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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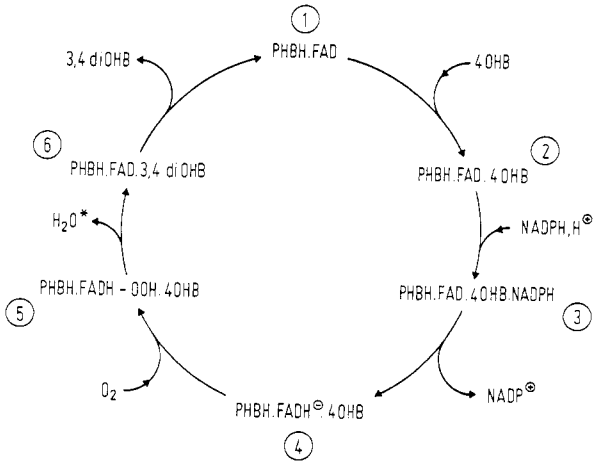

FIGURE 1: Catalytic cycle of *p*-hydroxybenzoate hydroxylase based on work by Entsch et al. (1976) and many others. PHBH, *p*-hydroxybenzoate hydroxylase; 4OHB, the substrate 4-hydroxybenzoate; $FADH^-$, reduced flavin bearing a negative charge at the N1 (Vervoort et al., 1991); FADH-OOH, flavin 4a-hydroperoxide 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 (Å²)				
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^α 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.

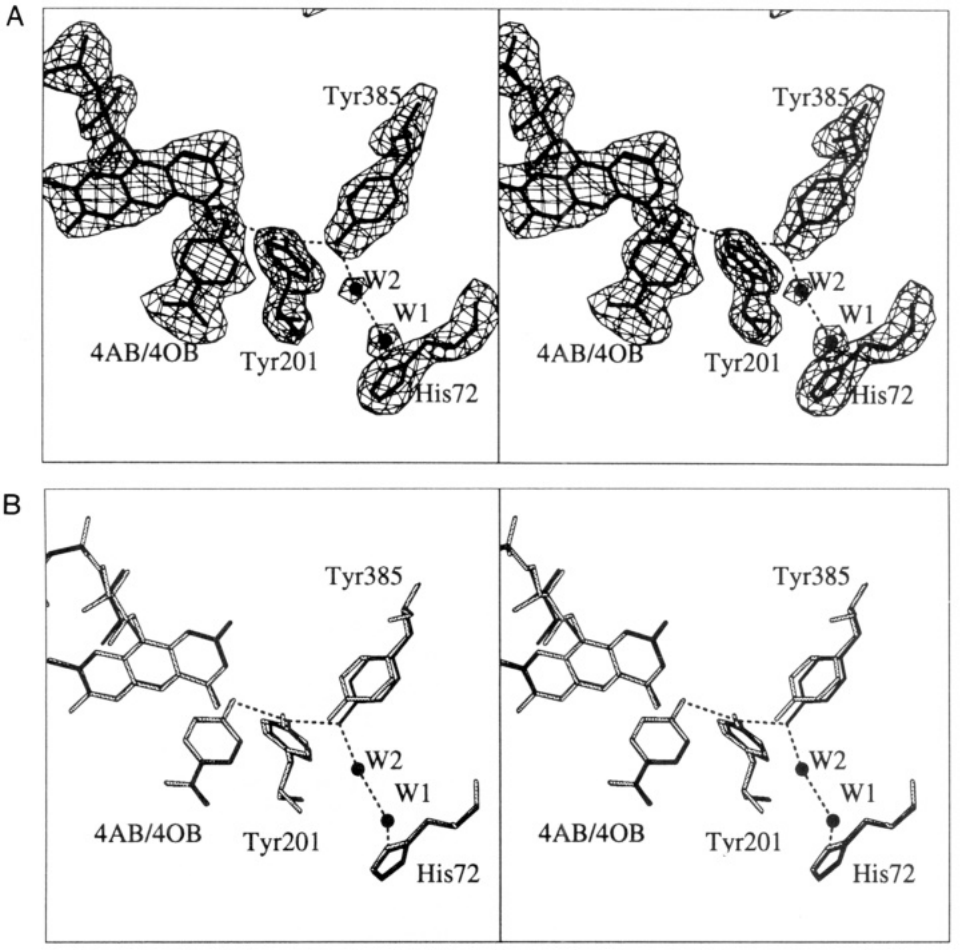

FIGURE 2: Structure of the 4-aminobenzoate complex. For clarity, only the flavin, the substrate (analogue), and the residues involved in the hydrogen-bonding network are shown. Abbreviations: 4OB, 4-hydroxybenzoate; 4AB, 4-aminobenzoate. (A) $F_o - F_c$ omit map, contoured at 4σ. The residues shown (His72, 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 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

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

	length of hydrogen bond (Å) for complex[a]				
hydrogen bond	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).

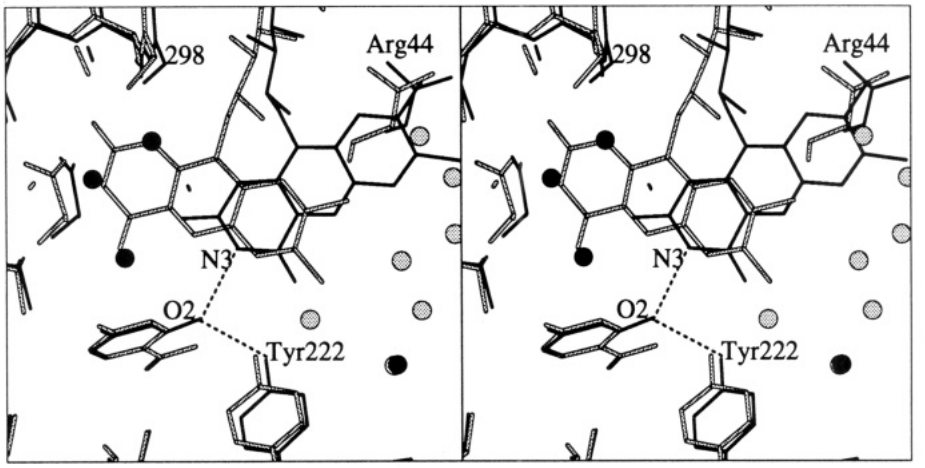

FIGURE 3: Superposition of the structures of the enzyme–substrate complex (Schreuder et al., 1989) and the 2,4-dihydroxybenzoate complex. The enzyme–substrate complex is drawn with open bonds, the 2,4-dihydroxybenzoate complex with solid bonds. Circles indicate bound water molecules. Broken lines indicate 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	(3.3) Wat172	(3.3) Wat172	(3.0) Wat209
	(3.1) N Leu299	(3.4) Wat186		
	(3.2) Wat230			
C2	(3.4) O Ala296		(3.4) Wat187	(3.2) Wat209
	(3.4) N Leu299			
O2	(3.1) O Ala296	(3.1) Wat172	(3.4) CB Ala45	(3.4) CB Ala45
	(2.9) N Leu299	(3.1) Wat182	(3.1) Wat187	(2.9) Wat209
	(3.5) CA Leu299	(2.9) Wat186	(3.2) Wat202	(3.2) Wat222
	(3.3) CB Leu299		(3.0) Wat206	(3.5) Wat224
	(3.1) N Asn300			
	(3.1) ND2 Asn300			
N3	(3.0) O Val47	(2.8) O2 DOB[c]	(2.9) O2 HAB[d]	(3.0) O2 HAB[d]
	(3.4) O Ala296			
C4	(3.5) CB Ala45			
O4	(3.2) N Gly46	(3.3) OH Tyr222	(3.4) OH Tyr222	
	(3.2) N Val47			
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	(2.8) Wat121
			(3.3) Wat115	
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

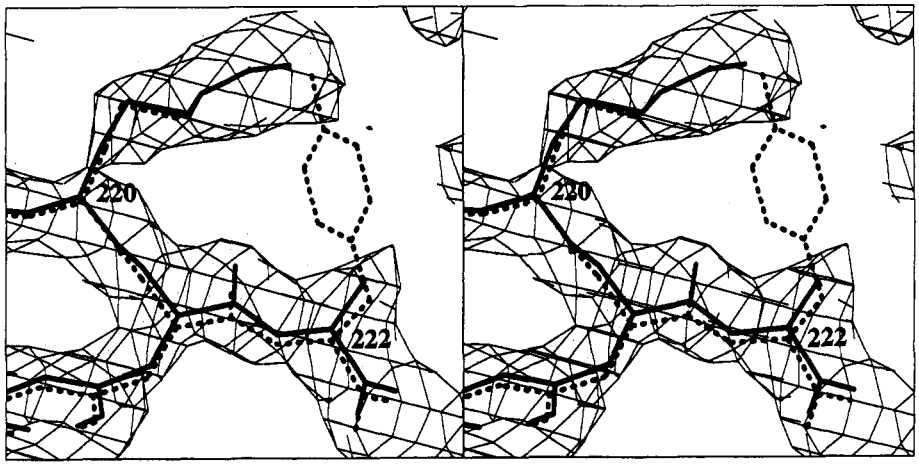

FIGURE 4: Electron density around the mutation site in the crystal structure of the Tyr222Ala mutant complexed with 2-hydroxy-4-aminobenzoate. The structure of the Tyr222Ala mutant is drawn with solid bonds; the structure of "wild-type" enzyme with 2-hydroxy-4-aminobenzoate is drawn in broken lines. 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-

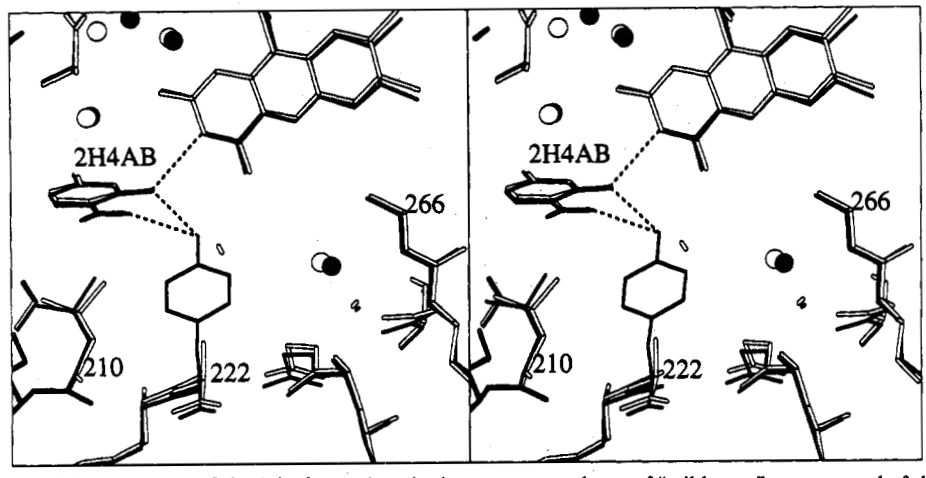

FIGURE 5: Superposition of the structures of the 2-hydroxy-4-aminobenzoate complexes of "wild-type" enzyme and of the Tyr222Ala mutant. "Wild-type" enzyme is drawn with solid lines, the Tyr222Ala mutant with open bonds. Dashed lines show hydrogen bonds between the OH of Tyr222 and the O2 and carboxyl 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

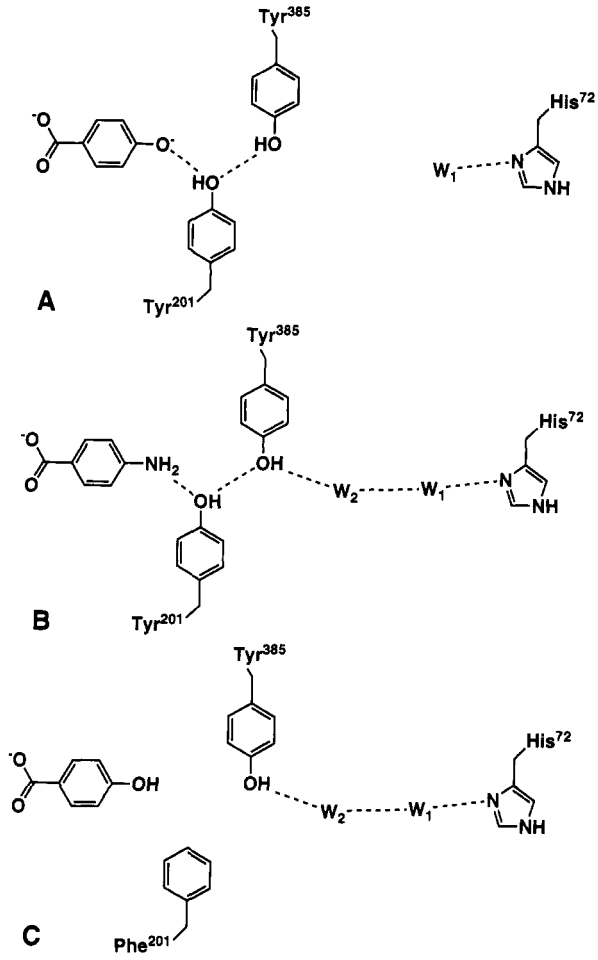

FIGURE 6: Schematic drawing of the active site hydrogen-bonding network in various structures of *p*-hydroxybenzoate hydroxylase. Protons cannot be determined by protein crystallography so their positions have been inferred. (A) 4-Hydroxybenzoate complex (Schreuder et al., 1989). Biochemical studies 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

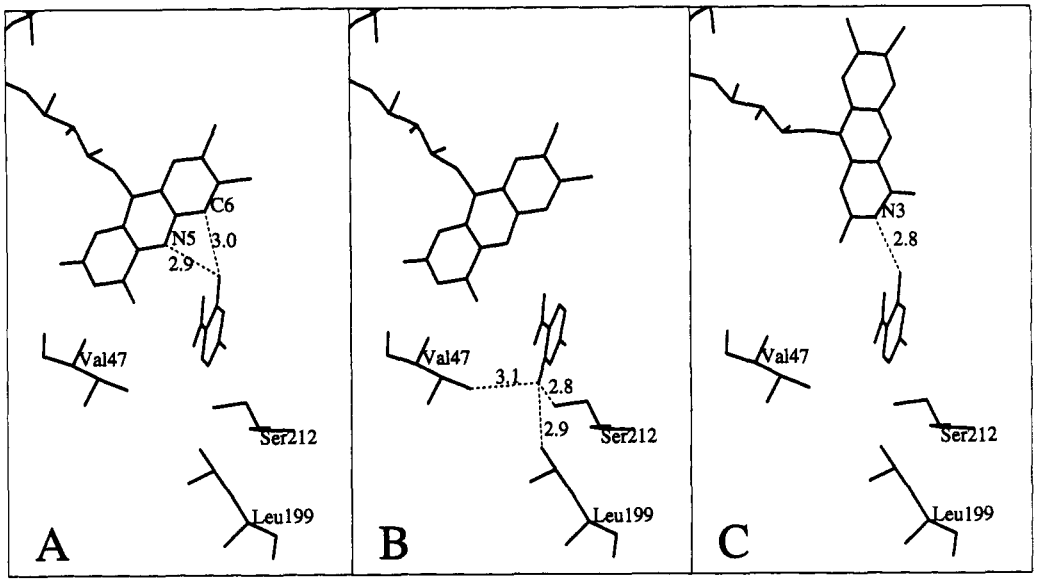

FIGURE 7: Theoretical contacts between the O2 of the 2,4-dihydroxybenzoate molecule and p-hydroxybenzoate hydroxylase with the flavin in the "in" position, and observed contacts with the flavin in the "out" position. The theoretical contacts of the observed orientation were obtained by superimposing 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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REFERENCES

Brünger, A. T. (1992) *XPLOR version 3.1. A system for X-ray crystallography and NMR,* Yale University Press, New Haven, CT.

DiMarco, A. A., Averhoff, B. A., Kim, E. E., & Ornston, L. N. (1993) *Gene 125,* 25–33.

Drenth, J., Hol, W. G. J., & Wierenga, R. K. (1975) *J. Biol. Chem. 250,* 5268–5269.

Engh, R. A., & Huber, R. (1991) *Acta Crystallogr. A47,* 392–400.

Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251,* 2550–2563.

Entsch, B., Nan, Y., Weaich, K., & Scott, K. F. (1988) *Gene 71,* 279–291.

Entsch, B., Palfey, B. A., Ballou, D. P., & Massey, V. (1991) *J. Biol. Chem. 266,* 17341–17349.

Eschrich, K., van Berkel, W. J. H., Westphal, A. H., de Kok, A., Mattevi, A., Obmolova, G., Kalk, K. H., & Hol, W. G. J. (1990) *FEBS Lett. 227,* 197–199.

Eschrich, K., van der Bolt, F. J. T., de Kok, A., & van Berkel, W. J. H. (1993) *Eur. J. Biochem. 216,* 137–146.

Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254,* 6657–6666.

Jones, T. A. (1985) *Methods Enzymol. 115,* 157–171.

Kabsch, W. (1976) *Acta Crystallogr. A32,* 922–923.

Kabsch, W. (1988) *J. Appl. Crystallogr. 21,* 916–924.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154,* 367–382.

Lah, M. S., Gatti, D., Schreuder, H. A., Palfey, B. A., & Ludwig, M. L. (1993) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) Walter de Gruyter, Berlin.

Lah, M. S., Palfey, B. A., Schreuder, H. A., & Ludwig, M. L. (1994) *Biochemistry 32,* 1555–1564.

Manstein, D. J., Pai, E. F., Schopfer, L. M., & Massey, V. (1986) *Biochemistry 25,* 6807–6816.

Messerschmidt, A., & Pflugrath, J. W. (1987) *J. Appl. Crystallogr. 20,* 306–315.

Meyer, E. (1992) *Protein Sci. 1,* 1543–1562.

Sanger, F., Nicklen, S., & Coulson, A. R. (1987) *Proc Natl. Acad. Sci. U.S.A. 74,* 4350–4354.

Schreuder, H. A., van der Laan, J. M., Hol, W. G. J., & Drenth, J. (1988a) *J. Mol. Biol. 199,* 637–648.

Schreuder, H. A., Hol, W. G. J., & Drenth, J. (1988b) *J. Biol. Chem. 263,* 3131–3136.

Schreuder, H. A., Prick, P. A. J., Wierenga, R. K., Vriend, G., Wilson, K. S., Hol, W. G. J., & Drenth, J. (1989) *J. Mol. Biol. 208,* 679–696.

Schreuder, H. A., Hol, W. G. J., & Drenth, J. (1990) *Biochemistry 29,* 3101–3108.

Schreuder, H. A., van der Laan, J. M., Swarte, M. B. A., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1992) *Proteins: Struct., Funct., Genet., 14,* 178–190.

Shoun, H., Beppu, T., & Arima, K. (1979) *J. Biol. Chem. 254,* 899–904.

Spector, T., & Massey, B. (1972) *J. Biol. Chem. 247,* 4679–4687.

van Berkel, W. J. H., & Müller, F. (1987) *Eur. J. Biochem. 167,* 35–46.

van Berkel, W. J. H., Müller, F., Jekel, P. A., Weijer, W. J., Schreuder, H., & Wierenga, R. K. (1988) *Eur. J. Biochem. 176,* 449–459.

van Berkel, W., Westphal, A., Eschrich, K., Eppink, M., & de Kok, A. (1992) *Eur. J. Biochem. 210,* 411–419.

van Berkel, W. J. H., van der Bolt, F. J. T., Eppink, M. H. M., de Kok, A., Rietjens, I. M. C. M., Vervoort, J., & Schreuder, H. A. (1993) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.), Walter de Gruyter, Berlin.

van der Laan, J. M., Schreuder, H. A., Swarte, M. B. A., Wierenga, R. K., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1989a) *Biochemistry 28,* 7199–7205.

van der Laan, J. M., Swarte, M. B. A., Groendijk, H., Hol, W. G. J., & Drenth, J. (1989b) *Eur. J. Biochem. 179,* 715–724.

Vervoort, J., van Berkel, W. J. H., Müller, F., & Moonen, C. T. W. (1991) *Eur. J. Biochem. 200,* 731–738.

Vervoort, J., Rietjens, I. M. C. M., van Berkel, W. J. H., & Veeger, C. (1992) *Eur. J. Biochem. 206,* 479–484.

Voorintholt, G., Kosters, M. T., Vegter, G., Vriend, G., & Hol, W. G. J. (1989) *J. Mol. Graphics 7,* 243–245.

Vrielink, A., Lloyd, L., & Blow, D. M. (1991) *J. Mol. Biol. 219,* 533–554.

Vriend, G. (1990) *J. Mol. Graphics 8,* 52–56.

Wierenga, R. K., de Jong, R. J., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1979) *J. Mol. Biol. 131,* 55–73.