the flavin ring out of the active site, allowing hydrogen-bond interaction between the 2-hydroxy group of the substrate analogs and N3 of the flavin. [A222]*p*-Hydroxybenzoate hydroxylase produces about 0.6 mol 2,3,4-trihydroxybenzoate from 2,4-dihydroxybenzoate/mol NADPH oxidized. This indicates that reduction of the Tyr222→Ala mutant shifts the equilibrium of flavin conformers towards the productive 'in' position.

[A222]*p*-Hydroxybenzoate hydroxylase converts 2-fluoro-4-hydroxybenzoate to 2-fluoro-3,4-dihydroxybenzoate. The regioselectivity of hydroxylation suggests that [A222]*p*-hydroxybenzoate hydroxylase binds the fluorinated substrate in the same orientation as wild-type. Spectral studies suggest that wild-type and [A222]*p*-hydroxybenzoate hydroxylase bind 2-fluoro-4-hydroxybenzoate in the phenolate form with the flavin ring preferring the 'out' conformation. Despite activation of the fluorinated substrate and in contrast to the wild-type enzyme, [A222]*p*-hydroxybenzoate hydroxylase largely produces hydrogen peroxide.

The effector specificity of *p*-hydroxybenzoate hydroxylase is not changed by the Tyr222→Ala replacement. This supports the idea that the effector specificity is mainly dictated by the protein–substrate interactions at the *re*-side of the flavin ring.

Keywords: aromatic hydroxylase; flavin mobility; monooxygenase; site-specific mutagenesis; substrate specificity.

p-Hydroxybenzoate hydroxylase is a member of the class of flavin-dependent monooxygenases (van Berkel and Müller, 1991). The enzyme catalyzes the conversion of 4-hydroxybenzoate to 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil bacteria (Stanier and Ornston, 1973).

The reaction mechanism of *p*-hydroxybenzoate hydroxylase has been studied in detail (Entsch and Ballou, 1989). In the first part of the reaction (Scheme 1), the substrate acts as an effector, strongly stimulating the rate of enzyme reduction by NADPH (Husain and Massey, 1979). After $NADP^+$ release, the reduced flavin rapidly reacts with oxygen to form the labile flavin (C4a)-hydroperoxide. This intermediate attacks the substrate to form the product 3,4-dihydroxybenzoate. Although many kinetic details of the reaction are known, it is unclear how the enzyme controls the reactivity of the flavin and the various reactants in order to achieve efficient catalysis (Entsch and van Berkel, 1995).

The crystal structure at 0.19-nm resolution of the enzyme-substrate complex (Schreuder et al., 1989) shows that the substrate is located in a relative hydrophobic environment, with its aromatic nucleus approximately perpendicular to the isoalloxazine ring of the FAD (Fig. 1). Site-specific mutagenesis studies on the enzymes from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* have addressed the role of the amino acid residues involved in substrate binding. The side chains of Tyr201 and Tyr385 form a H-bond network with the 4-hydroxy moiety of the substrate at the *re*-side of the flavin ring (Fig. 1). This H-bond network facilitates the deprotonation of the phenol required

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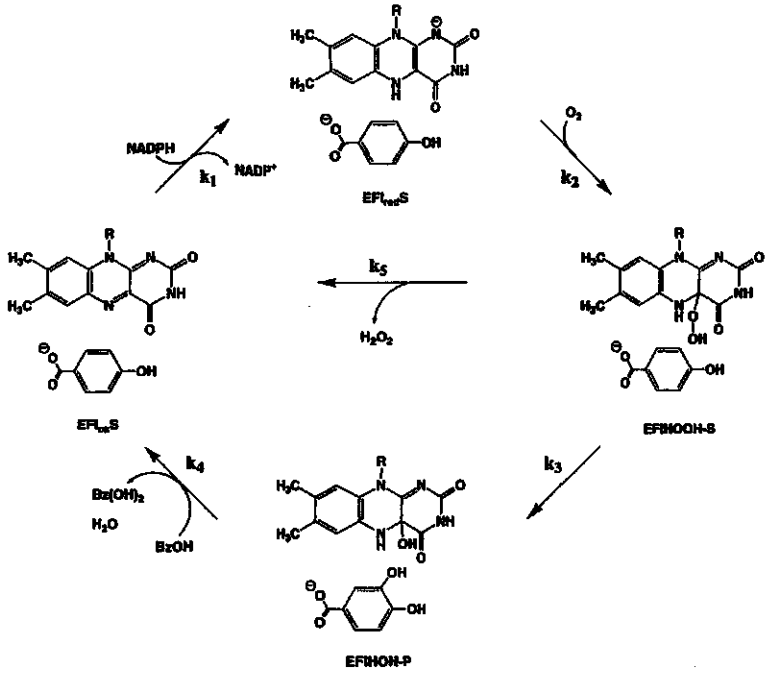
Abbreviations. [A222]*p*-hydroxybenzoate hydroxylase, *p*-hydroxybenzoate hydroxylase with Tyr222 replaced by Ala; similarly for Tyr222→Phe or Val.

Enzymes. p-Hydroxybenzoate hydroxylase (EC 1.14.13.2); catalase, hydrogen-peroxide oxidoreductase (EC 1.11.1.6); glucose oxidase, β-D-glucose: oxygen 1-oxidoreductase (EC 1.1.3.4); glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP 1-oxidoreductase (EC 1.1.1.49).

Scheme 1. Reaction cycle of *p*-hydroxybenzoate hydroxylase. BzOH, 4-hydroxybenzoate; $Bz(OH)_2$, 3,4-dihydroxybenzoate; k_1, reduction of enzyme-substrate complex; k_2, formation of flavin (C4a)-hydroperoxide; k_3, substrate hydroxylation; k_4, dehydration of flavin (C4a)-hydroxide and product release; k_5, uncoupling of hydroxylation.



for rapid hydroxylation (van Berkel and Müller, 1989; Schreuder et al., 1990, 1994; Entsch et al., 1991; Vervoort et al., 1992; Eschrich et al., 1993; Lah et al., 1994). Tyr201 and Tyr385 are also important for the effector specificity and in preventing the further hydroxylation of the aromatic product (Schreuder et al., 1989; Entsch et al., 1991; Eschrich et al., 1993).

Ser212, Arg214 and Tyr222 are located at the *si*-side of the flavin ring and point with their side chains to the carboxy moiety of the substrate (Fig. 1). Arg214 forms a salt bridge with the substrate. Arg214 substitutions have confirmed that this ion-pair interaction is indispensable for the tight binding of the substrate (van Berkel et al., 1992). Ser212 is located far away from the flavin. Preliminary studies on mutant Ser212→Ala suggest that the main role of Ser212 is to strengthen substrate binding (van Berkel et al., 1994a).

Tyr222 is located close to C6 of the flavin isoalloxazine ring in a solvent-accessible region. The hydroxyl group of Tyr222 is at short distance of one of the carboxyl oxygens of the substrate, suggesting a strong hydrogen bond (Schreuder et al., 1989). Recent crystallographic data suggest that Tyr222 is involved in flavin motion which may provide a path for the exchange of substrates and products during catalysis. This is concluded from the observation that in the crystal structures of mutant Tyr222→Ala complexed with 2-hydroxy-4-aminobenzoate (Schreuder et al., 1994) and mutant Tyr222→Phe complexed with 4-hydroxybenzoate (Gatti et al., 1994), the flavin ring is located outside the active site (Fig. 2). The 'out' position of the flavin is not solely induced by the Tyr222 substitutions. A comparable shift of the isoalloxazine ring was observed in the crystal structures of wild-type complexed with either 2,4-dihydroxybenzoate (Schreuder et al., 1994; Gatti et al. 1994) or 2-hydroxy-4-aminobenzoate (Schreuder et al., 1994) and in the crystal structure of the enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase reconstituted with arabino-FAD (van Berkel et al., 1994c).

Replacement of Tyr222 by Phe or Ala (Entsch et al., 1994; van Berkel et al., 1994a) results in inefficient hydroxylation of 4-hydroxybenzoate. Kinetic studies revealed that this is due to the instability of the flavin (C4a)-hydroperoxide and a diminished rate of oxygen transfer (Entsch et al., 1994). From crystallographic data it was argued that the uncoupling of hydroxylation in [F222]*p*-hydroxybenzoate hydroxylase is associated with the tendency of the flavin to occupy the 'out' position (Gatti et al., 1994). In this paper we report on the substrate and effector specificity of [A222]*p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. The results presented are discussed in relation to the corresponding properties of the wild-type enzyme and in relation to the flavin movement observed in the crystal structures. Some preliminary results have been reported elsewhere (van Berkel et al., 1994a).

MATERIALS AND METHODS

General. Chemicals used have been described elsewhere (Eschrich et al., 1993). 2-Fluoro-4-hydroxybenzoate was synthesized and purified as reported earlier (van Berkel et al., 1994b).

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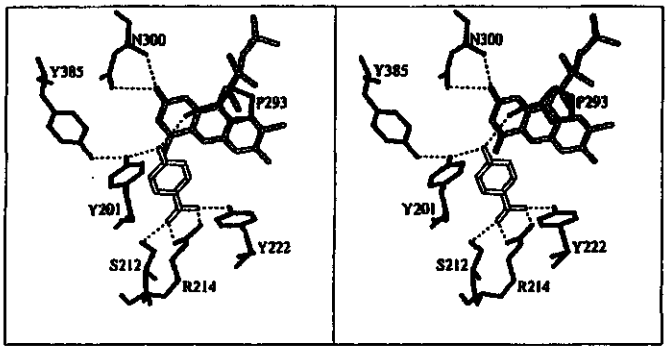

Fig. 1. Schematic representation of the active site of the enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Data according to the crystal structure refined to 0.19-nm resolution (Schreuder et al., 1989). The FAD is grey, the aromatic substrate is white, and protein residues are black. The *si* face of the flavin is the back of the page in this view; the *re* side is the front, as seen with the substrate phenol pointing out of the page.

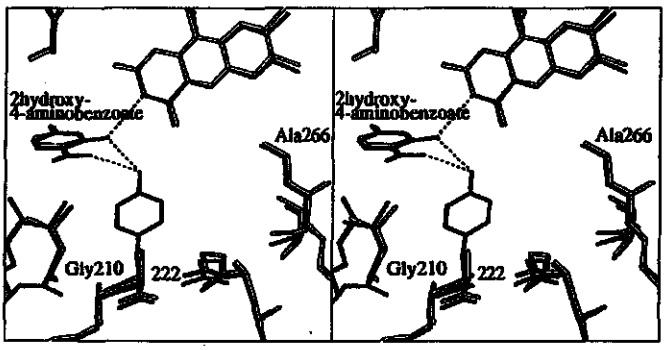

Fig. 2. Superposition of the structures of the 2-hydroxy-4-aminobenzoate complexes of wild-type and [A222]*p*-hydroxybenzoate hydroxylase. Data according to the crystal structures determined at 0.28-nm and 0.27-nm resolution (Schreuder et al., 1994). The wild type is drawn with solid lines and the Tyr222→Ala mutant with open bonds. Dashed lines show hydrogen bonds between the OH of Tyr222 and the O2 and carboxyl oxygen of the substrate analog and a hydrogen bond between the O2 of the analog and the N3 of the flavin ring. The flavin ring has the 'out' conformation in both complexes.

Optical (difference) spectra were recorded on an Aminco DW-2000 spectrophotometer. Fluorescence binding studies were performed on an Aminco SPF-500C spectrofluorimeter. ^{19}F-NMR spectra were recorded on a Bruker AMX 300 NMR-spectrometer (Peelen et al., 1993). Rapid-reaction kinetics were carried out using a High-Tech Scientific SF-51 stopped-flow spectrophotometer (Eschrich et al., 1993). All instruments were thermostatted at 25°C, unless stated otherwise.

Preparation of enzymes. Site-specific mutagenesis was performed according to the method of Kunkel et al. (1987), essentially as described elsewhere (van Berkel et al., 1992). Mutations were introduced into the *Escherichia coli* gene encoding the microheterogeneity-resistant Cys116→Ser mutant protein (Eschrich et al., 1990, 1993). The oligonucleotide GCCGCTAC-GYCGTACAGGTGC was used for construction of Tyr222 mutants, where Y is either C ([A222]*p*-hydroxybenzoate hydroxylase) or T ([V222]*p*-hydroxybenzoate hydroxylase). The mutations were confirmed by nucleotide sequencing using the M13 dideoxy-chain-termination method (Sanger et al., 1977). Mutant proteins were expressed in transformed *E. coli* TG2 and purified at pH 8.0 (Van Berkel et al., 1992). The expression and yield of the mutant proteins is in the same range as found for wild type (Eschrich et al., 1990; van Berkel et al., 1992). Initial analysis of the mutants revealed marginal differences between the kinetic and spectral properties of [A222] and [V222]*p*-hydroxybenzoate hydroxylase. From this it was decided to study the Tyr222→Ala mutant in further detail.

Analytical methods. Kinetic experiments were performed at 25°C in 100 mM Tris/sulfate pH 8.0 (Eschrich et al., 1993) unless stated otherwise. The anaerobic reduction of enzyme-substrate complexes by NADPH was followed at 450 nm using the stopped-flow spectrophotometer. Steady-state and rapid reaction kinetic parameters were determined as described (Eschrich et al., 1993).

The hydroxylation efficiency of mutant proteins was determined from oxygen consumption experiments performed in the absence or presence of catalase (Eschrich et al., 1993). Aromatic products were identified and quantified by reverse-phase HPLC (Entsch et al., 1991). Formation of 3-hydroxy-4-aminobenzoate from 4-aminobenzoate was determined colorimetrically (Entsch

Table 1. Dissociation constants of complexes between [A/V222]*p*-hydroxybenzoate hydroxylase and benzoate ligands. Dissociation constants (standard error < 10%) were determined fluorimetrically at 25°C in 100 mM Tris/SO_4 pH 8.0. The fluorescence quantum yield (Q) is expressed relative to that of uncomplexed wild-type enzyme (100%). All experiments were performed in duplicate.

Substrate (analog)	Dissociation constant (fluorescence quantum yield) of the complex		
	wild-type (Q=100)	A222 (Q=200)	V222 (Q=117)
	mM (%)		
4-Hydroxybenzoate	0.04 (20)	1.30 (36)	1.10 (39)
2-Hydroxybenzoate	0.20 (40)	0.18 (22)	0.13 (14)
2,4-Dihydroxybenzoate	0.09 (25)	0.08 (8)	0.07 (11)
3,4-Dihydroxybenzoate	0.28 (10)	1.00 (4)	1.50 (8)
4-Aminobenzoate	0.02 (12)	1.60 (20)	1.50 (21)
2-Hydroxy-4-aminobenzoate	0.03 (10)	0.02 (22)	0.02 (8)

et al., 1976). The same colorimetric procedure was used to measure the enzymatic conversion of 2-hydroxy-4-aminobenzoate. The orange color, formed upon complexation of 2,3-dihydroxy-4-aminobenzoate with potassium ferricyanide, was only transiently stable, probably due to air oxidation of the dihydroxylated product (Bhattacharyya and Seymour, 1950). ^{19}F-NMR product analysis was done as described (van Berkel et al., *1994b*).

Dissociation constants of enzyme/ligand complexes were determined fluorimetrically (van Berkel et al., 1992). Molar absorption coefficients of protein-bound flavin were determined by recording optical spectra in the absence and presence of 0.1% SDS (Entsch et al., 1991). Flavin perturbation difference spectra were recorded essentially as described previously (van Berkel et al., 1992). The ionization state of enzyme-bound 2-fluoro-4-hydroxybenzoate was measured by recording ultraviolet absorption difference spectra as a function of ligand concentration and of pH (Eschrich et al., 1993). Binding studies as a function of pH were performed in 40 mM Mes (pH 5–7) and 40 mM Hepes (pH 7–8). Buffers were adjusted to 50 mM ionic strength with 0.5 M sodium sulfate (Wijnands et al., 1984).

RESULTS

Binding studies. The side chain of Tyr222 in *p*-hydroxybenzoate hydroxylase interacts with the carboxy moiety of the substrate (Fig. 1). The effect of removing the aromatic side chain on substrate binding was tested by fluorimetric titration experiments. As can be seen from Table 1, replacement of Tyr222 with Ala or Val drastically decreases the affinity for 4-hydroxybenzoate, 4-aminobenzoate and 3,4-dihydroxybenzoate. Substrate analogs with a 2-hydroxy group interact more strongly with the Tyr222 mutants and the dissociation constants of these enzyme-ligand complexes are comparable to wild-type (Table 1). A plausible explanation for the relatively tight binding of the 2-hydroxybenzoate ligands is provided by crystallographic data. The structures of 2-hydroxy-4-aminobenzoate complexed wild-type and [A222]*p*-hydroxybenzoate hydroxylase show nearly indistinguishable binding modes for this substrate analog (Schreuder et al., 1994). In both 2-hydroxy-4-aminobenzoate complexes, the flavin ring occupies the 'out' conformation with the flavin N3 in close contact to the 2-hydroxy group of the substrate analog (Fig. 2). The same substrate–flavin interaction is present in the

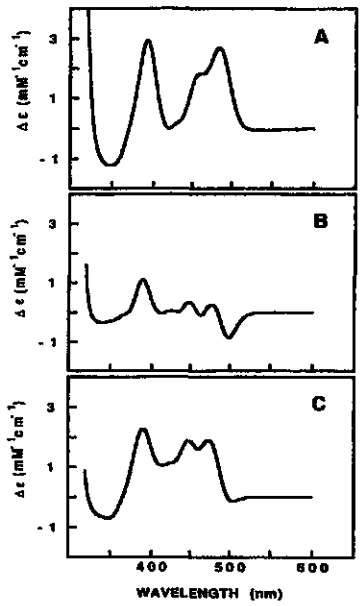

Fig. 3. Flavin perturbation difference spectra induced upon binding of substrates to wild-type or [A222]*p*-hydroxybenzoate hydroxylase. The absorption spectra were recorded at 25°C in 100 mM Tris/SO_4 pH 8.0. The enzyme concentration was about 40 μM. The absorption difference spectra are extrapolated to infinite substrate concentrations. (A) [A222]*p*-Hydroxybenzoate hydroxylase complexed with 2-hydroxy-4-aminobenzoate; (B) wild-type complexed with 4-aminobeazoate; (C) [A222]*p*-hydroxybenzoate hydroxylase complexed with 4-hydroxybenzoate.

structure of wild-type complexed with 2,4-dihydroxybenzoate (Schreuder et al., 1994; Gatti et al., 1994) supporting the view that in [A222]*p*-hydroxybenzoate hydroxylase, the absence of H-bond interaction between the side chain of residue 222 and the carboxy moiety of the substrate (cf. Fig. 1) is compensated by hydrogen-bond formation between the 2-hydroxy group of the substrate analogs and the N3 of the flavin ring (Fig. 2).

The flavin conformation observed in the crystal structures correlates with the optical difference spectrum induced upon substrate binding (Gatti et al., 1994). This property was used to study in more detail the interaction of the Tyr222 mutants with 4-hydroxybenzoate, 4-aminobenzoate, 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate. Replacement of Tyr222 by Ala or Val does not significantly change the optical properties of the uncomplexed enzyme. Unfolding experiments reveal molar absorption coefficients, $\varepsilon_{278} = 8.5$ mM^{-1} cm^{-1} and $\varepsilon_{450} = 10.2$ mM^{-1} cm^{-1} for the absorption maxima of the flavin in both Tyr222 mutants which are the same values as reported for the wild type (van Berkel et al., 1992). Binding of 2,4-dihydroxybenzoate or 2-hydroxy-4-aminobenzoate to wild-type or mutants results in nearly identical spectral perturbations. As an example, the flavin difference spectrum obtained upon binding of 2-hydroxy-4-aminobenzoate to [A222]*p*-hydroxybenzoate hydroxylase is presented in Fig. 3A. This difference spectrum reflects the flavin 'out' conformation (Gatti et al., 1994), which is in accordance with crystallographic data (Schreuder et al., 1994). In analogy, binding of 4-aminobenzoate (or 4-hydroxybenzoate)

Table 2. Catalytic properties of [A/V222]*p*-hydroxybenzoate hydroxylase with 4-hydroxybenzoate. Kinetic parameters were determined at 25°C in 100 mM Tris/SO_4 pH 8.0. Turnover rates (k_{cat}) are maximum values extrapolated to infinite concentrations of 4-hydroxybenzoate and NADPH. Reduction rates (k_{red}) and dissociation constants for NADPH (K_d NADPH) were determined from anaerobic stopped-flow experiments performed in the presence of 5 mM 4-hydroxybenzoate. Product, 3,4-dihydroxybenzoate. B_2OH, 4-hydroxybenzoate. Results for the wild-type enzyme are taken from van Berkel et al. (1992).

Enzyme	Product	k_{red}	k_{cat}	K_d NADPH	K_m NADPH	K_m B_2OH
	%	s^{-1}		mM		
Wild-type	98	300	55	0.15	0.07	0.02
A222	5	55	30	0.43	0.28	0.33
V222	9	51	10	0.59	0.39	0.18

Table 3. Catalytic properties of [A/V222]*p*-hydroxybenzoate hydroxylase with 2,4-dihydroxybenzoate. Apparent rate constants were determined at 25°C in 100 mM Tris/SO_4 pH 8.0, containing 0.5 mM EDTA, 0.2 mM NADPH and 2.5 mM 2,4-dihydroxybenzoate. Product, 2,3,4-trihydroxybenzoate.

Enzyme	k'_{red}	k'_{cat}	Product
	s^{-1}		%
Wild-type	1.1	0.7	87
A222	0.9	0.6	57
V222	0.3	0.2	64

to the wild type results in spectral perturbations reflecting the flavin 'in' conformation (Fig. 3B). It should be mentioned here that the shape of the difference spectrum of the wild-type enzyme complexed with 4-hydroxybenzoate is somewhat dependent on the pH of the solution (van Berkel and Müller, 1989). These results support the proposal that in the oxidized state, binding of 2-hydroxybenzoate ligands shifts the equilibrium of flavin conformers towards the 'out' position. Binding of 4-hydroxybenzoate or 4-aminobenzoate to the Tyr222 mutants is accompanied with spectral changes, intermediate between the changes observed for the 'in' and 'out' conformations (Fig. 3C). Although the affinity of [A/V222]*p*-hydroxybenzoate hydroxylase for 4-hydroxybenzoate and 4-aminobenzoate is rather weak, this suggests that removal of the aromatic side chain at position 222 influences the mutual orientation of these substrates and the flavin.

Catalytic properties with 4-hydroxybenzoate and 2,4-dihydroxybenzoate. In the presence of 4-hydroxybenzoate, [A222] and [V222]*p*-hydroxybenzoate hydroxylase largely act as oxidases forming hydrogen peroxide as the main product (Table 2). From rapid reaction studies, performed with [A222]*p*-hydroxybenzoate hydroxylase from *P. aeruginosa* at pH 6.6 and 4°C, it was suggested that this is due both to a slow rate of hydroxylation and the instability of the flavin hydroperoxide (Entsch et al., 1994). As can be seen from Table 2, replacement of Tyr222 by Ala or Val results in increased K_m values for the substrate and NADPH. Although the affinity of 4-hydroxybenzoate for the reduced states is unknown, this suggests that the oxidase activity of the Tyr222 mutants partially results from weak substrate binding (cf. Table 1).

HPLC analysis revealed that [A222] and [V222]*p*-hydroxybenzoate hydroxylase exclusively form 2,3,4-trihydroxybenzoate from 2,4-dihydroxybenzoate. The hydroxylation of 2,4-dihydroxybenzoate by the Tyr222 mutants is far more efficient than that of 4-hydroxybenzoate (cf. Table 2), yielding more than 50% of aromatic product per catalytic cycle (Table 3). This confirms that tight binding of substrates (cf. Table 1) is a prerequisite for efficient hydroxylation (van Berkel et al., 1992) and furthermore shows that the lack of an aromatic side chain at position 222 does not prohibit the reduced flavin from attaining the productive 'in' conformation. The percentage of hydroxylation of 2,4-dihydroxybenzoate by [A222]*p*-hydroxybenzoate hydroxylase is considerably lower than with the wild type (Table 3), confirming that Tyr222 is important for the stability and/or reactivity of the flavin hydroperoxide.

Effector specificity. The effector role of 4-hydroxybenzoate is not lost in the Tyr222 mutants. Table 2 shows that in the presence of 4-hydroxybenzoate, the maximal rate of reduction of the Tyr222 mutants by NADPH is about 50 s^{-1}. Binding of 4-hydroxybenzoate stimulates the rate of reduction of [A222] and [V222]*p*-hydroxybenzoate hydroxylase by about four orders of magnitude as deduced from the very slow reduction of the free mutants. As discussed elsewhere (Entsch and van Berkel, 1995), this suggests that the hydrogen-bond network connected to the phenolic moiety of the substrate is conserved in the Tyr222 mutants. In the presence of 2,4-dihydroxybenzoate, the apparent rate of turnover of the Tyr222 mutants is in the same range as found for the wild type (Table 3). With this substrate analog, reduction is rate limiting in catalysis (Table 3) and about two orders of magnitude slower than with 4-hydroxybenzoate (cf. Table 2).

From Tables 1–3 it is clear that replacement of Tyr222 by Ala or Val results in nearly identical properties. In view of the available crystallographic data (Schreuder et al., 1994), [A222]*p*-hydroxybenzoate hydroxylase was selected for further studies. Binding of 4-aminobenzoate hardly stimulates the rate of reduction of the Tyr222→Ala mutant (data not shown). Similar results were obtained for the wild type (Entsch et al., 1976) and other active-site mutants (Eschrich et al., 1993). As noted before (Entsch et al., 1991), the poor effector role of 4-aminobenzoate provides an important metabolic control function *in vivo*, preventing both the conversion of this growth factor and the wasteful utilization of valuable reducing equivalents. The substrate analog 2-hydroxy-4-aminobenzoate completely fails to stimulate the rate of reduction of wild-type and [A222]*p*-hydroxybenzoate hydroxylase (data not shown). Because of tight binding (Table 1), this compound is a strong competitive inhibitor. Crystallographic data suggest that the 4-amino group induces small changes in the H-bond network formed by the substrate and the side chains of Tyr201 and Tyr385 (Schreuder et al., 1994). Therefore, and in accordance with other mutagenesis studies (Entsch et al., 1991; Eschrich et al., 1993), this H-bond network presumably plays a crucial role in the as yet unknown conformational changes involved in the reduction reaction (Entsch and van Berkel, 1995). From the comparably poor effector roles of 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate in wild-type and [A222]*p*-hydroxybenzoate hydroxylase

Table 4. Hydroxylation efficiency of wild-type and [A222]*p*-hydroxybenzoate hydroxylase with substrate analogs. Hydroxylation efficiencies were determined at pH 8.0. For experimental details see Materials and Methods.

Substrate (analog)	Hydroxylation efficiency of	
	wild-type	A222
	%	
4-Hydroxybenzoate	98 ± 2	5 ± 2
4-Aminobenzoate	90 ± 5	3 ± 2
2,4-Dihydroxybenzoate	87 ± 5	57 ± 5
2-Hydroxy-4-aminobenzoate	80 ± 10	30 ± 10
2-Fluoro-4-hydroxybenzoate	95 ± 5	20 ± 5

we propose that, in the oxidized state, the type of substrate predominantly dictates the thermodynamically most favorable flavin conformation (cf. Fig. 3).

Conversion of 4-aminobenzoate and 2-hydroxy-4-aminobenzoate. Because 4-aminobenzoate and 2-hydroxy-4-aminobenzoate are very poor effectors for *p*-hydroxybenzoate hydroxylase, the enzymatic conversion of these potential substrates was tested by single-turnover experiments. As reported earlier (Entsch et al., 1976), wild-type enzyme efficiently converts 4-aminobenzoate to 4-amino-3-hydroxybenzoate (Table 4). In contrast, [A222]*p*-hydroxybenzoate hydroxylase almost completely fails to convert 4-aminobenzoate, resulting in hydrogen peroxide as the main product (Table 4). Quantification and identification of the product formed from 2-hydroxy-4-aminobenzoate was complicated by the instability of the dihydroxylated product (see Materials and Methods). Nevertheless, wild-type *p*-hydroxybenzoate hydroxylase forms more than 0.7 mol of the presumed product 2,3-dihydroxy-4-aminobenzoate/mol oxygen consumed (Table 4). This suggests that, upon reduction, the flavin ring in the wild type complexed with 2-hydroxy-4-aminobenzoate easily attains the productive 'in' conformation. Despite tight binding, hydroxylation of 2-hydroxy-4-aminobenzoate by [A222]*p*-hydroxybenzoate hydroxylase is far less efficient, yielding about 0.3 mol dihydroxylated product/mol oxygen consumed (Table 4). These results provide additional evidence that Tyr222 is essential for the stability and/or reactivity of the flavin hydroperoxide.

Studies with 2-fluoro-4-hydroxybenzoate. The binding mode of 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate observed in the crystal stuctures (Schreuder et al., 1994) prompted us to study the interaction between [A222]*p*-hydroxybenzoate hydroxylase and 2-fluoro-4-hydroxybenzoate. Flavin fluorescence titration experiments showed that [A222]*p*-hydroxybenzoate hydroxylase binds the fluorinated analog much more strongly than 4-hydroxybenzoate. At pH 8.0, binding of 2-fluoro-4-hydroxybenzoate results in an about 60% decrease of the fluorescence of protein-bound FAD. Fluorescence quenching follows simple 1:1 binding. From this a dissociation constant for the complex between [A222]*p*-hydroxybenzoate hydroxylase and 2-fluoro-4-hydroxybenzoate, $K_d = 80 \pm 5$ μM, is estimated. This value is more than one order of magnitude lower than the corresponding value for the complex between [A222]*p*-hydroxybenzoate hydroxylase and 4-hydroxybenzoate (cf. Table 1), showing that the electronegative fluorine substituent at the 2′-position of the aromatic ring considerably increases the substrate binding strength. Binding of 2-fluoro-4-hydroxybenzoate to [A222]*p*-hydroxybenzoate hydroxylase is somewhat weaker than with wild-type (Husain et al., 1980), presumably due to the absence of hydrogen-bond interaction with the carboxy moiety of the fluorinated substrate.

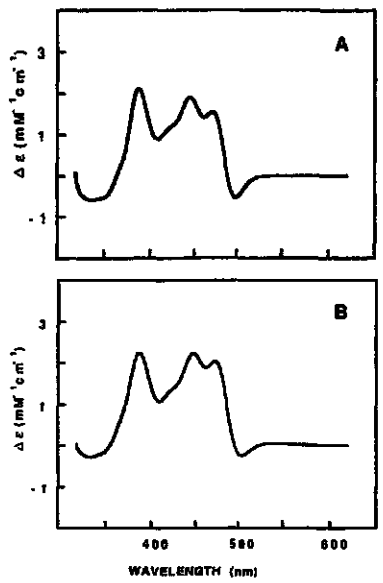

Fig. 4. Flavin perturbation difference spectra induced upon binding of 2-fluoro-4-hydroxybenzoate to wild-type and [A222]*p*-hydroxybenzoate hydroxylase. Absorption spectra were recorded at 25°C in 100 mM Tris/SO_4, pH 8.0. The enzyme concentration was about 40 μM. The absorption difference spectra are extrapolated to infinite substrate concentrations of 2-fluoro-4-hydroxybenzoate. (A) Wild-type; (B) [A222]*p*-hydroxybenzoate hydroxylase.

Studies with wild-type *p*-hydroxybenzoate hydroxylase have shown that 2-fluoro-4-hydroxybenzoate is mainly converted to 2-fluoro-3,4-dihydroxybenzoate (van Berkel et al., 1994b) and that enzyme reduction is tightly coupled to substrate hydroxylation (Husain et al., 1980). Because frontier orbital energy distribution calculations predict a comparable reactivity for 3′- or 5′-hydroxylation (Vervoort et al., 1992), this suggests that the fluorinated substrate binds to wild-type in a regioselective way with its 2-fluoro substituent pointing towards the flavin ring. In contrast to wild-type enzyme, conversion of 2-fluoro-4-hydroxybenzoate by [A222]*p*-hydroxybenzoate hydroxylase results in strong uncoupling of hydroxylation (Table 4). From oxygen consumption experiments and HPLC analysis it is deduced that [A222]*p*-hydroxybenzoate hydroxylase forms about 20% of fluorinated product per catalytic cycle. ^{19}F-NMR product analysis showed that [A222]*p*-hydroxybenzoate hydroxylase exclusively hydroxylates 2-fluoro-4-hydroxybenzoate at the 3′-position. This suggests that the fluorinated substrate binds to the Tyr222 mutant in the same orientation as in the wild type (van Berkel et al., 1994b). This conclusion is supported by substrate perturbation difference spectroscopy. As can be seen from Fig. 4, binding of 2-fluoro-4-hydroxybenzoate induces nearly identical spectral perturbations in the optical spectra of wild-type and [A222]*p*-hydroxybenzoate hydroxylase. As noted above, such spectral perturbations may reflect the equilibrium conformation of the flavin (Gatti et al., 1994). From the relatively high magnitude of the difference peaks at 390, 450 and 470 nm, and according to

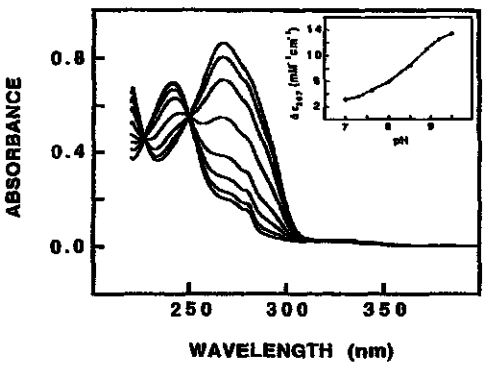

Fig. 5. Ultraviolet-absorption properties of free 2-fluoro-4-hydroxybenzoate. Absolute spectra of 65 μM 2-fluoro-4-hydroxybenzoate. From bottom to top: pH 7.0, pH 7.3, pH 7.6, pH 8.0, pH 8.5, pH 8.9, pH 9.2 and pH 9.5. From the molar absorptions at 267 nm an apparent pK_a value of 8.5 is estimated.

this proposal, we tentatively conclude that binding of the fluorinated substrate analog shifts the equilibrium of flavin conformers to the 'out' position.

Binding of 2-fluoro-4-hydroxybenzoate highly stimulates the rate of reduction of [A222]*p*-hydroxybenzoate hydroxylase by NADPH. At pH 8.0 and 25°C, the maximum reduction rate is 36 s^{-1} with an apparent K_d NADPH = 0.35 mM. The rate of reduction of [A222]*p*-hydroxybenzoate hydroxylase in the presence of 2-fluoro-4-hydroxybenzoate is comparable to the rate of reduction in the presence of 4-hydroxybenzoate (Table 2) and comparable to the rate of reduction of wild-type enzyme complexed with 2-fluoro-4-hydroxybenzoate (not shown), supporting the above proposal that replacement of Tyr222 by Ala does not change the effector specificity.

Substrate activation. Ionization of the phenolic moiety is expected to activate the substrate for electrophilic attack by the flavin hydroperoxide (Vervoort et al., 1992). In *p*-hydroxybenzoate hydroxylase, the activation of 4-hydroxybenzoate is presumably achieved through a network of hydrogen bonds connecting the hydroxy group of the substrate with the side chains of Tyr201 and Tyr385 (Lah et al., 1994; Schreuder et al., 1994). Upon binding to the oxidized wild-type enzyme, the phenolic pK_a of the substrate is decreased to about 7.2, compared to a pK_a of 9.3 free in solution (Shoun et al., 1979; Entsch et al., 1991; Eschrich et al., 1993).

The weak binding of 4-hydroxybenzoate to [A222]*p*-hydroxybenzoate hydroxylase does not allow the ionization state of the substrate in the mutant to be probed by absorption spectral analysis. To circumvent this experimental limitation, the ionization state of protein-bound 2-fluoro-4-hydroxybenzoate was studied in more detail. From the absorption spectra recorded in Fig. 5, a pK_a of 8.5 is estimated for the fluorinated substrate, free in solution. This is in perfect agreement with the value reported before (Husain et al., 1980). Upon titration of wild-type enzyme with 2-fluoro-4-hydroxybenzoate at pH 7.0, a large increase in absorption around 290 nm is observed, indicative for substrate deprotonation (Fig. 6A). From titration experiments as a function of pH and from extrapolating the absorption changes to saturating amounts of substrate, an apparent pK_a value of about 5.8 is estimated for protein-bound 2-fluoro-4-hydroxybenzoate (inset Fig. 6A). The strong decrease in pK_a suggests that

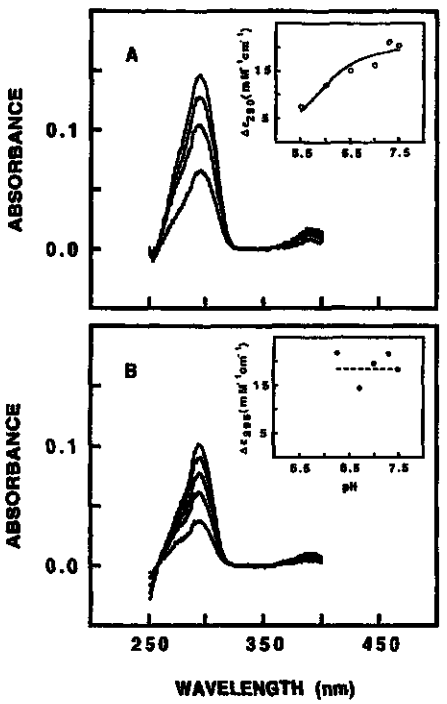

Fig. 6. Ultraviolet-absorption difference spectra observed upon binding of 2-fluoro-4-hydroxybenzoate to wild-type or [A222]*p*-hydroxybenzoate hydroxylase. Both sample and reference cell were titrated with 2-fluoro-4-hydroxybenzoate at 25°C. The sample cell contained 10 μM enzyme. For buffers used, see Materials and Methods. Difference spectra were computed by subtraction of the spectrum of free enzyme from the spectrum of enzyme after the addition of substrate. (A) Titration of wild type at pH 7.5; from bottom to top: difference spectrum at 12.5, 25, 50 and 100 μM 2-fluoro-4-hydroxybenzoate. (B) Titration of [A222]*p*-hydroxybenzoate hydroxylase at pH 7.5; from bottom to top: difference spectrum at 30, 59, 87, 115 and 143 μM 2-fluoro-4-hydroxybenzoate. In the insets the molar absorption differences at (○) 290 nm (wild-type) and (●) 295 nm ([A222]*p*-hydroxybenzoate hydroxylase) are extrapolated to infinite 2-fluoro-4-hydroxybenzoate concentration and plotted against pH. The data shown in the inset of Fig. 6A were best fitted with a theoretical curve for a pK_a value of 5.8 and a maximum difference absorption coefficient of $\Delta\varepsilon_{max}$ = 19.8 $mM^{-1}cm^{-1}$ at 290 nm.

the fluorinated substrate becomes activated upon binding. This is the expected result from theoretical considerations (Vervoort et al., 1992) and in accordance with the efficient hydroxylation observed (Husain et al., 1980). The ionization of 2-fluoro-4-hydroxybenzoate is also in accordance with the effector property of this substrate analog. The dissociation constant of the complex of wild-type and 2-fluoro-4-hydroxybenzoate is slightly dependent on the pH of the solution. From non-linear fitting and treating the data according to 1:1 binding, excellent fits are obtained yielding dissociation constants ranging from K_d = 20 μM at pH 7.5 to K_d = 45 μM at pH 6.0.

Binding of 2-fluoro-4-hydroxybenzoate to [A222]*p*-hydroxybenzoate hydroxylase at pH 7.5 also results in large absorption changes in the near ultraviolet (Fig. 6B). The shape and intensity of the difference peak are consistent with the formation

of the phenolate form of 2-fluoro-4-hydroxybenzoate (cf. Fig. 6A). From the absorption changes at 295 nm, the binding of 2-fluoro-4-hydroxybenzoate to [A222]*p*-hydroxybenzoate hydroxylase is described by simple binary complex formation, yielding a dissociation constant, $K_d = 90 \pm 10$ μM at pH 7.5. This value is in good agreement with the above reported value obtained from fluorescence quenching studies. As can be seen from the maximal molar absorption differences plotted in the inset of Fig. 6B, the degree of ionization of 2-fluoro-4-hydroxybenzoate is nearly independent between pH 6.3 and pH 7.5. In this pH range the dissociation constant for the complex between [A222]*p*-hydroxybenzoate hydroxylase and 2-fluoro-4-hydroxybenzoate gradually increases from $K_d = 90 \pm 10$ μM at pH 7.5 to $K_d = 240 \pm 30$ μM at pH 6.7. At lower pH values, estimation of the maximal absorption difference is complicated by the weak binding of the fluorinated substrate ($K_d = 720 \pm 90$ μM at pH 6.3). Nevertheless, from the data reported in Fig. 5 and Fig. 6B it is clear that upon binding to [A222]*p*-hydroxybenzoate hydroxylase, the pK_a of the phenol of 2-fluoro-4-hydroxybenzoate is lowered by at least 2. The above results indicate that under the conditions for maximal turnover (pH 7–8), removal of the aromatic side chain at position 222 does not dramatically influence the binding mode of the fluorinated substrate. This supports the proposal that inefficient hydroxylation by [A222]*p*-hydroxybenzoate hydroxylase is caused by the decreased stability and/or reactivity of the flavin (C4a)-hydroperoxide.

DISCUSSION

Tyr222 is strictly conserved in the *p*-hydroxybenzoate hydroxylases sequenced so far (Entsch and van Berkel, 1995). The hydroxy group of Tyr222 is at short distance of the carboxy moiety of the substrate, suggesting a strong hydrogen bond (Schreuder et al., 1989). It is clear from the present study that removal of the aromatic side chain of residue 222 strongly impairs the binding of 4-hydroxybenzoate and 4-aminobenzoate. In the 2,4-dihydroxybenzoate- or 2-hydroxy-4-aminobenzoate-complexed Tyr222→Ala mutant, this loss in binding energy is partially compensated by interaction of the 2-hydroxy group of the substrate analogs with the N3 of the flavin, promoting the flavin 'out' conformation. Flavin motion presumably also accounts for the relatively tight interaction between [A222]*p*-hydroxybenzoate hydroxylase and 2-fluoro-4-hydroxybenzoate. This suggests that in the oxidized enzyme, the hydrogen bonding capacity of the 2′-substituent of the substrate is of major importance in determining the equilibrium of flavin conformers. The substrate binding properties may provide an explanation why no crystals were obtained of [A222]*p*-hydroxybenzoate hydroxylase complexed with 4-hydroxybenzoate or 4-aminobenzoate (Schreuder et al., 1994). The impaired binding of these compounds likely increases the protein conformational flexibility inhibiting crystallization.

In wild-type *p*-hydroxybenzoate hydroxylase, the interior position of the flavin allows the efficient hydroxylation of substrates (Schreuder et al., 1992; Entsch and van Berkel, 1995). The flavin perturbation difference spectra presented in this study indicate that the Tyr222→Ala replacement shifts the equilibrium of flavin conformers in the direction of the 'out' position. In analogy with the Tyr222→Phe mutant (Gatti et al., 1994), this could explain the high degree of uncoupling observed. Moreover, the absence of an aromatic side chain at position 222 presumably facilitates the formation of hydrogen peroxide by increasing the solvent accessibility of the active site. The present study shows that [A222]*p*-hydroxybenzoate hydroxylase forms considerable amounts of 2,3,4-trihydroxybenzoate from 2,4-dihydroxybenzoate. This confirms that, upon reduction, the flavin ring in [A222]*p*-hydroxybenzoate hydroxylase can easily adopt the productive 'in' position. Furthermore, it is likely that a 2-hydroxy (or 2-fluoro) substituent in the aromatic ring of the substrate inhibits the uncoupling of hydroxylation in the Tyr222→Ala mutant by affecting the polarization of the oxygen–oxygen bond of the flavin (C4a)-hydroperoxide.

Replacement of Tyr222 by Ala in *p*-hydroxybenzoate hydroxylase does not change the effector specificity. Like the wild-type enzyme, catalytically competent reduction of the Tyr222→Ala mutant is induced upon binding of 4-hydroxybenzoate and 2-fluoro-4-hydroxybenzoate (Husain et al., 1980). With the substrate analogs 2,4-dihydroxybenzoate, 4-aminobenzoate and 2-hydroxy-4-aminobenzoate flavin reduction is slow to extremely slow, preventing rapid turnover. In analogy to the wild-type enzyme, [A222]*p*-hydroxybenzoate hydroxylase preferentially binds the phenolate form of 2-fluoro-4-hydroxybenzoate. The rapid reduction of [A222]*p*-hydroxybenzoate hydroxylase complexed with the deprotonated form of 2-fluoro-4-hydroxybenzoate is in accordance with the view that the effector specificity is tuned by the orientation of the H-bond network connecting the substrate with the protein surface (Entsch and van Berkel, 1995). The role of flavin mobility in the reduction process remains unclear. To gain more insight into the factors regulating flavin motion and its role in substrate binding and product release, we are addressing the dynamic properties of the flavin by time-resolved polarized fluorescence spectroscopy (Bastiaens et al., 1992).

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Chapter 7

^{19}F NMR study on the regiospecificity of hydroxylation of tetrafluoro-4-hydroxybenzoate by wild-type and Y385F *p*-hydroxybenzoate hydroxylase: Evidence for a consecutive oxygenolytic dehalogenation mechanism

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^{19}F NMR Study on the Regiospecificity of Hydroxylation of Tetrafluoro-4-hydroxybenzoate by Wild-Type and Y385F *p*-Hydroxybenzoate Hydroxylase: Evidence for a Consecutive Oxygenolytic Dehalogenation Mechanism†

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ABSTRACT: The regiospecificity of hydroxylation of tetrafluoro-4-hydroxybenzoate (F_4-POHB) by *p*-hydroxybenzoate hydroxylase (PHBH) and its active site mutant Y385F was investigated by ^{19}F NMR. Evidence is provided that the hydroxylation of F_4-POHB is not restricted to the C3 center of the aromatic ring but rather involves sequential oxygenation and dehalogenation steps. The catalytic efficiency of PHBH and Y385F with F_4-POHB was optimal near pH 6.5. Below pH 7.0, substantial substrate inhibition occurred. Dianionic F_4-POHB was a competent effector, highly stimulating upon binding the rate of flavin reduction by NADPH. Hydroxylation of F_4-POHB involved the formation of quinone intermediates as primary products of oxygenolytic defluorination. Ascorbate competed favorably with NADPH for the nonenzymatic reduction of these reactive intermediates and prevented the accumulation of nonspecific oxidation products. ^{19}F NMR showed that the initial aromatic product 2,5,6-trifluoro-3,4-dihydroxybenzoate (F_3-DOHB) was further converted to 5,6-difluoro-2,3,4-trihydroxybenzoate (5,6-F_2-TOHB). This reaction was most efficient with Y385F. F_3-DOHB was not bound in a unique regiospecific orientation as also 2,6-difluoro-3,4,5-trihydroxybenzoate (2,6-F_2-TOHB) was formed. The oxygenolytic dehalogenation of F_3-DOHB by PHBH and Y385F is consistent with the electrophilic aromatic substitution mechanism proposed for this class of flavoenzymes. Nucleophilic attack of the carbon centers of F_3-DOHB onto the distal oxygen of the electrophilic flavin C(4a)-hydroperoxide occurs when the carbon center has a relatively high HOMO density and is relatively close to the distal oxygen of the flavin C(4a)-hydroperoxide.

Flavoprotein aromatic hydroxylases are inducible enzymes involved in the biodegradation of aromatic compounds by soil microorganisms (Stanier & Ornston, 1973; van Berkel & Müller, 1991). Through the initial action of these NAD(P)H-dependent monooxygenases, many breakdown products of lignin can be metabolized. The enzymatic hydroxylation of an organic compound under physiological conditions requires the activation of oxygen. With flavoprotein monooxygenases, this is achieved through the formation of a transiently stable flavin C(4a)-hydroperoxide species (Entsch et al., 1976a; Vervoort et al., 1986; Massey, 1994).

In addition to the hydroxylation of their natural substrates, flavoprotein hydroxylases can mediate fortuitous dehalogenation reactions (Fetzner & Lingens, 1994). Examples of such reactions include the 3-hydroxylation of tetrafluoro-4-hydroxybenzoate (F_4-POHB)[1] by *p*-hydroxybenzoate hydroxylase (PHBH)[1] (Husain et al., 1980), the dechlorination of 2-chlorophenol by salicylate hydroxylase (Suzuki et al., 1991), and the defluorination of 2-fluorophenol by phenol hydroxylase (Peelen et al. 1995). The oxygenolytic cleavage of the carbon−halogen bond has been proposed to proceed through the formation of a quinonoid product intermediate (Husain et al., 1980). This intermediate presumably is reduced nonenzymatically by an additional equivalent of NAD(P)H, resulting in the dihydroxy product (Husain et al., 1980). A similar unusual reaction stoichiometry was reported for the *para*-hydroxylation of the wood preservative pentachlorophenol by pentachlorophenol hydroxylase (Xun et al., 1992). This enzyme belongs to an emerging group of flavoprotein hydroxylases which use a halogenated organic contaminant as the natural substrate (van Berkel et al., 1997; Wieser et al., 1997).

Relatively little is known about the structural features which determine the substrate specificity of flavoprotein aromatic hydroxylases. Only for PHBH, a three-dimensional model of the enzyme−substrate complex is known in atomic detail in the oxidized and two-electron reduced state (Schreuder et al., 1989, 1992; Gatti et al., 1996), as is the mode of binding of the aromatic product and several substrate analogs (Schreuder et al., 1991, 1994; Gatti et al., 1994). With the

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[1] Abbreviations: E(HOMO), energy of the highest occupied molecular orbital; HOMO, highest occupied molecular orbital; PHBH, *p*-hydroxybenzoate hydroxylase; Y201F, *p*-hydroxybenzoate hydroxylase with Tyr201 replaced by Phe; Y385F, *p*-hydroxybenzoate hydroxylase with Tyr385 replaced by Phe; POHB, 4-hydroxybenzoate; DOHB, 3,4-dihydroxybenzoate; TOHB, 3,4,5-trihydroxybenzoate; F_4-POHB, 2,3,5,6-tetrafluoro-4-hydroxybenzoate; F_3-DOHB, 2,5,6-trifluoro-3,4-dihydroxybenzoate; 2,6-F_2-TOHB, 2,6-difluoro-3,4,5-trihydroxybenzoate; 5,6-F_2-TOHB, 5,6-difluoro-2,3,4-trihydroxybenzoate; 5-F-TeOHB, 5-fluoro-2,3,4,6-tetrahydroxybenzoate; 6-F-TeOHB, 6-fluoro-2,3,4,5-tetrahydroxybenzoate.

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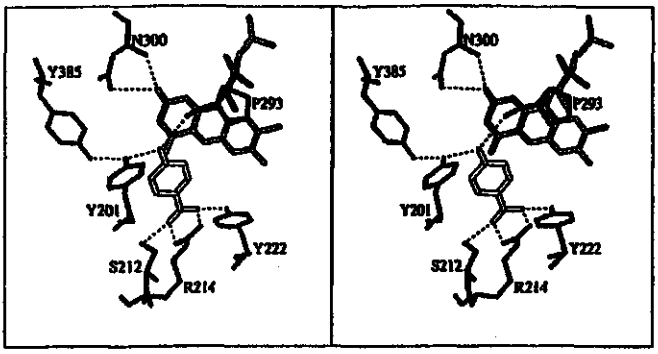

FIGURE 1: Schematic representation of the active site of PHBH complexed with POHB. Data according to the crystal structure at 1.9 Å resolution (Schreuder et al., 1989). The FAD is grey, the aromatic substrate is white, and the protein residues are black.

exception of *p*-mercaptobenzoate, which is hydroxylated at the nucleophilic highly reactive sulfur atom (Entsch et al., 1976b), all PHBH substrates have been proposed to become exclusively hydroxylated at the C3 position of the aromatic ring (Hussain et al., 1980). The specificity for C3 hydroxylation is in keeping with the mutual orientation of 3,4-dihydroxybenzoate (DOHB)[1] and the flavin prosthetic group observed in the crystal structure (Schreuder et al., 1988). Recent studies have shown that the active site mutant Y385F has lost the specificity of C3 hydroxylation (Entsch & van Berkel, 1995), leading to toxic 3,4,5-trihydroxybenzoate (TOHB).[1] Tyr385 is part of a hydrogen bond network, including Tyr201 and the 4OH of the substrate (Schreuder et al., 1989; Figure 1). Mutational analysis established that both tyrosines are involved in substrate activation by stimulating the deprotonation of the 4OH group (Entsch et al., 1991; Eschrich et al., 1993; Lah et al., 1994).

To gain more insight in the mechanism of flavin-mediated oxygenolytic dehalogenation of haloaromatic compounds and the role of substrate and enzyme dynamics (Peelen et al., 1993), we have addressed in the present study the regiospecificity of hydroxylation of F_4-POHB by PHBH. ^{19}F NMR was used for the unambiguous assignment of aromatic products, and mutant Y385F was selected as a tool to modulate the enzyme specificity. Because quinones are highly electrophilic compounds involved in several toxicological processes (Rietjens et al., 1997), the effect of ascorbate as chemical reducing agent was studied as well. Evidence is presented that the PHBH-mediated conversion of F_4-POHB involves several consecutive oxygenation and dehalogenation steps including hydroxylation at the substrate C2 position. The results are discussed in relation to the frontier orbital characteristics of the aromatic substrate and products and their orientation in the active site. A preliminary account of this work has been presented elsewhere (van Berkel et al., 1997).

MATERIALS AND METHODS

Materials. Mes, Hepes, Hepps, and Tris were from Sigma. NADH, NADPH, catalase, and superoxide dismutase were purchased from Boehringer Mannheim, Inc. F_4-POHB was prepared from pentafluorobenzoic acid (Aldrich) as described (Husain et al., 1980).

Enzyme Purification. PHBH and Y385F were purified as described before (Eschrich et al.,1993). *p*-Hydroxybenzoate 1-hydroxylase (decarboxylating) from the yeast *Candida parapsilosis* was isolated according to the procedure reported by van Berkel et al. (1994).

Enzyme Kinetics. The activity of PHBH was determined spectrophotometrically by measuring the F_4-POHB-stimulated oxidation of NADPH at 340 nm (25 °C). pH-dependent activity measurements were performed in 80 mM Mes (pH 5.6−6.6) and 80 mM Hepes (pH 7.0−8.0), in either the absence or the presence of 2 mM ascorbate. Buffers were brought to constant ionic strength (I = 0.1 M) with sodium sulfate as described (van Berkel & Müller, 1989). For estimation of steady state kinetic parameters, NADPH and F_4-POHB were varied using 0.15 mM (F_4-POHB) and 0.18 mM (NADPH) of the fixed substrate. Kinetic data were analyzed by nonlinear least-squares fitting routines (van Berkel et al., 1991; Eschrich et al., 1993). Rapid-reaction kinetics were carried out at 25 °C using a temperature-controlled single-wavelength stopped-flow spectrophotometer, type SF-51, from High-Tech Scientific Inc. with 1.3 ms deadtime. Rate constants for the anaerobic reduction of F_4-POHB complexed enzymes were estimated from kinetic traces recorded at 450 nm at variable concentrations of NADPH (Eppink et al., 1995).

Oxygen Uptake Experiments. Oxygen consumption was measured polarographically at 25 °C by using a Clark electrode. Assay mixtures contained 100 mM Mes, pH 6.0, or 50 mM potassium phosphate, pH 7.0, and varying amounts of aromatic substrate, NADPH, and enzyme. The degree of uncoupling of hydroxylation was determined with catalase (Eschrich et al., 1993). For the establishment of the reaction stoichiometry, the oxygen consumption experiments were performed in either the absence or the presence of 2 mM ascorbate. F_4-POHB (150 μM) was incubated with 50, 100, 150, 200, 250, 300, and 350 μM NADPH, respectively, and the reaction was started by the addition of 1.5 μM enzyme. At the end of the initial rapid phase of oxygen consumption (5−10 min), the residual amount of NADPH was determined by the addition of 1 mM POHB.

Substrate Binding Studies. Dissociation constants of binary complexes between oxidized enzyme and F_4-POHB were determined fluorimetrically using an Aminco SPF-500 spectrofluorimeter (van Berkel et al., 1992).

NMR Measurements. ^{19}F NMR measurements were performed on a Bruker AMX 300 or Bruker DPX-400 NMR spectrometer, essentially as described elsewhere (Peelen et al., 1993, 1995). Proton-decoupled spectra were obtained at 7 °C. Between 5000 and 15 000 scans were recorded, depending on the concentrations of the fluorinated products and the signal to noise ratio required. The sample volume was 2.0 mL. A coaxialary capillary contained 4-fluorobenzoate as a standard and ^2H_2O for locking the magnetic field. Concentrations of fluorinated products were calculated by comparison of the integral of the ^{19}F NMR resonances of the products to the integral of the ^{19}F NMR resonance of 4-fluorobenzoate. Chemical shifts are reported relative to $CFCl_3$. ^1H NMR analysis was carried out on a Bruker AMX 500 MHz spectrometer. Samples were prepared as described for ^{19}F NMR. At the end of the reaction the incubation mixtures were freeze-dried and dissolved in 0.5 mL ^2H_2O.

Product Identification. ^{19}F NMR product analysis revealed that the addition of ascorbate to the incubation mixtures was essential to prevent the accumulation of nonspecific oxidation products. Incubation mixtures (25 °C) contained 100 mM Mes, pH 6.0, or 50 mM potassium phosphate buffer, pH 7.0, 0.17 mM F_4-POHB, 0.05−0.3 mM NADPH, 1 mM ascorbate, 150 units of catalase, and 1.5 μM enzyme. The reaction was monitored spectrophotometrically by following the absorbance decrease at 340 nm until all NADPH was consumed. In time-dependent experiments, reactions with the fluorinated substrate were stopped by the addition of 1 mM POHB. Before recording the NMR spectra, samples were made anaerobic by five cycles of flushing with argon and degassing. Oxidative decarboxylation of fluorinated products was achieved by incubation with *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* (van Berkel et al., 1994). Initial incubations (total volume 2.0 mL, 25 °C) contained 50 mM potassium phosphate buffer, pH 7.0, 0.17 mM F_4-POHB, 0.3 mM NADPH, 1 mM ascorbate, 150 units of catalase, 30 μM FAD, and 1.5 μM Y385F. After analyzing the sample by ^{19}F NMR, 0.6 mM NADH, 50 units of superoxide dismutase, and 1 μM *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* were added, and the reaction was followed spectrophotometrically until no absorption decrease at 340 nm was observed. The sample was then again analyzed by ^{19}F NMR for the formation of fluorinated phenolic products.

Identification of fluorinated aromatic products was achieved by (a) the ^{19}F-^{19}F coupling patterns and (b) on the basis of the chemical shift values of the ^{19}F NMR resonances of a set of fluorinated benzene derivatives (Rietjens et al., 1993; Peelen et al., 1995). No proton coupling to the fluorine resonances was observed on comparison of ^1H decoupled ^{19}F NMR spectra with ^1H coupled ^{19}F NMR spectra. The quality of the spectra was good enough to observe small coupling constants (0.3−0.5 Hz). From the *ortho*-hydroxylation of fluorinated phenols by phenol hydroxylase from *Trichosporon cutaneum* (Peelen et al., 1995), it is known that substitution of a fluorine atom by a hydroxyl group results in a chemical shift of a fluorine substituent located at an *ortho* position of +2.2 (± 0.9) ppm, at a *meta* position of +1.0 (± 0.9) ppm, and at a *para* position of −5.4 (± 0.9) ppm.

Molecular Orbital Calculations. Molecular orbital calculations were performed on a Silicon Graphics Indigo 2 workstation with Insight (Biosym Technologies, CA). The semiempirical molecular orbital method was used, applying the AM1 Hamiltonian from the MOPAC program. All calculations were carried out, essentially as described by Peelen et al. (1995).

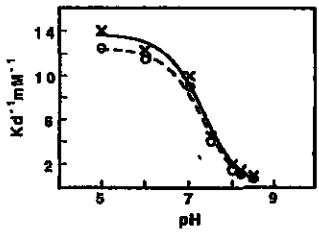

FIGURE 2: pH-dependent binding properties of PHBH and Y385F with F_4-POHB. The dissociation constants of the complexes were determined from flavin fluorimetric titration experiments: PHBH (×), Y385F (○).

RESULTS

Fluorescence Binding Studies. Previous studies have shown that PHBH preferentially binds F_4-POHB between pH 6 and 7 with an apparent pK_a of about 7.6 in the oxidized state (van Berkel & Müller, 1989). Because the pK_a of the fluorinated substrate free in solution is 5.3 (Husain et al., 1980), weak binding of F_4-POHB at pH 8 was tentatively ascribed to the ionization of one of the tyrosines in the active site (van Berkel and Müller, 1989). Titration of Y385F with F_4-POHB was accompanied by quenching of the fluorescence of protein-bound FAD and followed simple 1:1 binding. Figure 2 shows that the binding properties of F_4-POHB to Y385F were nearly indistinguishable from PHBH, excluding the possibility that the apparent pK_a observed originates from the deprotonation of Tyr385.

Catalytic Properties of PHBH with F_4-POHB. To identify the optimal conditions for the enzymatic conversion of F_4-POHB, steady state kinetic parameters were estimated as a function of pH. As can be seen from Table 1, Y385F catalyzed the F_4-POHB-stimulated oxidation of NADPH at approximately the same rate as PHBH. With both enzymes, substantial substrate inhibition occurred below pH 7.0 (Table 1). A similar type of inhibition was reported for other fluorinated substrate analogs (Husain et al., 1980). Table 1 also shows that the apparent K_m values for F_4-POHB and NADPH decreased with decreasing pH, resulting in an optimal catalytic efficiency near pH 6.5. This is clearly different from the reaction with the physiological substrate where optimal catalysis takes place around pH 8.0 (van Berkel & Müller, 1989).

The turnover rate of Y385F with F_4-POHB was about 1 order of magnitude higher than with POHB (Eschrich et al., 1993) where flavin reduction is rate limiting in catalysis (Entsch et al., 1991; Eschrich et al., 1993). This suggests that F_4-POHB is a better effector for Y385F than POHB. The effector role of F_4-POHB was addressed in more detail by studying the reductive half-reaction by anaerobic stopped flow experiments. At pH 7.0, 25 °C, flavin reduction of the F_4-POHB complexed enzymes was a simple monoexponential process. The extrapolated rate constants for reduction at infinite NADPH concentrations were 12.5 s^{-1} for PHBH (K_d of NADPH = 0.81 mM) and 19.3 s^{-1} for Y385F (K_d of NADPH = 0.79 mM), respectively. This

Table 1: Steady State Kinetic Parameters of PHBH and Y385F with F_4-POHB

enzyme	pH	apparent k_{cat}[a] (s^{-1})	apparent K_m F_4-POHB (μM)	apparent K_m NADPH (μM)	excess substrate inhibition (mM)
PHBH	6.0	3.7	36	51	>0.08
	6.5	3.8	43	73	>0.1
	7.0	4.1	67	145	>0.3
	8.0	4.0	156	280	>1.0
+ascorbate	6.0	3.0	36	30	>0.1
	6.5	3.2	68	66	>0.15
	7.0	3.5	102	112	>0.5
	8.0	3.4	120	225	>1.0
Y385F	6.0	3.9	43	47	>0.08
	6.5	4.1	36	67	>0.15
	7.0	5.1	66	127	>0.3
	8.0	4.1	154	154	>1.0
+ascorbate	6.0	2.7	41	36	>0.1
	6.5	3.1	51	49	>0.15
	7.0	3.5	68	105	>0.4
	8.0	3.0	112	152	>1.0

[a] Turnover numbers and apparent K_m values for F4-POHB and NADPH were determined from the initial velocity of NADPH consumption at 25 °C in air-saturated buffer, variable [F4-POHB] or [NADPH] and 0.2 mM of the fixed substrate. Experiments ($n = 2$) were performed in the absence or presence of 2 mM ascorbate. Turnover numbers are apparent maximum values extrapolated to infinite concentrations of F4-POHB and NADPH and taking into account substrate inhibition. The mean standard error of values of kinetic parameters was about 10%.

shows that the F_4-POHB stimulated rate of reduction of both enzymes is not rate limiting in catalysis (cf. Table 1) and confirms that F_4-POHB is a better effector for Y385F than POHB.

Stoichiometry of the Reaction. The stoichiometry of the reaction of PHBH and Y385F with F_4-POHB was studied by oxygen consumption experiments. In analogy with published results (Husain et al., 1980), PHBH fully coupled oxygen consumption to F_4-POHB hydroxylation. With Y385F, some uncoupling of hydroxylation was observed as evidenced by the formation of hydrogen peroxide. The efficiency of hydroxylation by Y385F was somewhat dependent on the reaction conditions yielding about 0.15 mol of hydrogen peroxide/mol of oxygen consumed at pH 7.0. This degree of uncoupling is in the same range as observed with the Y385F catalyzed conversion of POHB (Entsch et al., 1991; Eschrich et al., 1993).

When the reaction of PHBH (or Y385F) with F_4-POHB was performed with varying concentrations of NADPH (pH 7.0), about 2 equiv of NADPH was consumed/equiv of oxygen (Figure 3). Comparable results were obtained at pH 6.0 (data not shown). These results are consistent with an earlier proposal (Husain et al., 1980) that the utilization of 2 mol of NADPH/mol of oxygen can be ascribed to the initial formation of a quinonoid product intermediate. When the same set of experiments was repeated in the presence of ascorbate, nearly equal amounts of oxygen and NADPH were consumed (Figure 3). The reaction stoichiometry did not change when the concentration of ascorbate was varied beween 1 and 10 mM, indicating that 1 mM ascorbate is sufficient to compete favorable with NADPH for quinone reduction. In line with this, the presence of ascorbate significantly decreased the rate of NADPH consumption (Table 1). Interestingly, in the presence of ascorbate and at high NADPH/F_4-POHB ratios, more than 1 equiv of NADPH

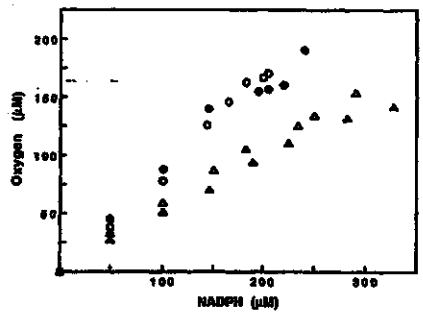

FIGURE 3: Reaction stoichiometry of the reaction of PHBH and Y385F with F_4-POHB as determined by oxygen consumption experiments. F_4-POHB (150 μM) was incubated with varying concentrations of NADPH in air-saturated buffer, pH 7.0, and the reaction was initiated by the addition of 1.5 μM enzyme: PHBH in the absence (Δ) or presence (O) of ascorbate, Y385F in the absence (▲) or presence (●) of ascorbate. The plotted *x*-axis values are the actual amounts of NADPH consumed. For other experimental details, see Materials and Methods.

and oxygen was consumed per substrate molecule (Figure 3). This prompted us to study the reaction stoichiometry in more detail by ^{19}F NMR.

Product Identification by ^{19}F NMR. Conversion of F_4-POHB by PHBH or Y385F did not result in the exclusive formation of 2,5,6-trifluoro-3,4-dihydroxybenzoate (F_3-DOHB). Especially with Y385F, several other fluorinated aromatic products were formed. Figure 4A shows the ^{19}F NMR spectrum of an incubation of Y385F in the presence of equimolar amounts of F_4-POHB and NADPH. The ^{19}F NMR resonances at −150.3, −157.1, and −171.1 ppm are assigned to F_3-DOHB, whereas the resonances at −153.1 ppm and −170.7 ppm are ascribed to residual F_4-POHB (Table 2). The resonance at −123.1 ppm originates from fluoride anion, released upon dehalogenation. The two additional main resonances at −154.7 and −177.2 ppm in Figure 4A showed identical integrals, and both resonances have the same *J* coupling, suggesting that these resonances are derived from an aromatic product with two fluorine substituents *ortho* positioned to each other. This indicates that the initial product F_3-DOHB was subject to further hydroxylation at the C2 position of the aromatic ring. The formation of 5,6-difluoro-2,3,4-trihydroxybenzoate (5,6-F_2-TOHB) was in good agreement with chemical shift predictions of −176.5 ppm for the C5 fluorine substituent and −156.1 ppm for the C6 fluorine substituent, respectively (Table 2). All together, the results presented in Figure 4A suggest that conversion of F_4-POHB by Y385F involves the consecutive formation of F_3-DOHB and 5,6-F_2-TOHB.

Further evidence for hydroxylation at the C2 center of the aromatic ring was obtained when the NADPH/F_4-POHB ratio in the incubation mixture of Y385F was increased. Addition of an extra equivalent of NADPH resulted in the complete depletion of F_4-POHB and in a nearly 2-fold increase in the intensities of the resonances assigned to 5,6-F_2-TOHB (Figure 4B). Moreover, some extra ^{19}F NMR resonances of relatively low intensity were observed at −153.6 ppm and −171.4 ppm, indicative for the formation of additional fluorinated products. The intensities of these two resonances were different, suggesting that they belong to different hydroxylation products. Furthermore, both these resonances

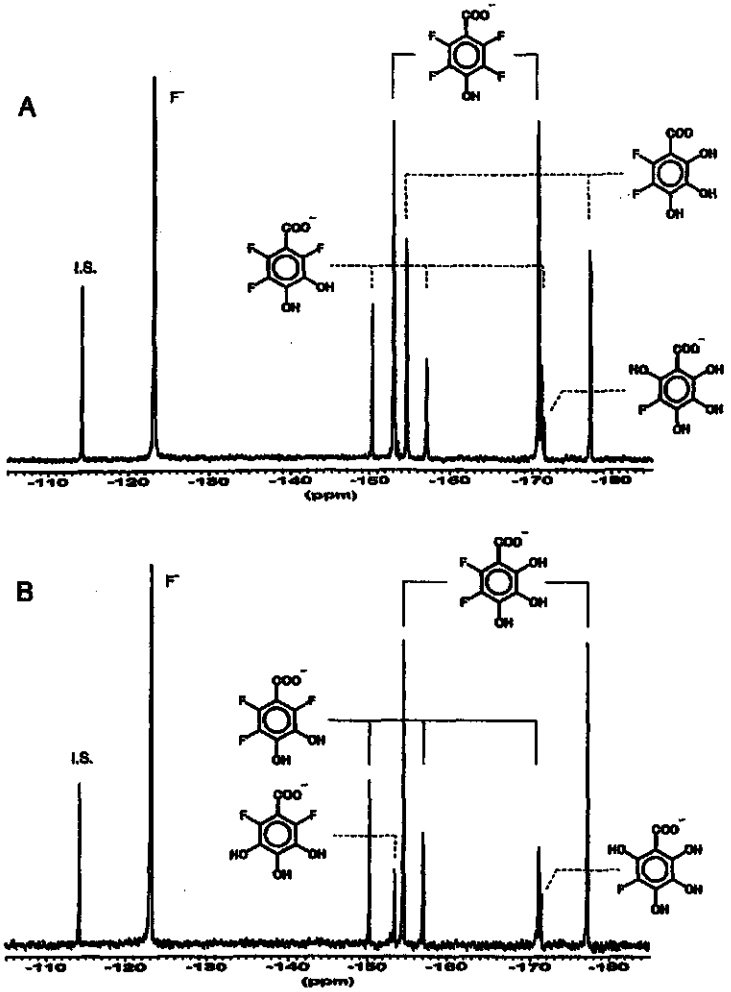

FIGURE 4: ^{19}F NMR product analysis of the reaction of F_4-POHB with Y385F: (A) NADPH/F_4-POHB ratio 0.9; (B) NADPH/F_4-POHB ratio 1.8. 170 μM F_4-POHB was incubated with NADPH in air-saturated buffer pH 7.0, containing 1 mM ascorbate. Resonances were identified on the basis of the results summarized in Table 2. The resonance at −114.2 ppm is from the internal standard, 4-fluorobenzoate.

showed no J_{p-p} coupling. Earlier studies have shown that Y385F hydroxylates the physiological product DOHB to TOHB (Entsch et al., 1991; Eschrich et al., 1993). On the basis of these data, the resonance at −153.6 ppm is assigned to 2,6-difluoro-3,4,5-trihydroxybenzoate (2,6-F_2-TOHB) (Table 2). The ^{19}F NMR resonance at −171.4 ppm (Figure 4B) could originate from either 5-fluoro-2,3,4,6-tetrahydroxybenzoate (5-F-TeOHB) or 6-fluoro-2,3,5,6-tetrahydroxybenzoate (6-F-TeOHB). On the basis of chemical shift values of the difluorinated products and theoretical considerations (Peelen et al., 1995), this resonance is assigned to 5-F-TeOHB (Table 2).

The identity of the reaction products was confirmed by ^1H-NMR. For this purpose, F_4-POHB was incubated with a 2-fold excess of NADPH either in the absence or presence of Y385F. In the presence of Y385F, no extra resonances were observed in the 6−8 ppm region of the ^1H NMR spectrum. This shows that no products with aromatic protons were formed. Moreover, in all ^{19}F NMR spectra, no ^1H proton coupling could be observed.

Further identification of fluorinated aromatic products was achieved by incubation of the reaction samples with *p*-hydroxybenzoate 1-hydroxylase from the yeast *C. parapsilosis*. Recent studies have shown that this NAD(P)H-dependent flavoenzyme catalyzes the oxidative decarboxylation of POHB, yielding 1,4-dihydroxybenzene (van Berkel et al., 1994). Figure 5A illustrates that *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* forms a single aromatic product from F_4-POHB which is ascribed to the formation of tetrafluoro-1,4-dihydroxybenzene. Figure 5B shows the ^{19}F NMR spectrum of F_4-POHB, successively incubated with Y385F and *p*-hydroxybenzoate 1-hydroxylase from *C. parap-*

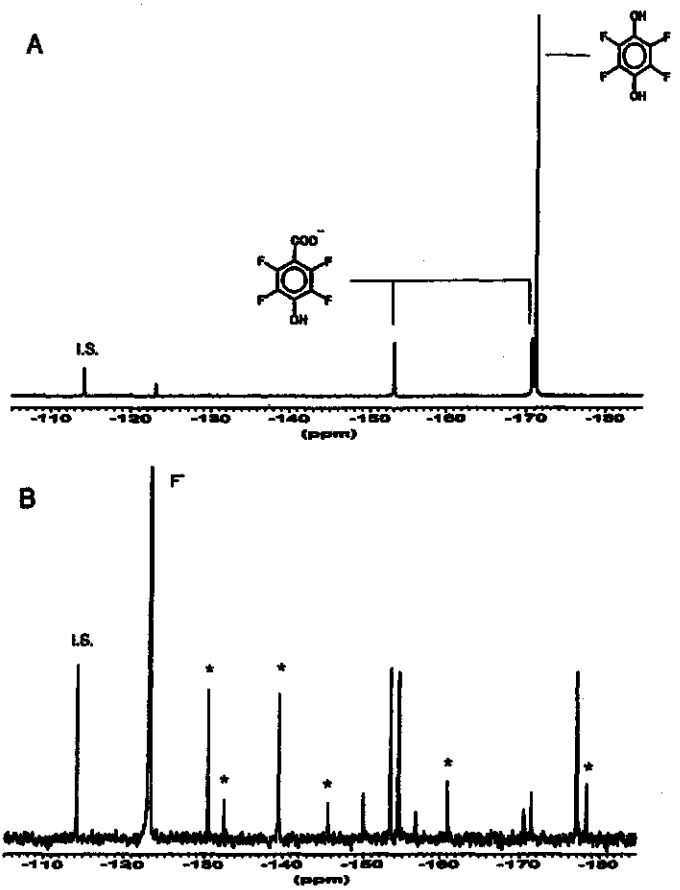

FIGURE 5: ^{19}F NMR spectra of the conversion of fluorinated POHB derivatives by *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis*: (A) conversion of F_4-POHB; (B) conversion of hydroxylation products obtained from the incubation of F_4-POHB with Y385F at a NADPH/F_4-POHB ratio of 1.8 (cf. Figure 4B). New resonances are indicated by an asterisk. The resonance at −114.2 ppm is from the internal standard, 4-fluorobenzoate.

silosis. This spectrum revealed six new resonances in relation to the same sample incubated in the absence of the yeast enzyme (cf. Figure 4B). From the difference spectrum, it could be established that three of the new resonances were derived from 2,5,6-trifluoro-1,3,4-trihydroxybenzene, one resonance from 5,6-difluoro-1,2,3,4-tetrahydroxybenzene, one resonance from 2,6-difluoro-1,3,4,5-tetrahydroxybenzene and one resonance from 5-fluoro-1,2,3,4,6-pentahydroxybenzene. This confirmed that all hydroxylation products formed by Y385F contained a carboxylic group. The polyhydroxylated fluorobenzene products formed by *p*-hydroxybenzoate 1-hydroxylase from *C. parapsilosis* were rather stable in the presence of ascorbate. This is concluded from the nearly identical ^{19}F NMR spectrum recorded after several hours.

Reaction Stoichiometry as Measured by ^{19}F NMR. The hydroxylation pattern of the enzymatic conversion of F_4-POHB was strongly dependent on the reaction conditions. With PHBH (pH 7.0), depletion of F_4-POHB resulted in nearly equal amounts of F_3-DOHB and 5,6-F_2-TOHB (Figure 6A, Table 3). With Y385F, the initial product F_3-DOHB was rather efficiently converted to 5,6-F_2-TOHB (Figure 6B) and, to a minor extent, to 2,6-F_2-TOHB (Table 3). Unlike Y385F, PHBH produced minor amounts of 5,6-F_2-TOHB at pH 6.0 (Table 3). During the conversion of F_4-POHB and in contrast to published data (Hussain et al., 1980), considerable higher amounts of fluoride anion were produced as expected from the amount of hydroxylated fluorinated aromatic products formed. From the concentrations of fluorinated aromatic substrate and products observed at the end of the reactions (Table 3), it could be calculated that, during the conversion of F_4-POHB, 10−20% of the fluorinated aromatic reaction products were lost and that the excess fluoride released increased with increasing concentrations of NADPH. Increasing the concentration of ascorbate in the incubation mixtures to 10 mM or addition of superoxide dismutase did not significantly change the amount of fluoride anion formed. On the other hand, conversion of F_4-POHB

Table 2: Chemical shifts of ^{19}F NMR Resonances of Identified Fluorinated Products Formed from the Conversion of F_4-POHB by PHBH and Y385F[a]

compound	chemical shift (ppm)	basis of identification
F_4-POHB	−153.1 (F2/F6)	2,3,5,6-tetrafluorophenol (F3/F5) (−149.3 ppm, $^3J_{FF}$ = 18.0 Hz)
	−170.7 (F3/F5)	2,3,5,6-tetrafluorophenol (F2/F6) (-170.5 ppm, $^3J_{FF}$ = 18.0 Hz)
F_3-DOHB	−150.3 (F2)	*o*-OH shifts F2 by +2.8 ppm ($^4J_{FF}$ = 5.1 Hz, $^3J_{FF}$ = 7.6 Hz)
	−171.1 (F5)	*m*-OH shifts F5 by −0.4 ppm ($^3J_{FF}$ = 24.2 Hz, $^3J_{FF}$ = 7.6 Hz)
	−157.1 (F6)	*p*-OH shifts F6 by −4.0 ppm ($^3J_{FF}$ = 24.2 Hz, $^4J_{FF}$ = 5.1 Hz)
2,6-F_2-TOHB	−153.6 (F2/F6)	*o*-OH shifts F6 by +3.5 ppm *p*-OH shifts F2 by −3.3 ppm
5,6-F_2-TOHB	−154.7 (F6)	*m*-OH shifts F6 by +2.4 ppm ($^3J_{FF}$ = 22.9 Hz)
	−177.2 (F5)	*p*-OH shifts F5 by −6.1 ppm ($^3J_{FF}$ = 22.9 Hz)
5-F-TeOHB	−171.4 (F5)	*o*-OH shifts F5 by +5.8 ppm

[a] The chemical shifts (as determined in 50 mM potassium phosphate, pH 7.0) are relative to $CFCl_3$. Chemical shifts were ascribed to specific fluorine substituents on the basis of relative peak intensities, proton-decoupled splitting patterns, and the knowledge that *meta* substituents influence chemical shifts far less than *para* or *ortho* substituents.

in the absence of ascorbate led to F_3-DOHB, several unidentified fluorinated reaction products, and only minor amounts of 5,6-F_2-TOHB (data not shown). This raised the possibility that the fluorinated quinone formed upon enzymatic attack of F_4-POHB undergoes a Michael addition by water at the 2-position, leading to fluoride elimination and, after reduction, the trihydroxy product. Because fluorinated benzoquinones are highly reactive species which become dehalogenated upon reaction with nucleophiles (den Besten et al., 1993), such a nonenzymatic mechanism could also explain the high amount of side-products formed in the absence of ascorbate.

In order to establish whether the conversion of F_3-DOHB to 5,6-F_2-TOHB was enzyme mediated, the following experiments were performed. F_4-POHB was completely converted by PHBH (cf. Figure 6A, Table 3) and the resulting mixture of F_3-DOHB and 5,6-F_2-TOHB was incubated in aerated buffer in the absence or presence of excess NADPH. ^{19}F NMR analysis revealed that in the absence of NADPH no reaction occurred. In the presence of NADPH, F_3-DOHB was further converted to 5,6-F_2-TOHB, providing direct evidence for an enzyme-mediated hydroxylation step. As already noted from the ^{19}F NMR experiments reported above, the extent of conversion of F_3-DOHB was strongly dependent on the reaction conditions. With PHBH, formation of 5,6-F_2-TOHB was most efficient at pH 7.0, but in contrast to pH 6.0, also minor amounts of 2,6-F_2-TOHB and 5-F-TeOHB were formed. Furthermore, the reaction of F_3-DOHB with PHBH was not complete, most probably as a result of product (5,6-F_2-TOHB) inhibition. This is in keeping with the results presented in Figures 3 and 6 and the fact that the NADPH left was readily consumed after the addition of 1 mM POHB. The reaction of F_3-DOHB with Y385F was rather efficient. At both pH 6.0 and 7.0, more than 80% of F_3-DOHB was converted to 5,6-F_2-TOHB. Again, additional formation of 2,6-F_2-TOHB and 5-F-TeOHB was only observed at pH 7.0.

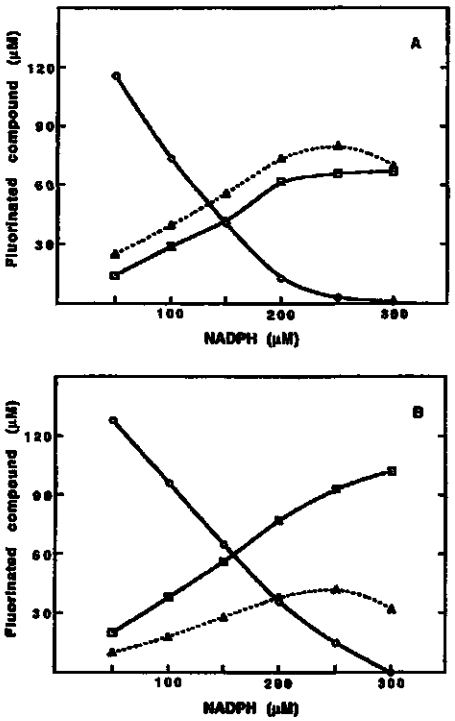

FIGURE 6: NADPH dependence of the conversion of F_4-POHB by PHBH and Y385F as determined by ^{19}F NMR. F_4-POHB (170 μM) was incubated with varying concentrations of NADPH in air-saturated buffer pH 7.0, containing 1 mM ascorbate and the reaction was initiated by the addition of 1.5 μM enzyme: (a) PHBH, (b) Y385F; F_4-POHB (○), F_3-DOHB (▲), 5,6-F_2-TOHB (□). The standard error in the concentrations of fluorinated compounds was about 5%. For other experimental details, see Materials and Methods.

Table 3: Conversion of F_4-POHB by PHBH and Y385F as determined by ^{19}F NMR[a]

fluorinated compound	pH	concentration of fluorinated product (μM) at NADPH/F_4-POHB ratio of 0.9 (1) or 1.8 (2) PHBH (1)	PHBH (2)	Y385F (1)	Y385F (2)
F_4-POHB	6.0	32		56	
	7.0	41	1	65	
F_3-DOHB	6.0	79	101	32	49
	7.0	56	68	28	32
2,6-F_2-TOHB	6.0				
	7.0		1	1	6
5,6-F_2-TOHB	6.0	30	41	52	89
	7.0	42	66	56	102
5-F-TeOHB	6.0				
	7.0		1	1	3
fluoride anion	6.0	191	245	203	327
	7.0	203	302	217	392

[a] The initial F_4-POHB concentration was 170 ± 8 μM and the enzyme concentration was 1.5 μM. All experiments (n = 2) were performed in the presence of 1 mM ascorbate. Concentrations of fluorinated products have a standard error <5%.

Further support for the consecutive hydroxylation at the C3 and C2 position of the aromatic ring was obtained by

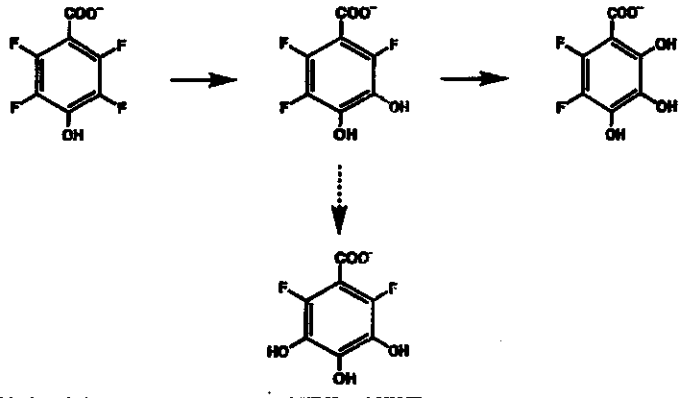

FIGURE 7: Proposed hydroxylation pathway of F_4-POHB by PHBH and Y385F.

Table 4: E(HOMO) and HOMO Density Characteristics of Dianionic POHB Derivatives[a]

		E(HOMO)	HOMO density on carbon center					
compound	dp	eV	C1	C2	C3	C4	C5	C6
POHB	4	0.44	0.29	0.01	0.21	0.05	0.21	0.01
F_4-POHB	4	−0.49	0.25	0.01	0.20	0.05	0.20	0.01
F_3-DOHB	4	−0.43	0.25	0.00	0.18	0.06	0.18	0.02
	3	−0.23	0.00	0.18	0.07	0.18	0.01	0.25
5,6-F_2-TOHB	4	−0.30	0.25	0.00	0.19	0.06	0.18	0.02
	3	−0.15	0.00	0.16	0.08	0.17	0.02	0.22
2,6-F_2-TOHB	4	−0.32	0.24	0.01	0.17	0.08	0.17	0.01
	3	−0.13	0.00	0.18	0.06	0.17	0.02	0.25

[a] Molecular orbital characteristics of dianionic forms of POHB derivatives of importance for nucleophilic attack on the flavin C(4a)-hydroperoxide, as calculated by the AM1 Hamiltonian of the MOPAC module of the Insight II package (Biosym, San Diego). Calculations were carried out with either a deprotonated 4-hydroxyl or 3-hydroxyl group (dp). eV, electronvolts.

recording ^{19}F NMR spectra at different time intervals. Spectra recorded of samples which were incubated for only 30 s after initiating the reaction of PHBH with equimolar amounts of F_4-POHB and NADPH showed nearly the exclusive formation of F_3-DOHB. With Y385F, the formation of F_3-DOHB as the first product was more difficult to assign because of its rapid conversion to 5,6-F_2-TOHB. The spectra recorded with time clearly showed the accumulation of 5,6-F_2-TOHB and rather slow formation of 2,6-F_2-TOHB and 5-F-TeOHB. All together, the above results from NMR product analysis are consistent with the hydroxylation pathway presented in Figure 7.

Frontier Orbital Characteristics of Fluorinated Hydroxylation Products. In order to explain the observed hydroxylation pattern from a chemical point of view, molecular orbital calculations were performed to investigate the intrinsic electronic characteristics of the fluorinated POHB derivatives. For PHBH it was reported that the turnover rates for the conversion of a series of fluorinated POHB analogs correlate with the *E*(HOMO) of their dianionic forms (Vervoort et al., 1992). From this it was suggested that a high *E*(HOMO) and HOMO density on C3 of the aromatic substrate will favor the electrophilic attack by the flavin C(4a)-hydroperoxide. Table 4 shows that the dianionic form of F_4-POHB has a relatively low *E*(HOMO) explaining the low turnover rate (Vervoort et al., 1992), but that the electron density at the C3 (C5) atom is comparable to that of POHB. This may explain the rather efficient C3 hydroxylation of F_4-POHB by PHBH at pH 6. In analogy to F_4-POHB, the fluorinated aromatic products formed from the enzymatic conversion of F_4-POHB showed considerable HOMO density (reactive electron density) at the fluorinated carbon atom *ortho* or *para* to a deprotonated hydroxyl group (Table 4). This suggests that the formation of 5,6-F_2-TOHB from F_3-DOHB results from the (partial) ionization of the 3OH of F_3-DOHB. In line with this proposal, formation of 2,6-F_2-TOHB must then result from the (partial) ionization of the 4OH of F_3-DOHB.

DISCUSSION

The flavoprotein-mediated oxygenolytic dehalogenation of haloaromatic substrates requires two NAD(P)H molecules per turnover (Husain et al., 1980; Suzuki et al., 1991; Xun et al., 1992; Peelen et al., 1995; Wieser et al., 1997). On the basis of studies of PHBH with fluorinated substrate analogs it was proposed that this unusual stoichiometry is due to the nonenzymatic reduction of a quinonoid species formed as the primary product of oxygenolytic dehalogenation (Husain et al., 1980). In the present study, the stoichiometry of the PHBH-catalyzed conversion of F_4-POHB was studied in the presence of ascorbate. Addition of ascorbate nearly restored the 1:1 reaction stoichiometry, indicative for the rapid chemical reduction of quinone intermediates by ascorbate. In line with earlier results on the oxygenolytic dehalogenation of *ortho*-fluorinated phenols (Peelen et al., 1995), ascorbate prevented the accumulation of nonspecific oxidation products. However, ^{19}F NMR analysis revealed that even in the presence of high concentrations of ascorbate, part of the fluorinated quinones were dehalogenated in a nonenzymatic process. These findings are of physiological relevance for flavoprotein hydroxylases which use a polyhalogenated aromatic compound as their natural substrate, because *in vivo* quinone reduction might compete with covalent binding to cellular macromolecules (den Besten et al., 1993; Rietjens et al., 1997).

The pH optimum of PHBH catalysis with F_4-POHB was around pH 6.5. This acidic shift in pH optimum with respect to the reaction with POHB is ascribed to the weak binding of dianionic F_4-POHB at pH 8.0. From mass spectral analysis, it was previously concluded that the conversion of F_4-POHB by PHBH results in the formation of F_3-DOHB

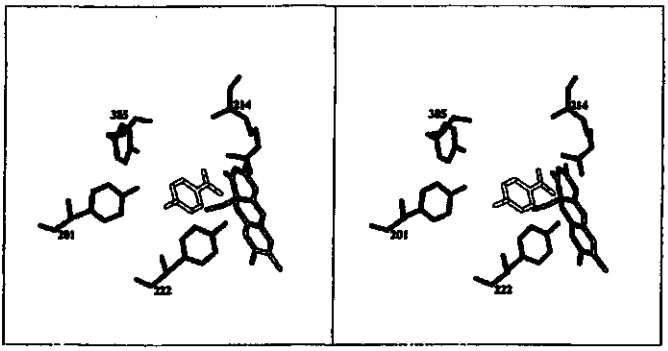

FIGURE 8: Stereo view of the flavin C(4a)-hydroperoxide model in the active site of the enzyme substrate complex of PHBH. Data according to Schreuder et al. (1990). In the depicted orientation, the distal peroxide oxygen is closest (2.4 Å) to the C3 and at 3.2 Å from the C2 of POHB. The FAD is grey, the aromatic substrate is white, and the protein residues are black.

as sole aromatic product (Husain et al., 1980). However, the ^{19}F NMR results presented in this paper provide clear evidence for a consecutive hydroxylation process leading to successive fluoride elimination at C3 and C2 of the substrate aromatic ring. The extent of C2 hydroxylation was dependent on the reaction conditions and the type of enzyme used. With PHBH, conversion of F_3-DOHB to 5,6-F_2-TOHB was less efficient than with Y385F, confirming that the hydroxylation of F_3-DOHB is enzyme mediated. The observed differences in the extent of C2 hydroxylation might be caused by the relative binding strengths of F_4-POHB and F_3-DOHB and the different degree of substrate and product inhibition. However, other factors like the effector properties of F_3-DOHB might also influence the outcome.

Fluoride elimination from F_4-POHB was not restricted to C2 and C3 of the aromatic ring. In the presence of excess NADPH, Y385F catalyzed the production of significant amounts of 2,6-F_2-TOHB from F_3-DOHB, a reaction nearly abolished in PHBH. These results are consistent with earlier observations (Entsch et al., 1991; Eschrich et al., 1993) that Y385F, unlike PHBH, can catalyze the C5 hydroxylation of DOHB, presumably by binding the physiological product with the 3OH rotated around the C1-C4 axis of the aromatic ring. From the structure of Y385F in complex with POHB it was deduced that the lost hydrogen bond between Tyr201 and Tyr385 in Y385F probably allows some displacement of Tyr201 (Lah et al., 1994). This would abolish the unfavorable contact between the 3OH of DOHB and the 4OH of Tyr201, presumably present in PHBH with DOHB bound in the flipped orientation (Lah et al., 1994). The predominant formation of 5,6-F_2-TOHB observed in the present study indicates that Y385F favors F_3-DOHB binding with the 3OH pointing toward the flavin. However, a similar reorientation of Tyr201 as envisioned from the Y385F structure, might induce the alternative binding mode of F_3-DOHB and lead to the formation of significant amounts of 2,6-F_2-TOHB. Moreover, the lost interaction between the side chains of Tyr201 and Tyr385 in Y385F might also explain the formation of small amounts of 5F-TeOHB from 5,6-F_2-TOHB (cf. Figure 4B).

As already pointed out before (Husain et al., 1980; Vervoort et al., 1992), *dianionic* F_4-POHB *is a rather slow* substrate for PHBH because of the deactivating effect of the fluorine substituents. The frontier orbital substrate characteristics presented in this paper suggest that C2 hydroxylation of F_3-DOHB is feasible when this substrate is activated at the C3 position, i.e., by deprotonation of the 3OH group. In keeping with their electron-withdrawing properties, the fluorine substituents of F_3-DOHB obviously stimulate the deprotonation of the 3OH moiety by delocalizing the negative charge. Upon deprotonation of the 3OH moiety, HOMO density and, thus, nucleophilic reactivity is redistributed and becomes located to a significant extent at the C2 center of F_3-DOHB. In line with this, the inability to deprotonate the 3OH group explains why DOHB is not a substrate for PHBH but rather an effector.

The enzymatic conversion of F_3-DOHB to 5,6-F_2-TOHB implies that F_3-DOHB binding induces the stabilization of the flavin C(4a)-hydroperoxide. Furthermore, it indicates that the peroxide bond of the oxygenated flavin intermediate becomes favorably oriented to allow C2 hydroxylation. Using the crystal structure of the reference compound 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (Bolognesi et al., 1978), Schreuder et al. (1990) have built a structural model of the flavin C(4a)-hydroperoxide in the active site of PHBH with bound POHB. In this model (Figure 8), the distal oxygen of the oxygenated flavin intermediate is in close vicinity (2.4 Å) of the C3 but also rather close (3.2 Å) to the C2 of the substrate. In the structure of the DOHB-complexed enzyme (Schreuder et al., 1989), the plane of the aromatic ring of DOHB is slightly rotated around the C1-C4 axis with respect to the plane of POHB, and the 3OH of the product is in short hydrogen bond distance of the carbonyl oxygen of Pro293. Because of steric constraints, this geometry will likely prevent the peroxide moiety of the oxygenated flavin to occupy the same orientation as modeled in the enzyme–substrate complex. From the conserved mode of binding of several substrate analogs (Schreuder et al., 1994), it is reasonable to assume that the binding mode of F_3-DOHB will resemble that of DOHB, leaving the salt bridge with Arg214 intact (cf. Figure 1). From this and the above considerations, we conclude that, in the F_3-DOHB complexed enzyme, the distal oxygen of the flavin C(4a)-hydroperoxide will approach the C2 atom. Moreover, enzyme dynamics will allow some rotational mobility of the substrate aromatic ring, facilitating a proper attack (see also below).

Several lines of evidence have indicated that the ionic state of the substrate is linked to the rate of flavin reduction by NADPH (Entsch & van Berkel, 1995). Our results are in accordance with this hypothesis. Unlike POHB binding (Entsch et al., 1991; Eschrich et al., 1993), binding of dianionic F_4-POHB highly stimulates the rate of reduction of Y385F. This supports the view that replacement of Tyr385 by Phe influences the ionization state of bound POHB (Eschrich et al., 1993). Crystallographic studies have indicated that deprotonation of POHB induces a conformational change in the active site loop extending from Pro293 to Ala296 (Gatti et al., 1996). It was inferred from these data that deprotonation of POHB might change the orientation of hydrogen bonds in the hydrogen bond network around the 4OH of the substrate. Binding of dianionic F_4-POHB might induce similar changes in the conformation of the active site loop. However, it seems obvious that in Y385F, the lost interaction between Tyr385 and Tyr201 will disrupt the potential chain of hydrogen bonds, which extends from the 4OH of POHB to His72 at the protein surface (Schreuder et al., 1994; Gatti et al., 1996). The conformation of the Pro293-Ala296 segment is probably also linked to the ionic state of F_3-DOHB. For instance, the deprotonated 3OH species of F_3-DOHB might be stabilized by hydrogen bond formation between the protonated 4OH and the carbonyl oxygen of Pro293. The deprotonation of the 3OH could be a transient one, i.e., under the influence of enzyme dynamics (Gatti et al., 1996). This suggests that the hydroxylation of the C2 center of F_3-DOHB predominantly is dictated by the HOMO density distribution of the π electrons in the substrate aromatic ring which is in accordance with the electrophilic aromatic substitution mechanism proposed for this class of flavoenzymes (Entsch et al., 1976a; Vervoort et al., 1992; Massey, 1994).

In conclusion, this paper demonstrates for the first time that C2 hydroxylation by PHBH is feasible when the chemical properties of the substrate favor (or allow) it. To get more insight in the dynamic factors which determine the regiospecificity of the hydroxylation reaction, we are presently addressing the mode of binding of fluorinated substrate analogs by ^{19}F NMR.

ACKNOWLEDGMENT

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Abbreviations and nomenclature

B-value	displacement of an atom from thermal motion, conformational disorder, and static lattice disorder
DOHB	dihydroxybenzoate
EFl_{ox}	oxidized flavoenzyme
EFl_{red}	reduced flavoenzyme
EPR	electron paramagnetic resonance
ϵ	molar absorption coefficient
E(HOMO)	energy of the highest occupied molecular orbital
FADHOH	flavin C(4a)-hydroxide
FADHOOH	flavin C(4a)-hydroperoxide
F_{obs}	fluorescence observed
F_3-DOHB	2,5,6-trifluoro-3,4-dihydroxybenzoate
F_4-POHB	2,3,5,6-tetrafluoro-4-hydroxybenzoate
G	Gauss
HOMO	highest occupied molecular orbital
I	ionic strength
I	inhibitor
k	rate constant
k_{cat}	turnover rate
K_d	dissociation constant
kDa	kilodalton
K_m	Michaelis constant
nm	nanometer
NMR	nuclear magnetic resonance
NO	nitric oxide
P	product
PHBH	*p*-hydroxybenzoate hydroxylase
pobA	gene encoding *p*-hydroxybenzoate hydroxylase
POHB	*p*-hydroxybenzoate
R	correlation coefficient
Rab proteins	(diverse group of) mammalial GTPases involved in intracellular transport

R factor	crystallographic refinement factor (degree of correspondence of calculated and observed amplitudes)
RP	reverse phase
R_{sym}	internal measure of the accuracy of a data set
S	substrate
5-F-TeOHB	5-fluoro-2,3,4,6-tetrahydroxybenzoate
6-F-TeOHB	6-fluoro-2,3,4,5-tetrahydroxybenzoate
TOHB	3,4,5-trihydroxybenzoate
2,6-F_2-TOHB	2,6-difluoro-3,4,5-trihydroxybenzoate
5,6-F_2-TOHB	2,6-difluoro-2,3,4-trihydroxybenzoate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
QAE	quaternary aminoethyl ion

Summary

Enzymes, which utilize molecular oxygen either to hydroxylate or to break the benzene nucleus, are known as monooxygenases or dioxygenases, respectively. Monooxygenases mostly contain a small organic molecule (prosthetic group or cofactor) such as heme, flavin, pterin or a transition metal ion in their active site, for oxygen activation. The dioxygenases mostly contain a metal ion.

This thesis focuses on *para*-hydroxybenzoate hydroxylase (PHBH), a flavoprotein monooxygenase involved in the mineralization of aromatic compounds in soil microorganisms. These organic molecules may originate from environmental pollution but also from natural sources since they are liberated during the biodegradation of lignin, one of the principal components of wood. Thus, understanding the action of flavoprotein monooxygenases is of importance for the so-called process of agrification, including the development of processes that enable the use of agro materials as sources for energy and industrial productions. PHBH has become a model for flavoprotein monooxygenases since its crystal structure is known and many aspects of the catalytic mechanism have been unravelled by rapid reaction techniques. In this thesis, the structure-function relationship of PHBH was addressed by the study of site-directed mutants.

Substrate binding

PHBH from *Pseudomonas fluorescens* is rapidly inactivated by mercurial compounds and the inactivation is strongly inhibited in the presence of 4-hydroxybenzoate. However, each PHBH subunit contains five sulfhydryl groups and it was unclear which cysteine(s) are protected from mercuration by substrate binding. Therefore, we selectively changed all cysteines into serines by site-specific mutagenesis and studied the recombinant enzymes for their catalytic properties and thiol reactivity (Chapter 3). By this approach, it was clearly established that the cysteine residues of PHBH are not essential in catalysis and that Cys211 was the main target of modification by spin-labeled *p*-mercuribenzoate. Spinlabelling of Cys211 resulted in 100-fold impaired binding of the aromatic substrate, 4-hydroxybenzoate, but had no effect on k_{cat}. This suggests that the covalently bound spin label is located at or near the substrate binding site. Moreover, it raises the possibility that modification of Cys211 perturbs flavin motion (see Chapter 5 and 6).

Substrate activation

The phenolic moiety of 4-hydroxybenzoate interacts in PHBH with the side chain of Tyr201, which is close to the side chain of Tyr385. Selective Phe replacements clearly revealed the

important role of this hydrogen bond network in substrate activation (Chapter 4). Both tyrosines are needed for the (transient) deprotonation of the phenolic moiety of 4-hydroxybenzoate, which stimulates the electrophilic attack of the flavin C(4a)-hydroperoxide. From anaerobic reduction experiments with a series of substrate analogs, it was established that both tyrosines are also important for the effector specificity.

In contrast to the wild-type enzyme, both Tyr->Phe mutants catalyzed the hydroxylation of the physiological product 3,4-dihydroxybenzoate to 3,4,5-trihydroxybenzoate. This showed that both tyrosines are also important for preventing the aromatic product from binding in the flipped orientation, i.e. with the C5 atom pointing towards the flavin ring. Together the results presented in Chapter 4 demonstrate that the tyrosine hydrogen bond network is important for substrate activation, for the effector specificity and for the regiospecificity of substrate hydroxylation. Moreover, the biocatalytic production of the anti-oxidant 3,4,5-trihydroxybenzoate (gallic acid) is of interest for the fine chemical industry.

Flavin movement and proton channel

In Chapter 5, the crystal structures of native PHBH complexed with the substrate analogues 4-aminobenzoate, 2,4-dihydroxybenzoate and 2-hydroxy-4-aminobenzoate, and the crystal structure of Y222A in complex with 2-hydroxy-4-aminobenzoate are described. These data revealed a new aspect of importance for the catalytic mechanism of PHBH. Binding of substrate analogues bearing a hydroxyl group at the 2-position leads to a displacement of the flavin ring outside the active site. From this it is proposed that FAD movement provides an entrance for the substrate to enter the active site and an exit for the product to leave.

The crystallographic data also revealed that binding of 4-aminobenzoate derivatives induces the binding of a water molecule next to Tyr385. As a result, a continuous hydrogen bonding network is formed between the 4-amino group of the substrate analogs and the side chain of His72, located near the protein surface. During normal catalysis, such an extended hydrogen bonding network may function as a proton channel, assisting substrate activation.

Substrate specificity

In Chapter 6, the role of Tyr222 in the active site of PHBH was investigated. This tyrosine interacts with the carboxy moiety of the substrate. Replacement of Tyr222 by Ala resulted in inefficient hydroxylation of 4-hydroxybenzoate, due to impaired substrate binding. However, Y222A tightly interacted with 2,4-dihydroxybenzoate. From crystallographic data (Chapter 5) it is inferred that this is due to the movement of the flavin ring out of the active site.

Y222A catalyzed the rather efficient hydroxylation of 2,4-dihydroxybenzoate to 2,3,4-trihydroxybenzoate. This indicates that reduction of Y222A by NADPH stimulates the flavin

ring to occupy the interior position in the active site. Spectral studies suggested that oxidized Y222A binds 2-fluoro-4-hydroxybenzoate in the phenolate form. From this and the reduction properties of Y222A it is concluded that the effector specificity of PHBH is mainly dictated by the protein-substrate interactions at the *re*-side of the flavin ring (Chapter 5). Altogether, Chapter 6 demonstrates that Tyr222 is crucial for the optimization of the substrate specificity of PHBH.

Regiospecificity of hydroxylation

Finally, in Chapter 7 it is shown that hydroxylation of tetrafluoro-4-hydroxybenzoate (F_4-POHB) by PHBH and Y385F is not restricted to C3, but rather involves sequential oxygenation and dehalogenation steps. The hypothesis presented to explain this observation relates to the electron withdrawing capacity of the fluorine substituents at C2 and C6 decreasing the pK_a of the 3-hydroxy moiety in the initially formed aromatic product (F_3-DOHB), facilitating its deprotonation, which is required for the activation of the C2 and C6 centre for an electrophilic attack by the flavin C(4a)-hydroperoxide cofactor.

The results of Chapter 7 also point at the perhaps unexpected mobility of the flavin C(4a)-hydroperoxide cofactor, able to reach not only the C3 position but clearly also able to interact with the C2 and C6 position of F_4-POHB. This also implies that C5 hydroxylation of F_3-DOHB should be possible. In wild-type PHBH, this hardly occurs due to a preferred orientation of F_3-DOHB in the active site. However, in Y385F hydroxylation at C5 is actually observed, confirming that Tyr385 is involved in the regiospecificity of hydroxylation (Chapter 4).

Substrate burying

In conclusion, this thesis shows that PHBH is highly optimized for its function by evolution. This optimization includes all residues involved in substrate binding and the dynamic movement of the flavin ring. Flavin movement opens the active site cavity to allow substrate binding and product release. The mobility of the flavin is also required for the efficient reduction of the enzyme by NADPH. Following reduction, the flavin swings back into the active site pocket in order to shield the hydroxylation site from solvent. The closure of the active site stabilizes the flavin C(4a)-hydroperoxide which becomes optimally oriented to allow efficient substrate attack.

Substrate burying is a recurrent property of flavoenzymes with a PHBH fold. In cholesterol oxidase, glucose oxidase and D-amino acid oxidase, the embedding of substrates is achieved by movement of an active site lid. The recently determined structure of phenol hydroxylase from yeast suggests that flavin movement might be a common feature of flavoprotein aromatic hydroxylases.

Samenvatting

Biochemie is de wetenschap die zich bezighoudt met de bestudering van biologische processen. In alle levende wezens spelen eiwitten een zeer belangrijke rol. Eiwitten zijn moleculen, die opgebouwd zijn uit aminozuren, waarvan er twintig bestaan. Door de aaneenschakeling van verschillende aminozuren, kunnen een oneindig aantal variaties in lengte en in samenstelling gemaakt worden. De code voor de volgorde van de specifieke aminozuren en de lengte van de aminozuurketen ligt opgeslagen in het DNA, de erfelijke informatie van elk organisme. De lineaire rangschikking van aminozuren in een polypeptideketen wordt de primaire structuur genoemd. Deze primaire structuur bepaalt de ruimtelijke structuur van een eiwit. Hierbij onderscheiden we de secundaire (o.a. α-helices en β-sheets), de tertiaire en quaternaire structuur. De ruimtelijke structuur van een polypeptideketen wordt aangeduid met de tertiaire structuur terwijl de assemblage van verschillende polypeptide ketens wordt aangeduid met de quaternaire structuur. Om de functie van een eiwit te kunnen begrijpen is het niet voldoende om de tertiaire structuur op te helderen. Ook inzicht in het dynamisch gedrag van aminozuren en liganden is noodzakelijk om het functioneren van het eiwit te leren begrijpen.

Eiwitten die een chemische reactie katalyseren worden enzymen genoemd. Enzymen bevatten soms een cofactor (bv. FMN, FAD) en zijn in staat bepaalde omzettingen die onder normale omstandigheden niet of zeer langzaam verlopen, snel en efficient te laten plaatsvinden. In dit proefschrift wordt ingegaan op de relatie tussen de structuur en functie van *p*-hydroxybenzoate hydroxylase (PHBH) uit *P. fluorescens*. Dit enzym is een flavine-afhankelijk monooxygenase dat voorkomt in bodem-microorganismen die betrokken zijn bij de aerobe afbraak van aromatische verbindingen. Aromaten komen van nature veel in de bodem voor door het rottingsproces van hout. Echter, door onzorgvuldig gebruik zijn met name in de eerste helft van deze eeuw ook veel recalcitrante niet-natuurlijke aromatische stoffen in de bodem terechtgekomen. Deze milieubelastende verbindingen worden langzaam maar zeker ook aangepakt door microorganismen omdat deze het vermogen bezitten de hiervoor geschikte enzymen aan te maken. In het kader van het milieu is het van belang om meer inzicht te verkrijgen in de precieze werking van de enzymen die kunnen fungeren als biologische schoonmaakmiddelen. PHBH is als model gekozen om de biologische activering van zuurstof te bestuderen omdat de kristalstructuur en vele aspecten van het katalytische mechanisme van dit enzym bekend zijn. De structuur-functie relatie van PHBH is in dit proefschrift onderzocht aan de hand van plaatsgerichte aminozuur veranderingen. Het onderzoek is uitgevoerd binnen de Wageningse onderzoeksschool Milieuchemie en Toxicologie (M&T).

Samenvatting

Inactivering van PHBH door aromatische kwikverbindingen kan sterk geremd worden door de aanwezigheid van het substraat 4-hydroxybenzoaat. In hoofdstuk 3 wordt aangetoond dat Cys211 verantwoordelijk is voor de kwikvergiftiging van het enzym waardoor het substraat geen toegang meer heeft tot het actieve centrum.

Activering van 4-hydroxybenzoaat door middel van deprotonering van de phenolische groep is van essentieel belang voor de electrofiele aanval van het flavine C(4a)-hydroperoxide intermediair op het substraat. In hoofdstuk 4 blijkt dat deze activering wordt bewerkstelligd door een waterstofbrugnetwerk wat loopt van His72 aan de buitenkant van het enzym via Tyr385 naar Tyr201. Beide tyrosines zijn ook belangrijk voor de stimulering van de reductiesnelheid en voor de substraatspecificiteit. Door de verandering van een van de tyrosine residuen wordt de regiospecifieke binding van het aromatisch produkt opgeheven. Hierdoor wordt 3,4-dihydroxybenzoaat verder omgezet tot cytotoxisch 3,4,5-trihydroxybenzoaat. Controversieel genoeg wordt deze verbinding ook toegepast als anti-oxidant.

Tot voor kort werd algemeen aangenomen dat prosthetische groepen in enzymen star gebonden zijn. Echter, de mobiliteit van het flavine molecuul blijkt een zeer belangrijk nieuw aspect te zijn voor het katalytisch mechanisme van PHBH (hoofdstuk 5). In aanwezigheid van bepaalde substraatanalogen beweegt het flavine naar een positie buiten het actieve centrum. Deze mobiliteit is essentieel voor het toelaten van het substraat en het vrijkomen van het product. Verder is het zeer aannemelijk dat de mobiliteit van het flavine ook belangrijk is voor een efficiënte reductie door NADPH. Deze laatste verbinding is een essentiële hulpstof (coenzym), waarvan onduidelijk is hoe het precies aan het enzym bindt.

De substraatspecificiteit van PHBH wordt mede bepaald door Tyr222 (hoofdstuk 6). De mutant Y222A bindt 4-hydroxybenzoaat slecht omdat er een H-brug interactie verloren gaat met de carboxyl groep van het substraat. Het substraatanaloog 2,4-dihydroxybenzoaat daarentegen wordt zeer stevig gebonden door een extra interactie met de flavine cofactor die uit het actieve centrum beweegt. Dit laatste verhindert echter niet de omzetting van 2,4-dihydroxybenzoaat wat er op wijst dat in de gereduceerde toestand het flavine weer naar binnen klapt. Uit de studies met de Y222A mutant kan ook worden afgeleid dat Tyr222 niet belangrijk is voor de effector specificiteit, m.a.w. voor de manier waarop substraat binding de juiste confrontatie van flavine en NADPH reguleert.

In hoofdstuk 7 is aangetoond dat hydroxylering van tetrafluoro-4-hydroxybenzoate (F_4-POHB) door PHBH niet gelimiteerd is tot de C3 positie van de aromatische ring, maar dat er opeenvolgende oxygeneringsstappen en defluorineringsstappen kunnen plaatsvinden. De unieke C2 hydroxylering wordt toegeschreven aan de reaktiviteit van het initieel gevormde produkt (F_3-DOHB) en de mobiliteit van het flavine hydroperoxide. Verder bevestigt de C5 hydroxylering

van F_3-DOHB door mutant Y385F dat Tyr385 betrokken is bij de regiospecificiteit van hydroxylering (zie hoofdstuk 4).

Concluderend kan gesteld worden, dat PHBH geoptimaliseerd is voor zijn functie tijdens de evolutie. Deze optimalisatie geldt voor alle aminozuren die betrokken zijn bij de substraatbinding en de beweeglijkheid van de flavine ring. Het naar buiten gaan van de flavine opent het actieve centrum waardoor het substraat naar binnen kan. Na enzymreductie (op een nog onbekende plaats) beweegt de flavine naar binnen om de hydroxyleringsplaats af te sluiten voor oplosmiddel. Hierdoor wordt het flavine hydroperoxide gepositioneerd en gestabiliseerd, wat efficiente hydroxylering mogelijk maakt. Na reoxidatie van het flavine gaat het actieve centrum weer open en het produkt wordt uitgewisseld voor een nieuw substraatmolecuul.

Het afschermen van het substraat is een eigenschap die gedeeld wordt door andere flavoproteinen met een soortgelijke domein structuur als PHBH. Echter, bij deze enzymen zorgt het eiwit zelf voor het dichtgaan van het actieve centrum. De recentelijk opgehelderde kristalstructuur van phenol hydroxylase suggereert dat flavine beweeglijkheid een typische eigenschap is van aromatische hydroxylases.

Curriculum vitae

Franciscus Jan Theodorus van der Bolt werd op 28 december 1963 geboren te Heerlen. In 1984 behaalde hij het VWO diploma aan het Grotius College te Heerlen. In 1986 begon hij met de studie biologie aan de Landbouwuniversiteit te Wageningen. Tijdens de doctoraalfase werden afstudeervakken gevolgd bij de vakgroepen Moleculaire Biologie (Prof. A. van Kammen), Fysische Plantenfysiologie (Prof. W. Vredenberg) en Biochemie (Prof C. Veeger). De stage vond plaats aan Universiteit van Arizona (Prof. W. Vermaas). In 1992 behaalde hij het doctoraal diploma.

In de periode van september 1992 tot en met september 1996 was hij werkzaam als onderzoeker-in-opleiding (OIO) bij de vakgroep Biochemie van de Landbouwuniversiteit te Wageningen. Het promotie-onderzoek werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk onderzoek (NWO) en heeft geleid tot dit proefschrift.

In 1996 heeft hij het ambachtelijke meubelbedrijf, gelegen aan de Streeperstraat 32 te Landgraaf, van zijn ouders overgenomen. Hier houdt hij zich vooral bezig met het ontwerpen en vervaardigen van meubels en of objecten van hout eventueel in combinatie met metaal, glas of andere materialen.

List of publications

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