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Crystal structure of *p*-hydroxybenzoate hydroxylase reconstituted with the modified FAD present in alcohol oxidase from methylotrophic yeasts: Evidence for an arabinoflavin

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Abstract

The flavin prosthetic group (FAD) of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* was replaced by a stereochemical analog, which is spontaneously formed from natural FAD in alcohol oxidases from methylotrophic yeasts. Reconstitution of *p*-hydroxybenzoate hydroxylase from apoprotein and modified FAD is a rapid process complete within seconds. Crystals of the enzyme–substrate complex of modified FAD-containing *p*-hydroxybenzoate hydroxylase diffract to 2.1 Å resolution. The crystal structure provides direct evidence for the presence of an arabityl sugar chain in the modified form of FAD. The isoalloxazine ring of the arabinoflavin adenine dinucleotide (a-FAD) is located in a cleft outside the active site as recently observed in several other *p*-hydroxybenzoate hydroxylase complexes.

Like the native enzyme, a-FAD-containing *p*-hydroxybenzoate hydroxylase preferentially binds the phenolate form of the substrate ($pK_a = 7.2$). The substrate acts as an effector highly stimulating the rate of enzyme reduction by NADPH ($k_{red} > 500\ s^{-1}$). The oxidative part of the catalytic cycle of a-FAD-containing *p*-hydroxybenzoate hydroxylase differs from native enzyme. Partial uncoupling of hydroxylation results in the formation of about 0.3 mol of 3,4-dihydroxybenzoate and 0.7 mol of hydrogen peroxide per mol NADPH oxidized. It is proposed that flavin motion in *p*-hydroxybenzoate hydroxylase is important for efficient reduction and that the flavin "out" conformation is associated with the oxidase activity.

Keywords: apo-flavoprotein; arabino-FAD; crystal structure; flavin conformation; flavoprotein oxidases; *p*-hydroxybenzoate hydroxylase; reconstitution

In 1985, Sherry and Abeles reported that alcohol oxidase isolated from methylotrophic yeasts contains 2 different forms of FAD. One form was identified as natural FAD and the other as an optical isomer differing only in the ribityl part of the ribityldiphosphoadenosine side chain. Subsequent studies by Bystrykh et al. (1989, 1991) revealed that the content of the modified flavin in alcohol oxidase from *Hansenula polymorpha* ranges from 5 to 95% of total flavin, dependent on the culturing conditions. Furthermore, it was demonstrated that conversion of natural FAD into modified FAD is autocatalyzed by the purified enzyme and strongly inhibited in the presence of reducing agents (Bystrykh et al., 1991). From this and from NMR structural analysis of the extracted flavin it was argued that the modified FAD most probably is an arabinoflavin adenine dinucleotide (Kellog et al., 1992). The stereochemical modification of FAD changes the catalytic properties of alcohol oxidase and may be of physiological relevance (Bystrykh et al., 1991). The lack of crystallographic data for the octameric alcohol oxidases does not allow rationalization of the changes in catalysis from a structural point of view. We therefore have started studying the interaction of the modified flavin with other flavoproteins for which structural data are available. In this paper we describe some properties of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* reconstituted with the stereochemi-

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Abbreviations: a-FAD, arabinoflavin adenine dinucleotide; a-FMN, arabinoflavin mononucleotide; alcohol oxidase, alcohol:oxygen oxidoreductase (EC 1.1.3.13); catalase, hydrogen-peroxide oxidoreductase (EC 1.11.1.6); cholesterol oxidase, 3β-hydroxysteroid:oxygen oxidoreductase (EC 1.1.3.6); glucose oxidase, β-D-glucose:oxygen 1-oxidoreductase (EC 1.1.3.4); *p*-hydroxybenzoate hydroxylase, 4-hydroxybenzoate, NADPH:oxygen oxidoreductase (3-hydroxylating) (EC 1.14.13.2).

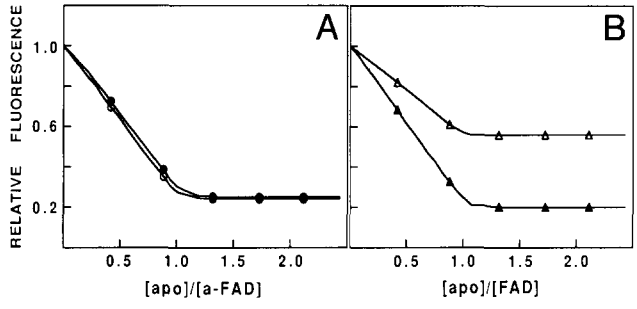

Fig. 1. Fluorescence titration of a-FAD with apo-*p*-hydroxybenzoate hydroxylase. Flavin (2 μM) was titrated with apo-*p*-hydroxybenzoate hydroxylase either in the absence or presence of 4-hydroxybenzoate. All experiments were performed at 25 °C in 100 mM Tris/sulfate, pH 8.0. Fluorescence emission was observed at 525 nm upon excitation at 450 nm. **A:** a-FAD in the absence (○) or presence (●) of 1 mM 4-hydroxybenzoate. **B:** Natural FAD in the absence (△) or presence (▲) of 1 mM 4-hydroxybenzoate.

cally modified FAD, obtained from *H. polymorpha* alcohol oxidase. By using the *p*-hydroxybenzoate hydroxylase apoprotein as a vehicle, direct crystallographic evidence is provided for the presence of an a-FAD in alcohol oxidases from methylotrophic yeasts.

Introduction of an optical isomer of FAD may give valuable additional information about the structure–function relationship of *p*-hydroxybenzoate hydroxylase. Recent crystallographic studies (Gatti et al., 1994; Schreuder et al., 1994) indicate that in *p*-hydroxybenzoate hydroxylase, flavin motion is a crucial factor for substrate binding and product release. As flavin motion requires a flexible ribityl side chain, a stereochemical modification in this side chain is expected to give more insight into the functional role of different flavin conformers. The crystal structure presented in this paper shows that the a-FAD bound to *p*-hydroxybenzoate hydroxylase is located in the "out" conformation. The rapid reduction of the arabinoflavin by NADPH lend us to propose that flavin motion in *p*-hydroxybenzoate hydroxylase is also important for an optimal positioning of the nicotinamide cofactor.

Results

Reconstitution of p-hydroxybenzoate hydroxylase

Reconstitution of holo *p*-hydroxybenzoate hydroxylase from the dimeric apoprotein and a-FAD is a rapid process. Upon addition to the apoprotein of an excess of a-FAD, the maximal activity is reached within a few seconds. The kinetics of reconstitution were not studied in detail. Activity measurements performed in the presence of nanomolar concentrations of flavin (Müller & van Berkel, 1982) indicate that binding of a-FAD is somewhat slower than with normal FAD.

The flavin fluorescence quantum yield of the a-FAD–apoprotein complex was determined from fluorescence titration experiments. Figure 1A shows that binding of the apoprotein results in a strong quenching of the fluorescence of a-FAD. The flavin fluorescence quantum yield of the a-FAD–apoprotein complex is much lower than that of the native enzyme (van Berkel & Müller, 1989). In contrast to the native enzyme (Fig. 1B), the presence of the aromatic substrate hardly influences the fluorescence quantum yield of protein-bound a-FAD (Fig. 1A).

Structural properties

The crystal structure of *p*-hydroxybenzoate hydroxylase complexed with a-FAD was solved and refined at 2.1 Å resolution (Kinemage 1). The final *R*-factor is 0.179 for 26,407 reflections between 8.0 and 2.1 Å. The almost (97.2%) complete 2.1-Å data allowed us to unambiguously establish the absolute configuration of the C2 carbon of the ribityl chain. The electron density map in Figure 2 clearly shows that the configuration at this position has changed from R to S, as has been inferred from NMR experiments (Kellog et al., 1992). The most important interaction between the modified flavin and the protein, the strong hydrogen bond with the OE1 of Gln 102, which is present both in the enzyme–substrate (Schreuder et al., 1989) and the 2,4-dihydroxybenzoate complex (Schreuder et al., 1994), is preserved in the a-FAD complex (see Fig. 3, Kinemage 1, and Table 1) despite the change in configuration. Table 1 also shows that whereas the O2′ of the natural flavin is buried between the side chain of Arg 44 and the flavin ring, it is more exposed in the a-FAD complex and contacts a bound water molecule (Wat 159).

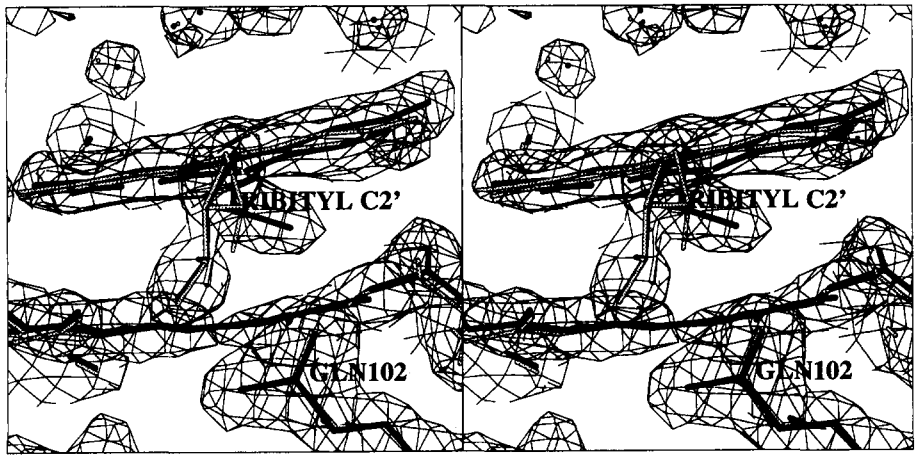

Fig. 2. Stereo diagram of the $2F_o - F_c$ electron density map of a-FAD-containing *p*-hydroxybenzoate hydroxylase in complex with 4-hydroxybenzoate. The final $2F_o - F_c$ electron density map was contoured at 1σ. The view is from the ribityl chain toward the flavin ring. The structure of the 4-hydroxybenzoate complex with a-FAD is drawn with solid bonds; the structure of the 2,4-dihydroxybenzoate complex with natural FAD is drawn with open bonds. Only the ribityl C2′ in the S-configuration (a-FAD complex, solid bonds) fits the electron density map. The side chain of Gln 102, which makes a hydrogen bond with the O2′ is visible in the lower right corner of the figure.

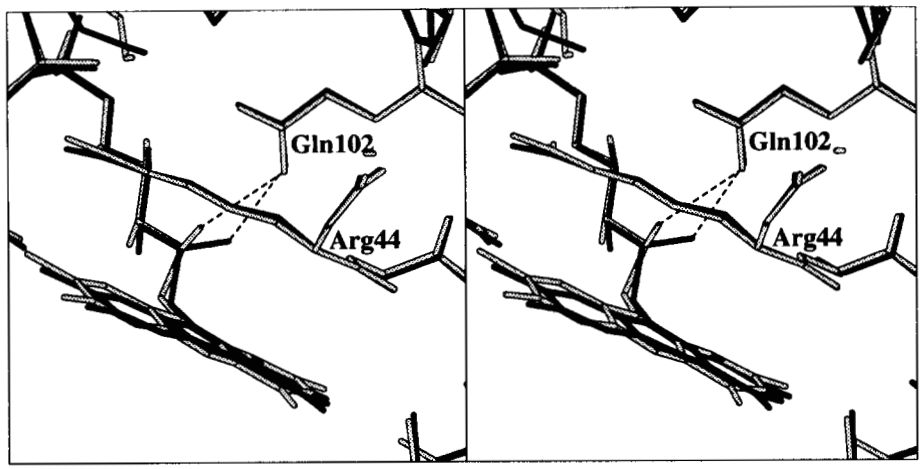

Fig. 3. Superposition of the structures of the a-FAD-containing enzyme–4-hydroxybenzoate complex and the native enzyme–2,4-dihydroxybenzoate complex. The structure of the 4-hydroxybenzoate complex with a-FAD is drawn with solid bonds; the structure of the 2,4-dihydroxybenzoate complex with natural FAD is drawn with open bonds. The view is from the flavin ring toward the ribityl chain. Broken lines indicate the hydrogen bond between the O2′ hydroxyl group and the OE1 of Gln 102, which is present in both complexes.

Binding of the modified flavin does not alter the overall structure of *p*-hydroxybenzoate hydroxylase. The RMS difference of the a-FAD-containing enzyme–substrate complex after superimposing the 391 C^{α} atoms present in the models are 0.22 Å with the native enzyme–substrate complex and 0.21 Å with the native 2,4-dihydroxybenzoate complex. These differences are of the same magnitude as the 0.2–0.3-Å mean coordinate error, which can be derived from Luzzati plots (Luzzati, 1952). The most important difference between the native enzyme–substrate complex and the a-FAD complex is that the flavin has moved to the "out" position (Fig. 4). The position of the flavin ring in the a-FAD-containing enzyme–substrate complex is almost identical to its position in the complex of the native enzyme with 2,4-dihydroxybenzoate (Schreuder et al., 1994). Figure 5 shows that in the latter complex, the flavin ring is approximately 0.7 Å further out, presumably because of a hydrogen bond contact of the 2-hydroxy group of the substrate analog (which is not present in the normal substrate) and the N3 of the flavin. The flavin ring is not completely planar in the a-FAD complex. The dimethylbenzene ring and the pyrimidine ring (the outer rings of the flavin ring system) make an angle of 7.1°. Interestingly, the flavin ring is not twisted as is the native flavin ring in the "in" position, but is bent like a butterfly. The geometry of the flavin ring is well within the range of butterfly conformations found in other flavoenzymes (Mathews, 1991).

Table 1. *Contacts (d < 3.4 Å) between the O2′ of the ribityl chain and protein or FAD atoms*[a]

a-FAD-containing 4-hydroxybenzoate complex	2,4-Dihydroxybenzoate complex[b]	4-Hydroxybenzoate complex[c]
	CD Arg 44 (3.2)	CD Arg 44 (3.4)
		CB Arg 44 (3.3)
OE1 Gln 102 (2.7)	OE1 Gln 102 (2.7)	OE1 Gln 102 (2.6)
	C9 flavin (3.3)	C9 flavin (3.2)
	C9A flavin (2.9)	C9A flavin (3.2)
N10 flavin (3.1)	N10 flavin (2.5)	N10 flavin (2.9)
O3′ ribityl (2.9)		
C4′ ribityl (2.9)	C4′ ribityl (3.1)	C4′ ribityl (3.1)
O4′ ribityl (3.4)	O4′ ribityl (2.9)	O4′ ribityl (3.1)
OW Water 159 (3.2)		

[a] Not mentioned are 1–2 and 1–3 contacts (i.e., with the ribityl C1′, C2′ and C3′ atoms). Distances in Å are given in parentheses.
[b] From Schreuder et al. (1994).
[c] From Schreuder et al. (1989).

Spectral properties

The visible absorption spectrum of free a-FAD is comparable to that of normal FAD (Table 2; Sherry & Abeles, 1985; Bystrykh et al., 1989). Binding to apo-*p*-hydroxybenzoate hydroxylase slightly influences the absorption characteristics of a-FAD. Table 2 shows that at pH 7.0, the maximum of the first absorption band is shifted from 450 to 458 nm and that the molar absorption coefficient of this band is higher than with the native enzyme. Upon binding of the aromatic substrate to the a-FAD-complexed enzyme, the intensity of the first absorption band increases with a concomitant shift of the absorption maximum to 455 nm. The intensity of the first absorption band of the a-FAD-containing enzyme–substrate complex is much higher than the corresponding band of the native enzyme–substrate complex (Table 2). Figure 6 shows a set of difference spectra recorded at pH 8.0, between the free a-FAD-containing enzyme and in the presence of increasing concentrations of 4-hydroxybenzoate. From the titration curve (inset, Fig. 6) simple 1:1 binding is observed and a dissociation constant for the a-FAD-complexed enzyme–substrate complex of about 50 μM is estimated. This value is in the same range as found for the corresponding complex of the native enzyme (van Berkel & Müller, 1989).

Bystrykh et al. (1989) reported that the CD spectrum of free a-FAD is different from natural FAD. In view of this, and because of the "out" position of the flavin observed in the crystal structure, it was of interest to study the CD properties of a-FAD-containing *p*-hydroxybenzoate hydroxylase. In the visible region, the CD spectrum of native *p*-hydroxybenzoate hydroxylase shows a negative Cotton peak around 450 nm and a positive Cotton peak around 365 nm (Fig. 7A; van Berkel & Müller, 1989). Upon binding of the aromatic substrate, the positive Cotton peak shifts to 380 nm, corresponding to the second

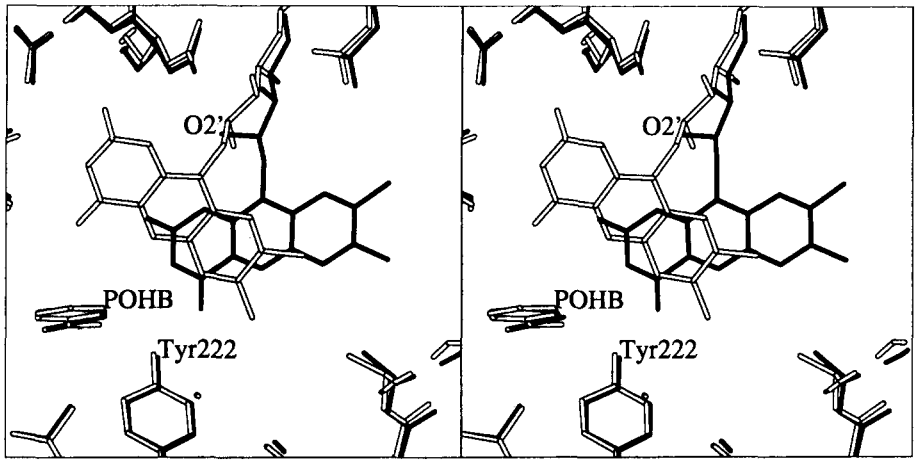

Fig. 4. Stereo diagram of the superposition of the native enzyme-substrate complex of *p*-hydroxybenzoate hydroxylase (Schreuder et al., 1989; white bonds), and the a-FAD-containing enzyme-substrate complex (this study; black bonds). The figure shows clearly how the flavin ring has moved to the "out" position.

absorption band of protein-bound FAD (Table 2). Furthermore, in the presence of the substrate both Cotton peaks become better resolved due to vibronic transitions in the 2 electronic absorption bands. Introduction of the a-FAD slightly changes the CD properties of *p*-hydroxybenzoate hydroxylase. Both Cotton peaks of the free enzyme and the enzyme-substrate complex are somewhat shifted with respect to the native enzyme (Fig. 7B). In line with the optical properties reported above, the main difference with the native enzyme is the increase in molar ellipticity of the first absorption band of the a-FAD-containing enzyme-substrate complex. At present it is not clear whether these differences in electronic transitions reflect the different flavin conformations observed in the crystal structures.

Native *p*-hydroxybenzoate hydroxylase preferentially binds the aromatic substrate in its phenolate form (Shoun et al., 1979; Entsch et al., 1991; Eschrich et al., 1993). Ionization of the 4-hydroxyl group is expected to activate the substrate for hydroxylation (Vervoort et al., 1992). Binding of the substrate to a-FAD-containing *p*-hydroxybenzoate hydroxylase also results in a large decrease in the phenolic pK_a. Figure 8 shows that binding of the substrate is accompanied by absorption changes around 290 nm and strongly dependent on the pH of the solution. The UV difference spectra observed are comparable with the corresponding spectra of the native enzyme (Eschrich et al., 1993). The estimated apparent pK_a value of 7.2 for protein-bound substrate (Fig. 8), compared to the pK_a of 9.3 for the substrate free in solution, strongly suggests that introduction of the a-FAD does not perturb the hydrogen bonding network formed by the 4-hydroxy moiety of the substrate and the side chains of Tyr 201 and Tyr 385 (Schreuder et al., 1989; Lah et al., 1994). This is in full accordance with the crystal structure presented above.

Catalytic properties

The catalytic properties of *p*-hydroxybenzoate hydroxylase reconstituted with a-FAD differ from native enzyme. Table 3 shows that replacement of normal FAD with a-FAD decreases the turnover rate (k_{cat}) and changes the reaction stoichiometry. Partial uncoupling of substrate hydroxylation results in a relatively high oxidase activity (production of hydrogen peroxide). Such an impaired hydroxylation capacity has also been observed with various mutant enzymes (Entsch et al., 1991, 1994; van Berkel et al., 1992, 1994; Eschrich et al., 1993) and with native enzyme reconstituted with artificial flavins (Entsch et al., 1980,

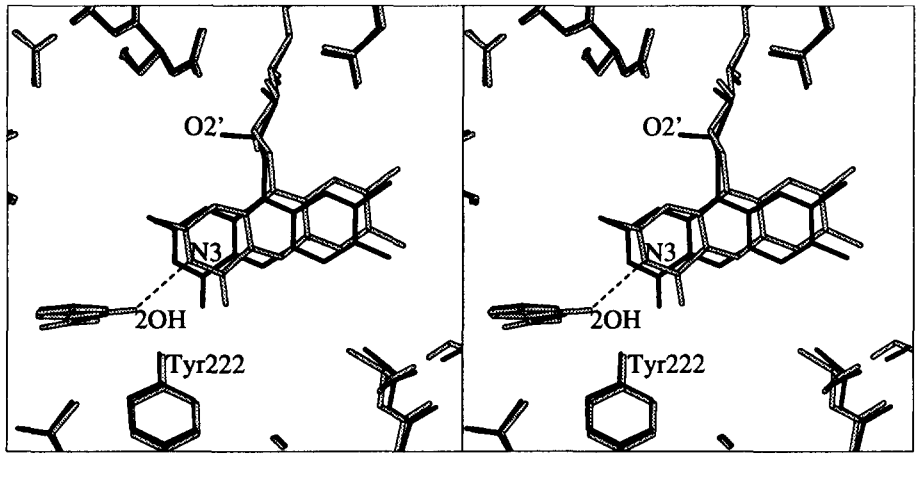

Fig. 5. Stereo diagram of the superposition of native 2,4-dihydroxybenzoate-complexed *p*-hydroxybenzoate hydroxylase (Schreuder et al., 1994; gray bonds) and the a-FAD-containing enzyme-substrate complex (black bonds). The flavin ring occupies the "out" position in both complexes, but the flavin ring is slightly further out in the 2,4-dihydroxybenzoate complex, presumably because of a hydrogen bond contact, indicated by a broken line, between the 2-hydroxy group of the substrate analog and the N3 of the flavin ring.

Table 2. *Absorption properties of free and protein-bound a-FAD*[a]

Sample	A_{max} (nm)	ϵ (mM^{-1} cm^{-1})	A_{max} (nm)	ϵ (mM^{-1} cm^{-1})
FAD	376	9.2	450	11.3
a-FAD	376	9.2	450	11.3
Native free enzyme[b]	373	8.5	450	10.2
a-FAD-containing free enzyme	374	8.6	458	11.3
Native ES complex[b]	380	9.1	450	10.2
a-FAD containing ES complex	378	9.1	455	12.3

[a] Molar absorption coefficients were determined at 25 °C in 50 mM sodium phosphate, pH 7.0. ES complex, enzyme–substrate complex.
[b] From van Berkel et al. (1992).

1987; Claiborne & Massey, 1983). Table 3 shows that the apparent K_m for *p*-hydroxybenzoate is in agreement with the K_d derived above, suggesting that partial uncoupling of hydroxylation is not due to weak substrate binding. Partial uncoupling of hydroxylation in the a-FAD-containing enzyme therefore most probably results from nonproductive decomposition of the C(4a)-hydroperoxyflavin intermediate (Entsch et al., 1976).

Anaerobic reduction of free *p*-hydroxybenzoate hydroxylase by NADPH is a very slow process. The rate of reduction is orders of magnitude stimulated in the presence of the aromatic substrate, acting as an effector (Nakamura et al., 1970; Howell et al., 1972). Anaerobic reduction of free a-FAD-containing *p*-hydroxybenzoate hydroxylase by NADPH ($k'_{red} = 0.1$ s^{-1} at 1 mM NADPH, pH 8.0, 25 °C) is considerably faster than with the native enzyme ($k'_{red} < 0.002$ s^{-1}; van Berkel et al., 1992) as measured under the same experimental conditions. In line with this, the free a-FAD-containing enzyme possesses considerable NADPH oxidase activity. From activity experiments performed in the absence of the aromatic substrate, apparent values of $k_{cat} = 0.1$ s^{-1} and K_m NADPH = 1.9 mM (pH 8.0, 25 °C) are estimated. Wasteful consumption of NADPH is not observed with the native free enzyme, probably because of a nonoptimal geometry of the flavin with respect to the nicotinamide ring (van Berkel & Müller, 1991).

The substrate is a very strong effector for the a-FAD-complexed enzyme. In Figure 9, the rate of reduction of the a-FAD-containing enzyme–substrate complex at 25 °C is plot-

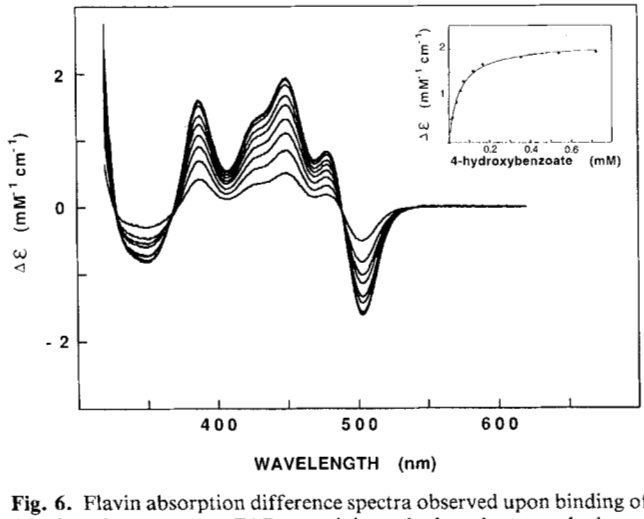

Fig. 6. Flavin absorption difference spectra observed upon binding of 4-hydroxybenzoate to a-FAD-containing *p*-hydroxybenzoate hydroxylase. The absorption spectra were recorded at 25 °C in 100 mM Tris/sulfate, pH 8.0. The enzyme concentration was 30 μM. Difference spectra are plotted between free enzyme as a reference and the same solution containing variable amounts of 4-hydroxybenzoate. The inset shows the molar absorbance difference at 450 nm as a function of the substrate concentration.

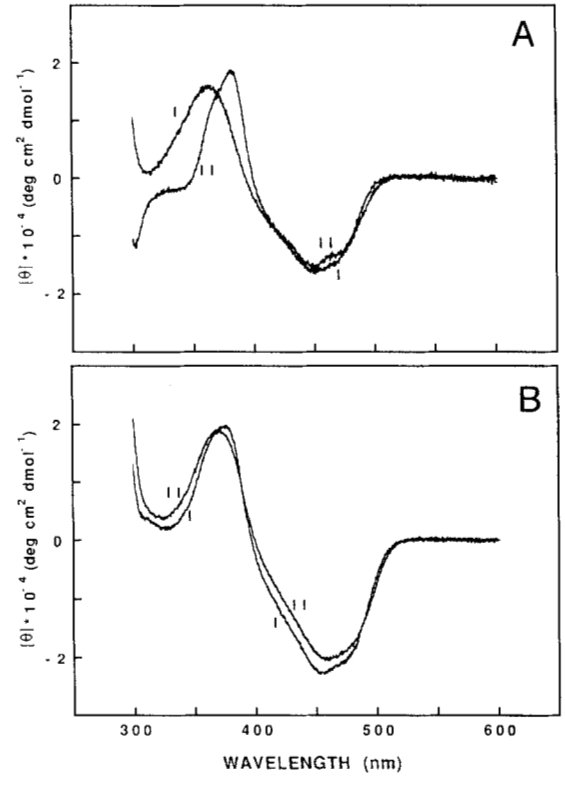

Fig. 7. CD spectra of native and a-FAD-containing *p*-hydroxybenzoate hydroxylase. The CD spectra were recorded at 25 °C in 100 mM Tris/sulfate, pH 8.0. The enzyme concentration was 40 μM. **A:** Native enzyme in the absence (I) or in the presence of 1 mM 4-hydroxybenzoate (II). **B:** a-FAD-complexed enzyme in the absence (I) or in the presence of 1 mM 4-hydroxybenzoate (II).

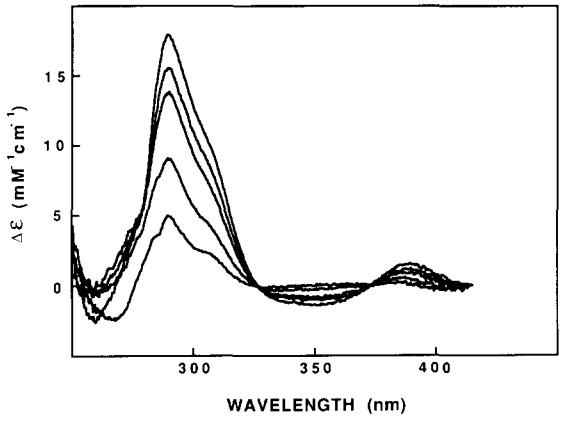

Fig. 8. UV absorption difference spectra upon binding of 4-hydroxybenzoate to a-FAD-containing *p*-hydroxybenzoate hydroxylase. Both cuvettes contained 1.0 mL 20 μM a-FAD-complexed enzyme in one compartment and 1.0 mL 200 μM 4-hydroxybenzoate in the same buffer in the other compartment. The temperature was 25 °C. For buffers used see Materials and methods. Before mixing the solutions in the 2 compartments of the sample cell, a baseline was recorded. From bottom to top: difference spectrum of a-FAD-containing *p*-hydroxybenzoate hydroxylase at pH 6.7, pH 7.2, pH 7.7, pH 8.0, and pH 8.3, respectively. From the molar absorption differences at 290 nm an apparent pK_a value of 7.2 for protein-bound substrate is estimated.

ted as a function of the concentration NADPH. The dissociation constant for NADPH as derived from this plot is in the same range as found for the native enzyme-substrate complex (Table 3). The maximal rate of reduction in the presence of 4-hydroxybenzoate is higher than found for native enzyme (Howell et al., 1972; van Berkel et al., 1992; Table 3). Interestingly, an enhanced rate of reduction with respect to the native enzyme-substrate complex has so far only been observed with *p*-hydroxybenzoate hydroxylase-containing 2-thio-FAD (Claiborne & Massey, 1983).

Discussion

This is, to our best knowledge, the first paper describing the crystal structure of a flavoenzyme reconstituted with a stereochemical analog of FAD. The high quality of the crystals shows that the immobilization technique used to prepare apo-*p*-hydroxybenzoate hydroxylase (Müller & van Berkel, 1982) is a convenient method to replace the flavin while retaining the native state of the enzyme. By using the apoenzyme of *p*-hydroxybenzoate hydroxylase as a vehicle and because the modified FAD was taken from alcohol oxidase, clear crystallographic evidence is provided for the presence of an a-FAD in alcohol oxidases from methylotrophic yeasts. The electron density map of the modified flavin containing enzyme-substrate complex shows that the absolute configuration of the C2 carbon of the ribityl chain has changed from R to S, in perfect agreement with an earlier proposal based on NMR studies (Kellog et al., 1992). Replacement of natural FAD with a-FAD in *p*-hydroxybenzoate hydroxylase does not alter the overall structure of the protein but induces the flavin ring to occupy the "out" position (Kinemage 1). This "out" conformation has been observed before in a number of crystal structures including complexes of wild-type enzyme with 2-hydroxybenzoate analogues (Schreuder et al., 1994), in mutant Tyr 222 Phe complexed with 4-hydroxybenzoate (Gatti et al., 1994), and in mutant Tyr 222 Ala complexed with 2-hydroxy-4-aminobenzoate (Schreuder et al., 1994). Superpositions have shown that in complexes with 2-hydroxybenzoate analogues, the "in" conformation is destabilized by a short contact between the 2-OH group of the substrate analogue and flavin C6, whereas the "out" conformation is stabilized by a strong hydrogen bond of the 2-OH group of the substrate analogue with the N3 of the flavin. These destabilizing/stabilizing interactions do not explain the "out" conformation in the crystals with a-FAD presented here because these crystals contain the natural substrate 4-hydroxybenzoate, which does not possess a 2-OH group. Here, the "out" conformation must have been caused by the R to S transition of the C2 carbon of the ribityl chain because this is the only difference between the crystals with the a-FAD and the crystals of the native enzyme-substrate complex (Schreuder et al., 1989). In order to assess the contacts of

Table 3. *Kinetic parameters of a-FAD complexed p-hydroxybenzoate hydroxylase*[a]

Enzyme	Product (%)	K_d NADPH (μM)	k_{red} (s^{-1})	k_{cat} (s^{-1})	K_m (μM) NADPH	K_m (μM) BzOH
Native	98 ± 2	150	300	55	70	20
a-FAD complexed	33 ± 5	115	530	11	7	56

[a] Kinetic parameters were determined at 25 °C in 100 mM Tris/SO_4^{2-}, pH 8.0. Turnover rates are maximum values (k_{cat}) determined at 0.26 mM oxygen. Dissociation constants for NADPH (K_d NADPH) were determined from rapid reaction experiments. Data for native *p*-hydroxybenzoate hydroxylase are taken from van Berkel et al. (1992). Product, 3,4-dihydroxybenzoate; BzOH, 4-hydroxybenzoate.

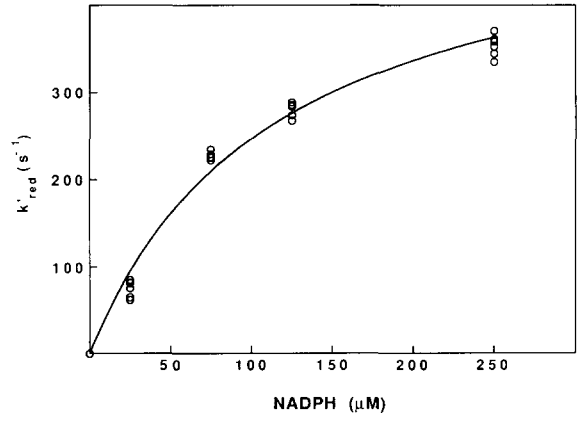

Fig. 9. Reduction of a-FAD-containing *p*-hydroxybenzoate hydroxylase by NADPH. All experiments were performed in 100 mM Tris/sulfate pH 8.0. Enzyme (18 μM) was anaerobically mixed with an equal volume of variable concentrations of NADPH in the stopped-flow spectrophotometer. Both solutions contained 1 mM 4-hydroxybenzoate. The rate of reduction of a-FAD-containing *p*-hydroxybenzoate hydroxylase at 25 °C is plotted as a function of the concentration of NADPH, as monitored at 450 nm.

a-FAD in the "in" conformation, we inverted the conformation of the ribityl C2 carbon in the structure of the native enzyme–substrate complex. The result indicates that (without adaptation of the protein) the ribityl 2-OH group would be involved in 2 short contacts: one with the CA of Leu 299 (3.0 Å) and one with the CB of Leu 299 (2.5 Å). The hydrogen bond with the side chain of Gln 102 would still be present. Only small (0.5–1.0 Å) rearrangements are needed to relieve the short contacts. These rearrangements, however, may be hindered because Leu 299 is part of helix H10. These observations point to a small difference in binding energy between the "in" and "out" conformation because the a-FAD binds in the "out" conformation in spite of apparently minor adaptations necessary to bind the "in" conformation.

The shift of the equilibrium position toward the "out" conformation, as observed in the crystal structure, explains perfectly the observed formation of considerable amounts of hydrogen peroxide during catalysis because the hydroperoxyflavin intermediate can only hydroxylate the substrate in the "in" position (Schreuder et al., 1994). A dynamic equilibrium of flavin conformers probably is also of relevance for the native enzyme because the entrance of the substrate binding site is blocked with the flavin in the "in" position and movement of the flavin to the "out" position seems to be necessary to provide a path for the substrate to enter the active site (see Gatti et al., 1994; Schreuder et al., 1994). The situation is less clear for the NADPH binding and reduction step. We have previously argued (Schreuder et al., 1994) that the flavin would most likely not be reduced in the "out" position because 2,4-dihydroxybenzoate (which also pushes the flavin toward the "out" conformation), stimulates the reduction much less than the normal substrate. However, the present data strongly suggest that the flavin is rapidly reduced in the "out" conformation. Clearly, additional data (e.g., site-directed mutants with altered NADP binding properties or a crystal structure of a complex with an NADPH analog) are needed to settle this point.

No crystal structure of methanol oxidase is available, but amino acid sequence comparisons have revealed that this enzyme belongs to the glucose-methanol-choline (GMC) oxidoreductase family (Cavener, 1992). Within this class of flavoenzymes, 2 crystal structures are known. The crystal structures of glucose oxidase from *Aspergillus niger* (Hecht et al., 1993) and cholesterol oxidase from *Brevibacterium sterolicum* (Vrielink et al., 1991; Li et al., 1993) show that the O2′ of the ribityl chain of the FAD makes a hydrogen bond with the amide nitrogen of a conserved Asn residue (Asn 107 in glucose oxidase and Asn 119 in cholesterol oxidase). The conserved Asn (Asn 98 in methanol oxidase from *H. polymorpha*; Ledeboer et al., 1985) seems to fulfill the same role as Gln 102 in *p*-hydroxybenzoate hydroxylase. Analysis of hypothetical short contacts of the ribityl O2′ after inverting the configuration of the C2 carbon reveals that for the 2 flavoprotein oxidases with known 3-dimensional structure, the short contacts would be less severe than with *p*-hydroxybenzoate hydroxylase. The shortest non-hydrogen bond-type contacts are 3.0 Å with the Cγ of Met 561 for glucose oxidase and 3.1 Å with the Cβ of Phe 487 for cholesterol oxidase, in contrast to the short contact of 2.5 Å with the Cβ of Leu 299 in *p*-hydroxybenzoate hydroxylase. These observations and the general occurrence of a-FAD in methanol oxidase suggest that the active site of this enzyme is likely to be able to accommodate the a-FAD without too many rearrangements.

Materials and methods

General

Biochemicals and chromatography resins used have been described elsewhere (van Berkel et al., 1992). Optical (difference) spectra were recorded at 25 °C, on a computer-controlled Aminco DW-2000 spectrophotometer. Fluorescence experiments were performed on an Aminco SPF-500C spectrofluorimeter at 25 °C. CD spectra were recorded on a Jobin Yvon Mark V dichrograph, essentially as described elsewhere (Benen et al., 1991). Rapid-reaction kinetics were carried out using a temperature-controlled single-wavelength stopped-flow spectrophotometer, type SF-51, from High-Tech Scientific Inc., with a 1.3-ms dead-time. The instrument was interfaced to an IBM microcomputer for data acquisition and analysis.

Purification procedures

Crude a-FAD as extracted from alcohol oxidase (Kellog et al., 1992) was a gift of Dr. L.V. Bystrykh. a-FAD was separated from natural FAD by HPLC using a Microspher C18 (20 × 300-mm) column. The elution solvent contained 15% MeOH, 85 mM ammonium bicarbonate, pH 3.7. The flow rate was 2 mL/min. Using a 10-μL sample loop, the following elution times are observed: a-FAD (4.2 min), a-FMN (5.7 min), FMN (6.3 min), and FAD (6.9 min). Micromolar quantities of pure a-FAD were obtained from repeated 50-μL injections. Purified a-FAD was desalted by reverse-phase chromatography and stored at −20 °C. FAD used in fluorescence experiments was purified by gel filtration on Biogel P-2 (Müller & van Berkel, 1982).

p-Hydroxybenzoate hydroxylase from *P. fluorescens*, as cloned in *Escherichia coli*, was purified as described (van Berkel et al., 1992). The apoprotein of *p*-hydroxybenzoate hydroxylase was prepared by covalent chromatography (Müller & van Berkel, 1982). The residual activity of the apoprotein was less than 0.2%.

Analytical methods

Fluorescence titration experiments were performed in 100 mM Tris/sulfate, pH 8.0. Flavin fluorescence emission was observed at 525 nm upon excitation at 450 nm (Müller & van Berkel, 1982). Molar absorption coefficients of protein-bound flavin were determined at 25 °C by recording absorption spectra in 50 mM sodium phosphate, pH 7.0, either in the absence or presence of 0.5% SDS (de Jong et al., 1992). Protein concentrations were determined using the following molar absorption coefficients: holo *p*-hydroxybenzoate hydroxylase, ϵ_{450} = 10.2 mM^{-1} cm^{-1} (van Berkel et al., 1992); apo-*p*-hydroxybenzoate hydroxylase, ϵ_{280} = 74 mM^{-1} cm^{-1} (Müller & van Berkel, 1982). The molar ellipticity of protein-bound flavin was determined by recording CD spectra in the visible region. The enzyme concentration was 40 μM in 50 mM potassium phosphate, 0.5 mM EDTA, pH 7.0. The ionization state of enzyme-bound 4-hydroxybenzoate was measured by recording UV absorption difference spectra as a function of substrate concentration and of pH (Eschrich et al., 1993).

p-Hydroxybenzoate hydroxylase activity was routinely assayed in 100 mM Tris/sulfate, pH 8.0, containing 0.5 mM EDTA, 150 μM NADPH, 150 μM 4-hydroxybenzoate, and

10 μM FAD (Müller & van Berkel, 1982). Kinetic parameters of *p*-hydroxybenzoate hydroxylase were determined at pH 8.0, essentially as described elsewhere (Eschrich et al., 1993). The hydroxylation efficiency of *p*-hydroxybenzoate hydroxylase was estimated from oxygen consumption experiments, either in the absence or presence of catalase (Eschrich et al., 1993). The product 3,4-dihydroxybenzoate was identified and quantified by reverse-phase HPLC (Entsch et al., 1991).

Crystallization

Crystals of a-FAD-containing *p*-hydroxybenzoate hydroxylase in complex with 4-hydroxybenzoate were obtained using the hanging drop method. The protein solution contained 10 mg/mL enzyme in 50 mM potassium phosphate buffer (pH 7.0). The reservoir solution contained 50% saturated ammonium sulfate, 0.04 mM FAD, 0.15 mM EDTA, 0.1 mM reduced glutathione, 1 mM 4-hydroxybenzoate, 60 mM sodium sulfite, and 50 mM potassium phosphate buffer, pH 7.0. Drops of 4 μL protein solution and 4 μL 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 3 days.

Data collection

X-ray diffraction data were collected using a Siemens multiwire area detector and graphite monochromated CuKα radiation from an 18-kW Siemens rotating anode generator, operating at 45 kV and 80 mA. The crystal-detector distance was 11.6 cm and the 2θ angle was 25°. Data were processed using the XDS package (Kabsch, 1988). The space group is $C222_1$ and the cell dimensions: $a = 72.1$ Å, $b = 146.4$ Å, and $c = 88.45$ Å differ only slightly from the native crystals: $a = 71.5$ Å, $b = 145.8$ Å, and $c = 88.2$ Å (Schreuder et al., 1989). A total of 102,196 observations yielded 26,934 unique reflections with an *R*-sym of 6.9%. The data set is 97.2% complete to 2.1 Å.

Refinement

A starting electron density map was calculated based on the structure of the *p*-hydroxybenzoate hydroxylase-substrate complex (Schreuder et al., 1989), after correcting for the slightly different cell dimensions (Schreuder et al., 1994). However, the $2F_o - F_c$ and $F_o - F_c$ electron density maps clearly indicated that the flavin does not occupy the "in" position as in the enzyme-substrate complex, but that it occupies the "out" position as observed in several other *p*-hydroxybenzoate hydroxylase complexes (Gatti et al., 1994; Schreuder et al., 1994). We decided therefore not to use the structure of the enzyme-substrate complex as starting model, but to use the structure of the enzyme-2,4-dihydroxybenzoate complex (Schreuder et al., 1994) instead. The starting *R*-factor, after correcting for slightly different cell dimensions, was 0.277 for data between 8.0 and 2.1 Å. The $2F_o - F_c$ and $F_o - F_c$ maps clearly indicate that the absolute configuration of the C2′ of the ribityl-chain differs from normal FAD, as has been suggested earlier on the basis of NMR experiments (Kellog et al., 1992). The ribityl chain was built and the model was inspected and corrected where necessary with the graphics program FRODO (Jones, 1985). 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. For the FAD we used the parameters as described by Schreuder et al. (1994). The topology definition for the C2 of the ribityl chain was changed from R to S. The model was refined with energy minimization and temperature factor refinement. Water molecules were assigned by searching $F_o - F_c$ maps for peaks of at least 4σ, which were between 2.0 and 5.0 Å of other protein or water atoms. Water molecules with temperature factors after refinement in excess of 70 Å^2 were rejected.

The final structure was obtained after 4 cycles of map inspection and refinement and contains 284 water molecules. The final *R*-factor is 0.179 for 26,407 reflections between 8.0 and 2.1 Å. The RMS deviations are 0.008 Å for bond lengths and 1.4° for bond angles. All non-glycine residues have ϕ, ψ angles within, or close to allowed regions. The only exceptions are Arg 44 and Asp 144, which also deviate in the structures of other *p*-hydroxybenzoate hydroxylase complexes from *P. fluorescens* (Schreuder et al., 1989, 1994). The coordinates of the refined a-FAD-containing enzyme-substrate complex will be deposited in the Brookhaven Protein Data Bank.

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