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Analysis of the Active Site of the Flavoprotein *p*-Hydroxybenzoate Hydroxylase and Some Ideas with Respect to Its Reaction Mechanism†

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Received January 17, 1989; Revised Manuscript Received November 16, 1989

ABSTRACT: The flavoprotein *p*-hydroxybenzoate hydroxylase has been studied extensively by biochemical techniques by others and in our laboratory by X-ray crystallography. As a result of the latter investigations, well-refined crystal structures are known of the enzyme complexed (i) with its substrate *p*-hydroxybenzoate and (ii) with its reaction product 3,4-dihydroxybenzoate and (iii) the enzyme with reduced FAD. Knowledge of these structures and the availability of the three-dimensional structure of a model compound for the reactive flavin 4a-hydroperoxide intermediate has allowed a detailed analysis of the reaction with oxygen. In the model of this reaction intermediate, fitted to the active site of *p*-hydroxybenzoate hydroxylase, all possible positions of the distal oxygen were surveyed by rotating this oxygen about the single bond between the C4a and the proximal oxygen. It was found that the distal oxygen is free to sweep an arc of about 180° in the active site. The flavin 4a-peroxide anion, which is formed after reaction of molecular oxygen with reduced FAD, might accept a proton from an active-site water molecule or from the hydroxyl group of the substrate. The position of the oxygen to be transferred with respect to the substrate appears to be almost ideal for nucleophilic attack of the substrate onto this oxygen. The oxygen is situated above the 3-position of the substrate where the substitution takes place, at an angle of about 60° with the aromatic plane, allowing strong interactions with the π electrons of the substrate. Polarization of the peroxide oxygen–oxygen bond by the enzyme may enhance the reactivity of flavin 4a-peroxide.

Flavoprotein monooxygenases have been the subject of intensive studies. Members of this class of enzymes are *p*-hydroxybenzoate hydroxylase and other phenolic hydroxylases, bacterial luciferase, and microsomal flavin-dependent monooxygenase. These enzymes share the unique property that they are able to break the double bond of molecular oxygen and incorporate one oxygen in a substrate without the help of a metal ion as a catalyst (Bruice, 1984b). The other oxygen of the dioxygen molecule is converted into water. The way in which the flavin prosthetic group is able to activate molecular oxygen has received much attention [see, for instance, Wessiak et al. (1984b) and Bruice (1984a)].

p-Hydroxybenzoate hydroxylase is a well-studied example of a flavin-containing monooxygenase. It converts *p*-hydroxybenzoate into 3,4-dihydroxybenzoate with the help of molecular oxygen and NADPH. From biochemical studies (Entsch et al., 1976a; Husain & Massey 1979; Shoun et al., 1979b), the reaction scheme shown in Figure 1 has emerged.

The amino acid sequence of the enzyme from *Pseudomonas fluorescens*, which contains 394 residues, is known (Hofsteenge et al., 1983; Weijer et al., 1982; Wijnands et al., 1986). Chemical modification studies (Van Berkel et al., 1984; Wijnands et al., 1986), nuclear magnetic resonance studies (Vervoort, 1986), and studies of model reactions (Anderson et al., 1987; Bruice, 1984b; Wessiak et al., 1984a) have given further insights in the catalytic pathway. In our laboratory, the structure of the *Pseudomonas fluorescens* *p*-hydroxybenzoate hydroxylase enzyme–substrate (ES)[1] complex in the oxidized state was elucidated by Wierenga et al. (1979) and has recently been refined at 1.9-Å resolution (Schreuder et al., 1989). Lower resolution structures of the substrate-free complex, the reduced enzyme–substrate (ES_{red}) complex, and the enzyme–product (EP) complex have been obtained (Van der Laan, 1986; Schreuder et al., 1987, 1988a). Some of these crystal structures will now be analyzed to find out which factors might contribute to the catalysis of the hydroxylation step.

We will particularly focus our attention on the flavin 4a-peroxide intermediate (Figure 1, intermediate 5; Figure 2a) and the flavin 4a-hydroxide. The first intermediate has been shown to occur a few milliseconds before the actual hydroxylation step, the second intermediate a few milliseconds after the hydroxylation reaction (Entsch et al., 1976a). The

†This research has been carried out under the auspices of The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for Scientific Research (NWO).

[1] Abbreviations: enzyme, *p*-hydroxybenzoate hydroxylase; substrate, *p*-hydroxybenzoate; product, 3,4-dihydroxybenzoate; ES, enzyme–substrate; ES_{ox}, oxidized ES; ES_{red}, reduced ES; EP, enzyme–product; Sol, solvent molecule.

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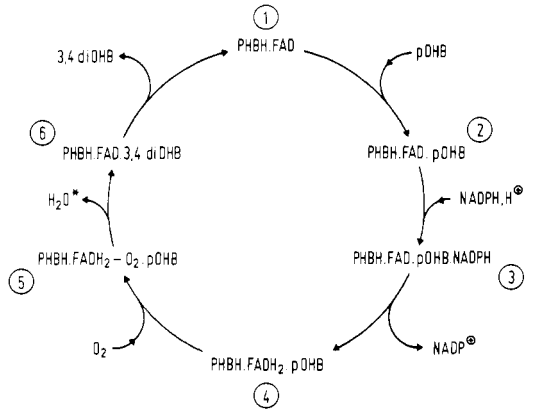

FIGURE 1: Catalytic cycle of *p*-hydroxybenzoate hydroxylase. Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate, the substrate; 3,4-diOHB, 3,4-dihydroxybenzoate, the reaction product. The $FADH_2 \cdot O_2$ intermediate is almost certain flavin 4a-hydroperoxide (Figure 2a).

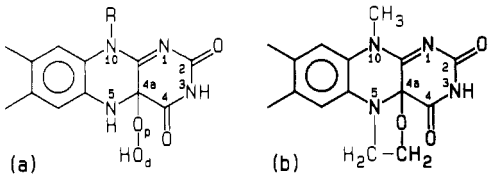

FIGURE 2: (a) Flavin 4a-hydroperoxide; (b) 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin, which is model for (a). Bold lines indicate the part of (b) which was used in the modeling study. Abbreviations: O_p, proximal oxygen; O_d, distal oxygen.

flavin 4a-peroxide intermediate has been observed by ^{13}C NMR in the related enzyme bacterial luciferase (Vervoort et al., 1986). At the actual hydroxylation step, a very short-lived strongly absorbing intermediate could be detected under certain conditions (Entsch et al., 1976a). The nature of this intermediate has been subject of considerable debate (Entsch et al., 1976a; Visser, 1983; Wessiak et al., 1984a,b; Bruice, 1984b; Anderson et al., 1987), but no conclusive evidence of its structure has been presented yet. Since flavin 4a-hydroperoxide is shown to be able to transfer oxygen to a variety of compounds and the flavin 4a-peroxide anion transfers dioxygen to a phenolate anion (Bruice, 1984b; Kemal & Bruice, 1979; Muto & Bruice, 1980, 1982), we assume in this study the flavin 4a-hydroperoxide to be the hydroxylating agent. The strongly absorbing intermediate might then represent a later occurring intermediate like the biradical proposed by Anderson et al. (1987).

Since the oxygenated flavin intermediates have lifetimes too short to be investigated by conventional X-ray crystallography, they have been studied by model building with the help of a model compound, determined by Bolognesi et al. (1978) (Schreuder et al., 1988b). Here we shall describe the investigation of possible conformations of the flavin 4a-peroxide, which gives further insight in the reaction mechanism and may serve as a basis for further experiments.

Materials and Methods

The recently refined crystal structures of *p*-hydroxybenzoate hydroxylase (Schreuder et al., 1989) were used for the present analysis. The gold cyanide position was obtained by calculating a difference Fourier between F_{obs} of the gold cyanide data set used for solving the phase problem and F_{obs} from a 2.5-Å data set of the ES_{ox} complex. Both data sets were collected by Wierenga et al. (1979). The phases used were from a partially refined structure of the ES_{ox} complex (R = 26.6%; Prick et al., unpublished results).

Table I: Geometry of a Number of Peroxides of the Type XOOY As Reported in Literature Compared with the Model Used in This Study

X	Y	r(O–O) (Å)	X–O–O angle (deg)	ref
Ph_3C	Ph_3C	1.480	107.5	*a*
Me_3C	Me_3C	1.480	103.9	*b*
Me_3Si	Me_3Si	1.481	106.6	*b*
PhCO	PhCO	1.46	111, 109	*c*
C_8H_{12}	H	1.442	112.0	*d*

	r(C–O) (Å)	X–O–C angle (deg)	ref
flavin 4a-epoxide	1.432	108.4	*e*

	r(O–O) (Å)	X–O–O angle (deg)	ref
flavin 4a-peroxide model	1.47	108.4	*f*

[a] Glidewell et al. (1979). [b] Käss et al. (1977). [c] Sax & McMullan (1967). [d] Belitskus & Jeffrey (1965). [e] Bolognesi et al. (1978). [f] Constructed for this study.

As a model for the flavin 4a-hydroperoxide intermediate (Figure 2a), the crystal structure of 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (Figure 2b) was taken as determined by Bolognesi et al. (1978). A detailed account of the fitting of this model to the active site of the *p*-hydroxybenzoate hydroxylase EP complex has been given before (Schreuder et al., 1988b). In contrast to the earlier modeling study where only the position of the proximal oxygen was used, the position of the carbon of the ethylene bridge, connected to the C4a-oxygen, was taken as the approximate position of the distal oxygen. The O–O bond length was adjusted on the basis of the geometry in a number of published structures (Table I). The peroxide model arrived at was compared with the known structures of a number of peroxides to check whether the model was reasonable (Table I).

The model of the flavin 4a-hydroperoxide was superimposed onto the flavin ring in the known crystal structures of complexes of *p*-hydroxybenzoate hydroxylase according to the method of Kabsch (1976) to obtain an initial position and by the method of Rao and Rossmann (1973) to optimize the superposition. In the last step, atoms deviating more than 3 times the standard deviation were excluded from the calculations to find the best superposition.

All possible orientations of the distal oxygen were scanned with the help of a computer program which rotated the distal oxygen about the C4a-proximal oxygen bond over 360° in steps of 1°. For each step, all distances with the 100 nearest protein atoms were calculated. Potentially interesting positions were printed.

The results were analyzed with programs from the Groningen BIOMOL protein structure package and also on an Evans & Sutherland PS390 picture system running FRODO software (Jones, 1985).

Results

Position of Gold Cyanide inside the Proposed Nicotinamide Binding Pocket. Although the crystal structure of *p*-hydroxybenzoate hydroxylase with bound NADPH has proved to be elusive, the position of the gold cyanide ion provides some clues. Gold cyanide is a very strong competitive inhibitor with respect to NADPH (Müller et al., 1983). It is bound in a pocket next to the flavin ring at a position normally occupied by two solvent molecules (Figure 3), just above Pro293 at the re side of the flavin. In studies with a radioactively labeled flavin analogue, Manstein et al. (1986) found that NADPH binds also to the re side of the flavin in *p*-hydroxybenzoate hydroxylase. In the crystal structure of glutathione reductase,

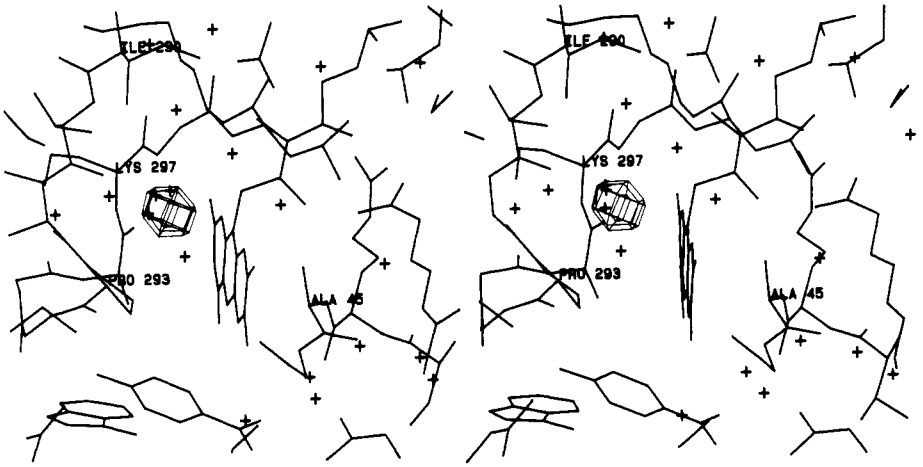

FIGURE 3: $F_{obs}[Au(CN)_2^-] - F_{obs}(ES)$ difference Fourier map indicating the position of the gold cyanide ion in the active site. It is bound just above Pro293 next to the flavin ring. (+) indicates the position of bound water molecules.

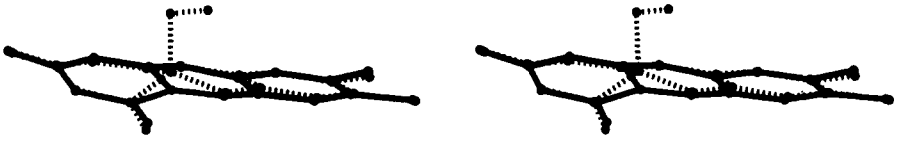

FIGURE 4: Superposition of the flavin 4a-hydroperoxide model (dashed lines) and the flavin ring in the 1.9-Å structure of the *p*-hydroxybenzoate hydroxylase ES complex (solid lines). The structures of both compounds have been determined independently by crystallographic techniques (Bolognesi et al., 1978; Schreuder, 1989).

the nicotinamide moiety of NADPH binds with its ring parallel to the flavin ring (Pai & Schultz, 1983), and the observation of a charge-transfer complex between NADPH and FAD in *p*-hydroxybenzoate hydroxylase (Husain & Massey, 1979) points to binding of the nicotinamide parallel to the flavin ring. In the ES complex, Pro293 is a little too close to the flavin ring to allow a parallel packing of flavin and nicotinamide rings, and, hence, the main chain must move somewhat upon nicotinamide binding in this pocket. An analogous main chain movement upon NADPH binding has been observed in glutathione reductase (Karplus & Schultz, 1987).

Positioning of a Model Compound in the Active Site. The flavin 4a-hydroperoxide model was superimposed onto the flavin ring in the three known structures of *p*-hydroxybenzoate hydroxylase as described under Materials and Methods. The results are summarized in Table II.

The flavin 4a-hydroperoxide model deviates less from the flavin rings as experimentally observed in the *p*-hydroxybenzoate hydroxylase structures than from a completely planar flavin ring. The striking resemblance between the flavin compounds becomes clear by examining Figure 4 where the superposition of the ring systems is shown. The fact that the flavin ring is bound by the enzyme in a conformation very similar to the independently determined flavin 4a-hydroperoxide model makes the occurrence of such an intermediate during the catalytic reaction by *p*-hydroxybenzoate hydroxylase very likely.

Survey of Possible Positions of the Distal Oxygen. Since bond angles and lengths are more or less fixed but torsional freedom exists about single bonds, all possible positions of the distal oxygen, O_d, were assessed by rotating around the C4a-proximal oxygen bond in steps of 1°. The value of the rotation angle α as found in the model determined by Bolognesi et al. (1978) was taken as 0°. The flavin 4a-hydroperoxide model was fitted to the active site of the 1.9-Å structure of the ES_{ox} complex. The ES_{ox} complex was chosen since its crystal structure is virtually identical with the crystal structure of the ES_{red} complex (Van der Laan, 1986; Schreuder et al., 1987), and it has been determined at the highest resolution

Table II: Root-Mean-Square Differences between All Equivalent Atoms after Superposition of the Flavin 4a-Hydroperoxide Model and Several Flavin Rings As Found in the Crystal Structures of *p*-Hydroxybenzoate Hydroxylase Complexes[a]

complex	resolution (Å)	rms[b] differences (Å)	atoms deviating >3σ
reduced ES[c]	2.3	0.19	C4a, O4
oxidized ES	1.9	0.17	C4a
enzyme–product	2.3	0.17	C4a, N1, N5
planar flavin		0.23	C4a, O4

[a] For comparison, also the root-mean-square differences with a planar flavin are given. [b] rms; root-mean-square. [c] ES, enzyme–substrate.

(1.9 versus 2.3 Å for the ES_{red} and also 2.3 Å for the EP complex). To obtain an estimate of the severity of the clashes which occurred during this rotation, for each 1° step the "total contact violations" were calculated. The "total contact violations" are defined as the sum of all distances by which the minimum allowed distance of a protein atom to the distal oxygen was violated. In order to allow for some flexibility of the protein molecule, we used a minimum allowed contact distance of 3.0 Å for the distance of the O_d to a carbon atom, 2.6 Å for the distance to a nitrogen, and 2.4 Å for the distance to an oxygen atom. So, for example, if a carbon atom was at a distance of 2.5 Å from the distal oxygen while the minimum allowed distance was 3.0 Å, this would add 0.5 Å to the "total contact violations". The higher the "total contact violations", the more severe the clashes with protein atoms. Distances to the flavin ring itself were not taken into account. The energies associated with positions of the oxygen above the flavin ring are probably comparable with energies of eclipsed conformations in hydrocarbons. A definition of the rotation angle α and a plot of the total contact violations are given in Figure 5. We will now briefly discuss features of three positions of the O_d atom.

Around $\alpha = 0°$, the flavin 4a-hydroperoxide intermediate has a staggered conformation. That is, O_d is trans with respect to C10a and is in between C4 and N5 (position 3 in Figure 5). The contact violation is due to a short contact of 2.4 Å

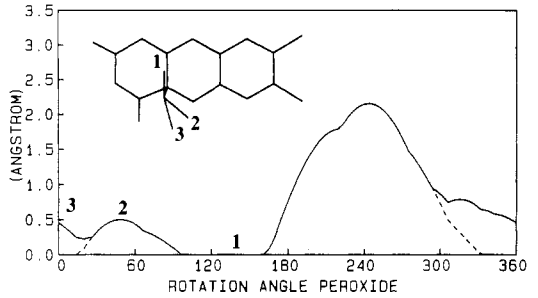

FIGURE 5: Total contact violations when the distal oxygen in the flavin model is rotated over 360° about the C4a–O_p bond. The original position of the distal oxygen derived from the model compound by Bolognesi et al. (1978) was taken as $\alpha = 0°$ (position 3 in the inset). The total contact violations are defined as the sum of the distances by which the minimum allowed distances are violated (see text). Solid line, contact violations including those with the substrate; dashed line, violations excluding those with the substrate. At position 1 near $\alpha = 140°$, the distal oxygen is near the peptide nitrogens of Lys297 and Gly298. No contact violations with the protein are present in this position. Some short contacts with Pro293 are present when the distal oxygen is situated above the N5 of the flavin (position 2). Around $\alpha = 0°$ (position 3), the distal oxygen is close to the C3 of the substrate, which reacts with this oxygen.

with the C3 of the substrate molecule. This is precisely the carbon atom of the substrate which reacts with the oxygen. It is clear that for this value of the rotation angle, the enzyme brings the reactants very close together and this may play a crucial role in enhancing the reaction rate.

The contact violations around $\alpha = 60°$ (position 2 in Figure 5) are due to short contacts with the C^{α} and C^{β} of Pro293. These clashes occur in the area above the flavin N5. Biochemical experiments by Manstein et al. (1986) indicate that the nicotinamide part of NADPH binds at this side of the flavin ring and is able to transfer a hydride ion to the N5 of the flavin ring. If a nicotinamide ring is able to bind in this region, an oxygen must be able to approach the flavin ring here as well. The shortest distance between Pro293 and the distal oxygen is 2.6 Å. This is rather short, but some flexibility in the protein molecule can be expected. As mentioned before, in glutathione reductase, main chain movements were observed upon NADPH binding (Karplus & Schultz, 1987).

Near $\alpha = 140°$ (position 1 in Figure 5), the distal oxygen is in a favorable position without any short contacts to protein atoms. Moreover, O_d is close to the main chain nitrogens of Lys297 and Gly298 and might form hydrogen bonds. This position is 1.2 Å away from the position of a bound solvent molecule, H_2O717 in Figure 6. Rotating beyond $\alpha = 180°$ is not possible without major protein rearrangements because of severe clashes with protein atoms (Figure 5), the most important one with the C^{α} of Ala296.

The rotation survey demonstrates the presence of a cavity near the N5, N10, and N1 atoms of the flavin ring, which is also the position where the nicotinamide part of NADPH is supposed to bind. It also shows that no space is available above the remaining part of the pyrimidine moiety of the flavin ring.

Situation in the Active Site Prior to the Hydroxylation. The fitting of the flavin 4a-hydroperoxide model and the survey of all possible positions of the distal oxygen give a clear impression how the atomic arrangement in the active site is likely to be at the hydroxylation step. This situation is based on model building and not on observation, but since a rotation of the distal oxygen by 20° results in a movement of only 0.5 Å, the atomic arrangement in the active site does not change drastically over a rather broad range of rotation angles. It is therefore expected that our model will not differ too much from the real situation. In Table III, the distances of the distal

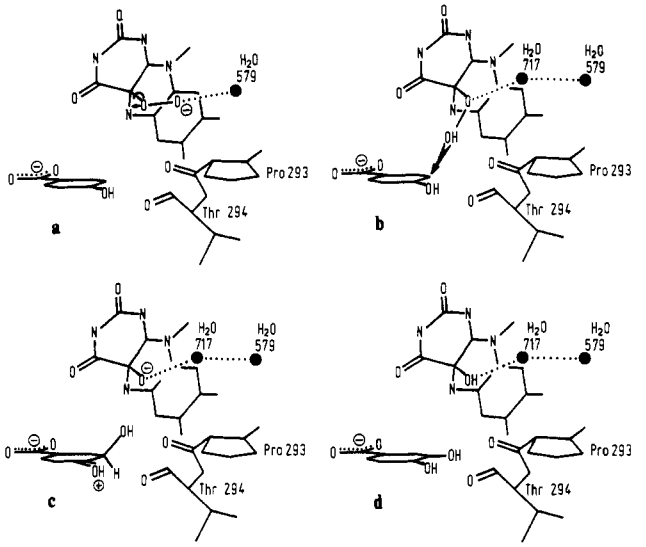

FIGURE 6: Possible scheme of events during catalysis by *p*-hydroxybenzoate hydroxylase. For details, see Figures 7 and 8. (a) Situation around $\alpha = 140°$ (see Figure 5). The negatively charged distal oxygen is situated near the main chain nitrogens of Lys297 and Gly298 (Table III) at the N-terminus of helix H10. Water molecule 579 could serve as a proton donor for the peroxide anion. (b) Situation in the active site with the distal oxygen closest to the C3 of the substrate where the substitution takes place. The distal oxygen seems to be almost ideally positioned for interaction with the π electrons of the substrate. (c) Oxygenated substrate intermediate. This intermediate of the electrophilic aromatic substitution reaction is well-known from organic chemistry (Solomons, 1976). An intermediate with a protonated 4-OH group is drawn. Other proposals include an intermediate with a deprotonated 4-OH group (Figure 8b; Entsch et al., 1976a) and a neutral radical (Anderson, 1987). (d) Product and flavin 4a-hydroxide. The flavin 4a-hydroxide decomposes into oxidized flavin and a water molecule. After the product has left the active site, the enzyme is ready for the next cycle.

Table III: Atoms within 3.5 Å of the Distal Oxygen of the Flavin 4a-Hydroperoxide Model in Two Situations[a]

situation i		situation ii	
atom	distance to distal oxygen (Å)	atom	distance to distal oxygen (Å)
Pro293 C	3.4	Ala296 CA	3.4
Pro293 O	2.5	Lys297 N	3.1
Ala296 CA	3.1	Gly298 N	3.3
pOHB[b]	3.2	Sol579	3.3
pOHB C3	2.4	Sol717[c]	1.2
pOHB O4	3.2	FAD N1	2.7
pOHB C4	2.9	FAD C2	3.1
FAD C4	3.0	FAD C4	3.4
FAD N5	2.5	FAD C10	2.3
FAD O4	3.3	FAD N5	3.4

[a] (i) At $\alpha = -21°$ (Figure 5), when the distal oxygen is closest to the C3 of the substrate where the substitution takes place. (ii) At $\alpha = 138°$ (Figure 5), when the distal oxygen is above the flavin ring near the N-terminus of helix H10. [b] *p*-Hydroxybenzoate. [c] The distal oxygen probably replaces the bound solvent molecule.

oxygen and its nearest neighbors are tabulated for the position where the distance between the oxygen and the C3 of the substrate, where the substitution occurs, is minimal.

In view of the nucleophilic attack of the π electrons of the substrate on the distal oxygen, the position of the distal oxygen with respect to the aromatic ring of the substrate is very interesting. In Figure 6b, the situation is given when the distal oxygen is closest to the C3 of the substrate. The line C3–O_d makes an angle of 57° with the plane of the aromatic ring. This appears to be very close to the angle required for the reaction intermediate with an sp^3 hybridized C3 atom as indicated in Figure 6c.

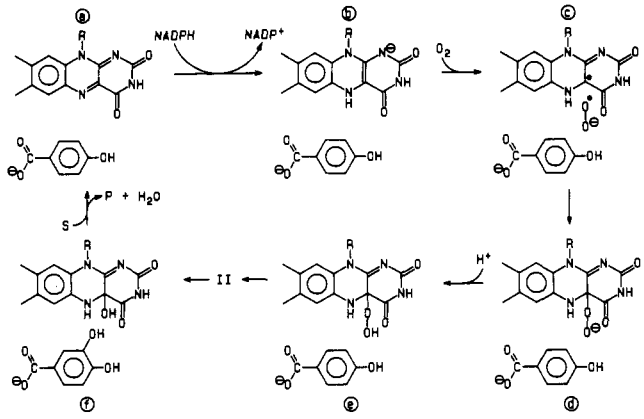

FIGURE 7: Scheme of the reaction of *p*-hydroxybenzoate hydroxylase, based on biochemical, spectroscopic, and model studies (Entsch et al., 1976a; Husain & Massey, 1979; Anderson, 1982; Wessiak et al., 1984b; Bruice, 1984b; Vervoort, 1986). This scheme is also compatible with the crystal structures. The exact nature of the strongly absorbing intermediate II is still not clear.

Discussion

The various aspects of the oxygen reactions in *p*-hydroxybenzoate hydroxylase can now be discussed in some detail. In the Discussion, we will follow the scheme in Figure 7, which is based on publications by various authors (Entsch et al., 1976a; Husain & Massey, 1979; Anderson, 1982; Wessiak et al., 1984b; Bruice, 1984b; Vervoort, 1986).

Fit of Models of Oxygenated Flavin Intermediates. The flavins in the crystal structures of the ES_{ox}, ES_{red}, and EP complex are not completely planar (Schreuder, 1988, 1989). In particular, the oxidized flavin ring appears to be slightly twisted. As shown in Table II, these slightly twisted flavins deviate less from the flavin 4a-peroxide analogue than does a completely planar flavin. The similarity of the analogue and the flavin, as it is bound to *p*-hydroxybenzoate hydroxylase, is in agreement with the notion that the active site of this enzyme is complementary to the flavin 4a-hydroperoxide intermediate (Schreuder et al., 1988b).

Absence of a Postulated Enzyme-Bound Base. Entsch et al. (1976a) postulated the presence of a base in the active site which would remove a proton from the 4-hydroxyl group of the substrate molecule and donate it to the flavin 4a-peroxide anion (Figure 7d). Examination of the residues in the neighborhood of the 4-OH of the substrate did not reveal any basic amino acid residue which could perform such a function. Alternatively, the distal oxygen could directly receive a proton from the 4-OH of the substrate. Their interatomic distance of 3.2 Å (Table III) is sufficiently short to allow a direct transfer of the 4-OH proton from the substrate to the flavin 4a-peroxide. The pK_a of the 4-OH group of the *p*-hydroxybenzoate molecule in solution is 9.32 (Handbook of Chemistry and Physics, 1977), while, as one referee pointed out, a pK_a of 13–14 can be inferred for the flavin 4a-peroxide. This is based on a pK_a of ca. 10 of the flavin 4a-hydroxide (Bruice et al., 1983) and the notion that the pK_a's of percarboxylic acids are about 4–5 units above those of the corresponding carboxylic acids. This pK_a difference makes it possible for the 4a-peroxyflavin to accept a proton from the substrate.

The Rotation "Experiment". The results of the rotation experiment open the possibility that movement of the distal oxygen occurs during catalysis by *p*-hydroxybenzoate hydroxylase. If molecular oxygen enters the active site via the same channel as the nicotinamide moiety of NADPH presumably does, the dioxygen molecule is above the flavin ring when it encounters the reactive C4a position. Model reactions (Anderson, 1982; Bruice, 1984b) indicate that first one electron is transferred from the reduced flavin to the dioxygen, resulting in a superoxide anion (Figure 7c). This superoxide anion could be bound by the amide nitrogens of Lys297 and Gly298. Also, the dipole moment of helix H10 will attract a negatively charged ion to this pocket. After spin inversion, a covalent intermediate could be formed with the distal oxygen above the flavin ring. Our modeling indicates that such an intermediate fits in the active site (Figure 6a; Table III). The distal oxygen of the 4a-flavoperoxide anion (Figures 6a and 7d) is in this situation near an active-site water molecule (Sol579 in the 1.9-Å structure of the ES complex) which is in contact with the bulk solvent. This water molecule would be able to donate a proton to the flavin 4a-peroxide anion to form a flavin 4a-hydroperoxide (Figure 7e). The N1 of the flavin does not act as a proton donor since the N1 of the reduced flavin in *p*-hydroxybenzoate hydroxylase is negatively charged and does not carry a proton (Entsch et al., 1976a; Moonen, 1983; Vervoort, 1986).

The flavin 4a-hydroperoxide is at this point in an "eclipsed" conformation. Rotation of the distal oxygen toward the hydroxylation position would relieve the strain caused by this conformation. The rotation "experiment" shows that space is available for such a movement.

Hence, the following sequence of events could be possible: (i) the flavin 4a-peroxide is formed with the distal oxygen above the flavin ring; (ii) the distal oxygen takes up a proton from a solvent molecule; and (iii) it moves toward the hydroxylation site by a rotation about the C4a–O_p bond.

The Hydroxylation Step. Substitution ortho to a hydroxyl group and experiments with different substrates (Husain et al., 1980) indicate that the hydroxylation reaction proceeds via an electrophilic aromatic substitution mechanism.

Other mechanisms like the involvement of a flavin 4,4a-epoxide (Visser, 1983) or an unknown mechanism similar to the reaction between the flavin 4a-peroxide anion and a phenolate anion (Bruice, 1984b; Kemal & Bruice, 1979; Muto & Bruice, 1980, 1982) seem less likely. The flavin 4,4a-epoxide does not fit in the active site as well as the flavin 4a-peroxide does, and the positioning of the reacting oxygen with respect to the substrate becomes less optimal. A reaction between the flavin 4a-peroxide anion and the phenolate anion of the substrate would bring two formal negative charges (the distal oxygen of the peroxide anion and the ionized 4-hydroxyl group of the phenolate anion) almost within van der Waals distance of each other. Probably for this reason only para substitution and no ortho substitution is observed in the model reaction.

The ionization state of the 4-OH group of the substrate molecule which affects the reaction is not clear. On the basis of a pK_a of 9.32 for this hydroxyl group in solution (*Handbook of Chemistry and Physics*, 1977), one would expect the hydroxyl group to be uncharged. The crystal structure gives no reason for a lowering of the pK_a below 7.5, necessary for the hydroxyl group to become ionized. However, biochemical experiments on *p*-hydroxybenzoate hydroxylase from *Pseudomonas putida* (Shoun et al., 1979a) and on the enzyme from *Pseudomonas fluorescens* using *p*-mercaptobenzoate as substrate (Entsch et al., 1976b) indicate that the 4-OH and 4-SH, respectively, of the bound substrate are deprotonated. Recently, more extensive studies on *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* indicate that not the hydroxyl group of the substrate, but the hydroxyl group of a neighboring tyrosine residue is ionized (van Berkel & Müller, 1989). This leaves two possibilities for the actual hydroxylation

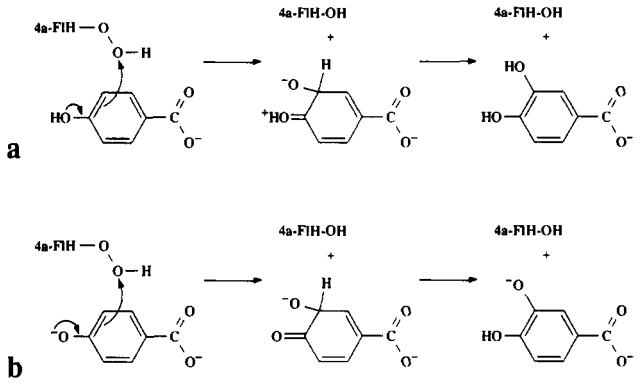

FIGURE 8: Two possible pathways for the hydroxylation step, based on Solomons (1976) and Bruice (1984b). (a) Starting from the substrate with an unionized hydroxyl group. (b) Starting from the substrate in the enolate form. Note that in these schemes, the proton of the distal oxygen moves to the proximal oxygen during transfer. In Figure 6, this proton stays on the O_d during transfer. Since this proton almost certainly mediates a hydrogen bond between the O_p and the former O_d, it is difficult to decide to which oxygen it is exactly bound.

step, which are depicted in Figure 8.

The reaction given in Figure 8a is preferred on the basis of the crystal structure. Two partial negatively charged main chain carbonyl oxygens near the 4-OH position seem to favor a neutral or positive charge at this position over a negative charge, particularly when the neighboring Tyr201 bears a negative charge (van Berkel & Müller, 1989). If Tyr201 is deprotonated, it could of course take the 4-OH proton from the reaction intermediate.

However, recent NMR experiments on crystalline serine protease and serine protease in solution (Smith et al., 1989) indicate an unexpected pK_a shift of almost 1 unit of a histine residue, which changes the hydrogen bonding scheme in the active site of the serine protease. The authors attribute this pK_a shift to the 1.0–1.3 M Li_2SO_4 used as a precipitant. For *p*-hydroxybenzoate hydroxylase, 35% saturated ammonium sulfate (1.4 M) is used for crystallization and may cause similar unexpected artifacts. Nevertheless, we would like to point out that the enzyme in crystalline form is fully active (Wierenga et al., 1979; Schreuder et al., 1988a), although about 40 000 times slower than free in solution.

In the active site of *p*-hydroxybenzoate hydroxylase, many hydrogen bond donors and acceptors are close together, and small rearrangements of the protein can lead to different hydrogen bonding schemes. An example is the hydrogen bonding scheme involving the substrate, Tyr201 and Tyr385. In the early models by Wierenga et al. (1982), before the model was completely refined, the hydroxyl groups of both tyrosines made hydrogen bonds with the hydroxyl group of the substrate. In the final model, only Tyr201 is directly hydrogen bonded to the substrate hydroxyl group.

Spectroscopic studies revealed a strongly absorbing transient intermediate (intermediate II; Figure 7) occurring close to the actual hydroxylation step (Entsch et al., 1976a). The exact nature of this intermediate is not clear and cannot be deduced from the crystal structures. A biradical as proposed by Anderson et al. (1987) may be a good candidate.

It is clear that further experimental evidence is necessary to find out along which path the hydroxylation reaction of *p*-hydroxybenzoate hydroxylase exactly proceeds. The protonation state of the bound substrate might be found by NMR experiments using ^{13}C-labeled *p*-hydroxybenzoate. The gene of *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa* has been cloned and is virtually identical with the enzyme from *Pseudomonas fluorescens* (Entsch et al., 1988), which allows testing of the various hypotheses by protein engineering.

Activation of the Substrate and the Flavin 4a-Peroxide. Catalysis of the hydroxylation reaction itself requires activation of two groups: the aromatic ring of the substrate and the distal oxygen of the flavin 4a-hydroperoxide. The aromatic ring of the substrate is activated by its 4-OH or 4-O^- group.

The reaction intermediate in the scheme of Figure 8a is stabilized by the formation of hydrogen bonds and perhaps also by compensation of the positive charge of the ionized hydroxyl group by the partial negative charges of two nearby carbonyl oxygens. Such stabilization increases the rate of the electrophilic aromatic substitution reaction (Solomons, 1976).

Ionization of the 4-OH group to 4-O^- in the scheme of Figure 8b renders the 3-position of the substrate much more nucleophilic, thus enhancing the reaction rate. The answer to the question which of the two schemes actually occurs must await further experimental evidence.

In general, the reactivity of the distal oxygen of the flavin 4a hydroperoxide intermediate can be enhanced by polarization of the oxygen–oxygen bond (Kemal et al., 1977) and by the electron-withdrawing properties of the flavin (Bruice, 1984b). In *p*-hydroxybenzoate hydroxylase, the dipole of helix H10 and hydrogen bonds of amide nitrogens with the N1, the O2, and the O4 exert a positive electrostatic field on the flavin ring and may thus enhance its electronegativity. Also, the position of Sol717 is interesting. One side of the water molecule is near two peptide nitrogens, and the other side is close to the proximal oxygen (Figure 6b). This causes the positive end of the dipole of this water molecule to be near the proximal oxygen. In contrast to the proximal oxygen, the distal oxygen is near two carbonyl oxygens with partial negative charges, those of Pro293 and Thr294. It is likely that this arrangement will cause a polarization of the oxygen–oxygen bond in the flavin 4a-hydroperoxide, such that the distal oxygen tends to become more positive and the proximal oxygen more negative.

Alternative Substrates. *p*-Hydroxybenzoate hydroxylase seems to be quite discriminating with respect to the substrates it accepts. Alternative substrates which are known to be hydroxylated include 2,4-dihydroxybenzoate and *p*-aminobenzoate. These compounds are highly activated toward electrophilic aromatic substitution, supporting the notion that the reaction proceeds via such a mechanism.

The pK_a of 9.12 for the 4-OH group of 2,4-dihydroxybenzoate (Mattoo, 1959) does not differ significantly from the pK_a for the 4-OH group of *p*-hydroxybenzoate, and its ionization state will probably be identical with that of *p*-hydroxybenzoate. It does not seem likely that the *p*-amino group of *p*-aminobenzoate gets deprotonated upon binding to the active site, and for this substrate, a reaction scheme similar to that of Figure 8a seems more plausible.

p-Mercaptobenzoate is also a substrate, but it is oxidized at the sulfur instead of at the aromatic ring (Entsch et al., 1976b). The sulfur is easier oxidized than the aromatic ring, and our model shows that the distal oxygen is very close to the para substituent of the substrate (3.2 Å for the *p*-hydroxybenzoate molecule, Table III), which offers a straightforward explanation for the observed reaction.

Finally, compounds not activated towards electrophilic aromatic substitution like benzoate and 6-hydroxynicotinate are not substrates for the enzyme.

CONCLUSIONS

The present study shows a remarkable similarity between the flavin ring in the crystal structures of three different

complexes of *p*-hydroxybenzoate hydroxylase and the flavin 4a-hydroperoxide model compound determined by Bolognesi et al. (1978). This indicates that the active site of *p*-hydroxybenzoate hydroxylase is complementary to the flavin 4a-hydroperoxide intermediate.

It does not seem to be necessary to search for an exotic reaction mechanism to explain the reaction of a peroxide anion with an aromatic ring. The rotation search shows that either an active-site water molecule or the hydroxyl group of the substrate might donate a proton to the flavin peroxide anion.

The almost ideal position of the distal oxygen, at an approximate angle of 60° with the plane of the aromatic ring of the substrate, is certainly important in enhancing the reaction rate. The ionization state of the substrate and the neighboring tyrosines requires further investigations before firm conclusions can be drawn.

The reactivity of the flavin 4a-peroxide is increased by a polarization of the O_d–O_p peroxide bond by the environment created by the protein. All these factors working together may explain why it is virtually impossible to perform the same reaction without the enzyme (Bruice, 1984b).

Acknowledgments

We thank Drs. J. M. van der Laan, J. Vervoort, and W. J. H. van Berkel for many helpful discussions. We are indebted to Prof. F. Müller for the critical reading of the manuscript and helpful suggestions. We thank Roelof Kloosterman for the drawings.

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Reaction of Porcine Pancreatic Elastase with 7-Substituted 3-Alkoxy-4-chloroisocoumarins: Design of Potent Inhibitors Using the Crystal Structure of the Complex Formed with 4-Chloro-3-ethoxy-7-guanidinoisocoumarin†

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Received June 9, 1989; Revised Manuscript Received November 27, 1989

ABSTRACT: The crystal structure of the acyl enzyme formed upon inhibition of porcine pancreatic elastase (PPE) by 4-chloro-3-ethoxy-7-guanidinoisocoumarin has been determined at a 1.85-Å effective resolution. The chlorine atom is still present in this acyl enzyme, in contrast to the previously reported structure of the 7-amino-4-chloro-3-methoxyisocoumarin–PPE complex where the chlorine atom has been replaced by an acetoxy group. The guanidino group forms hydrogen bonds with the carbonyl group and side-chain hydroxyl group of Thr-41, and the acyl carbonyl group has been twisted out of the oxyanion hole. Molecular modeling indicates that the orientation of the initial Michaelis enzyme–inhibitor complex is quite different from that of the acyl enzyme since simple reconstruction of the isocoumarin ring would result in unfavorable interactions with Ser-195 and His-57. Molecular models were used to design a series of new 7-(alkylureido)- and 7-(alkylthioureido)-substituted derivatives of 3-alkoxy-7-amino-4-chloroisocoumarin as PPE inhibitors. All the 3-ethoxyisocoumarins were better inhibitors than those in the 3-methoxy series due to better interactions with the S_1 pocket of PPE. The best ureido inhibitor also contained a *tert*-butylureido group at the 7-position of the isocoumarin. Due to a predicted interaction with a small hydrophobic pocket on the surface of PPE, this isocoumarin and a related phenylthioureido derivative are among the best irreversible inhibitors thus far reported for PPE ($k_{obs}/[I]$ = 8100 M^{-1} s^{-1} and 12 000 M^{-1} s^{-1}). Kinetic studies of the stability of enzyme–inhibitor complexes suggest that many isocoumarins are alkylating the active site histidine at pH 7.5 via a quinone imine methide intermediate, while at pH 5.0, the predominant pathway appears to be simple formation of a stable acyl enzyme derivative.

There is considerable interest in the design of inhibitors for elastases, which are thought to play a major role in the tissue destruction associated with arthritis, pancreatitis, and pulmonary emphysema (Janoff & Dearing, 1980; Powers & Bengali, 1986). Modern methods of rational drug design utilize computer graphics to model the binding of inhibitors to the active site of the targeted enzyme and can lead to the design of new inhibitors through visualization of preferred interactions between the enzyme and inhibitors (Gund et al., 1987). These methods should be very fruitful with elastases because the crystal structures of porcine pancreatic elastase (PPE)[1] and human leukocyte elastase (HLE) have been determined at atomic resolution and are available for molecular modeling (Bode et al., 1989).

Another requirement for successful inhibitor design is an X-ray structure of a complex between the targeted enzyme and one or more members of the class of inhibitors being investigated. These structures can then serve as "leads" for further modeling, design, and synthesis. Fortunately, several enzyme–inhibitor complexes are available with the elastases, including two peptide chloromethyl ketone complexes with HLE (Wei et al., 1988; Navia et al., 1989), two peptide fluoro ketones with PPE (Takahashi et al., 1988, 1989), and two benzoxazinones with PPE (Radhakrishnan et al., 1987).

Heterocyclic compounds such as 3-alkoxy-7-amino-4-chloroisocoumarins exhibit potent inhibitory activity toward a number of serine proteases including HLE and PPE (Harper & Powers, 1985; Kam et al., 1988). Many isocoumarins simply form acyl enzymes with serine proteases as a result of nucleophilic attack of the Ser-195 hydroxyl group on the isocoumarin carbonyl group. However, some isocoumarins are mechanism-based inhibitors in which a reactive group is unmasked during the acylation reaction and subsequently reacts with other residues in the active site of the enzyme, leading to irreversible inhibition. The structure of the acyl enzyme

†Supported by grants from the National Institutes of Health to J.C.P. (HL 29307 and HL 34035) and from the Robert A. Welch Foundation (A328) and the Texas Agricultural Experiment Station to E.F.M. Computational resources were provided by Associate Provost for Computing, Dr. John Dinkel (Texas A&M).

[1] Abbreviations: CDI, 1,1′-carbonyldiimidazole; EIC, 7-amino-4-chloro-3-ethoxyisocoumarin; GIC, 4-chloro-3-ethoxy-7-guanidinoisocoumarin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLE, human leukocyte (neutrophil) elastase; IC, isocoumarin; MIC, 7-amino-4-chloro-3-methoxyisocoumarin; NA, *p*-nitroanilide; PPE, porcine pancreatic elastase; Suc, succinyl.

0006-2960/90/0429-3108$02.50/0